Functional and Regulatory Properties of H^+ Pumps at the Tonoplast and Plasma Membranes of *Zea mays* Coleoptiles

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A microsomal membrane fraction ($6000 \times g$ supernatant of a cell homogenate), isolated from coleoptiles of *Zea mays*, was separated by isopycnic sucrose density gradient centrifugation in the presence of EDTA and without a prior pelleting step to avoid irreversible sticking of different membrane species. The membrane fractions were characterized by assaying commonly used marker enzymes, and the levels of activity investigated of ATP hydrolysis, ATP-dependent H^+ transport, and co- and countertransport of ions, such as Cl^-, fumarate^{2-}, K^+ and Li^+. The following results were obtained:

(1) ATP hydrolysis is performed by different enzymes associated with different membranes:
- vacuolar acid phosphatase (AP; inhibited by molybdate);
- Golgi phosphatase, revealing IDPase, pNPase and ATPase activity (not inhibited by molybdate);
- ATPase activity of residual submitochondrial particles (sensitive to azide);
- a H^+-translocating ATPase at tonoplast membranes ($Km_{(ATP)} = 0.29$ mM; pH 7.5; stimulated by uncouplers and completely inhibited by NO_3^-);
- the H^+-translocating ATPase of the plasmalemma ($Km_{(ATP)} = 0.39$ mM at pH 6.5, inhibited by vanadate, but not by NO_3^-).
  The latter activity is evident only after an osmotic shock, indicating that PL vesicles primarily exist as inside-in-vesicles.

(2) ATP-fueled H^+ pumps are localized at tonoplast (TO) and plasmalemma (PL) vesicles, they differ to some extent in their properties:

(a) The PL H^+ pump has a very narrow pH optimum and exhibits highest levels of activity at pH 6.5 with a pronounced increase of activity between pH 7.5 and 6.5 (properties, obviously important *in vivo* for the regulation of active H^+-extrusion by certain growth substances, which affect the cytoplasmic pH (Hager and Moser, Planta 1984, in press); in contrast, TO H^+ pumps show a considerably wider pH optimum with highest levels of activity around pH 7.5.

(b) In variance to the PL H^+ pump the activity of TO pumps ($Km_{(ATP)} = 0.24$ mM) is regulated *via* the oxidation state of essential thiol groups. Their oxidation to the S–S-form (*e.g.* by blue light in the presence of a flavin) causes an inactivation, whereas a re-reduction by GSH or cystein restores the activity [51].

(c) The ATP-fueled H^+ transport into TO vesicles depends on an anion co-transport; most effective is Cl^-, but there is also a stimulation by organic ions, C_4 and C_5 dicarboxylates, such as malate, succinate, fumarate, 2-oxoglutarate and aspartate; NO_3^- is inhibitory.

(d) H^+-transport into sealed PL vesicles is also anion dependent. In this case, however, NO_3^- is as effective as Cl^-.

(3) The TO membranes contain a H^+/K^+ exchange mechanism responsible for a secondary active K^+ uptake into the vacuole. This mechanism could be the reason for a lower (ATP dependent) acidification of TO vesicles in the presence of K^+ compared with Li^+. – Similar effects are observed with plasmamembrane vesicles, but in this case there is still the question whether a H^+/K^+-exchange, a K^+ channel, or both are acting.

Abbreviations: AP, acid phosphatase; BSA, bovine serum albumin (fract. V, defatted); CCO, cytochrome *c* oxidase; CCR, cytochrome *c* reductase; DTT, DL-dithiothreitol; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol-bis (*β*-aminoethyl ether) N, N, N′, N,′-tetraacetic acid; ER, endoplasmatic reticulum; FC, fusicoccin; FMN, flavin mononucleotide; GS, glucan synthetase; GSH, glutathione (reduced form); HEPES, N-2-hydroxyethyl-piperazine-N′-2-ethanesulfonic acid; HPLC, high pressure liquid chromatography; IAA, indole-3-acetic acid; IDP, inosine 5′-triphosphate; NEM, N-ethylmaleimide; NR, neutral red; pHMB, *p*-hydroxymercuribenzoate; PL, plasmalemma; pNP, *p*-nitrophenyl phosphate; TO, tonoplast; TRIS, tris (hydroxymethyl) aminomethane.

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Introduction

During recent years a large body of evidence has accumulated which indicates that electrogenic proton pumps localized at cell membranes are responsible for maintaining the membrane potential [1, 2] and are the major driving force for primary and secondary active ion and solute transports through membranes [3–5]. In addition, H^+ pumps are thought to be directly involved in inducing elongation growth [6, 7]. It has been suggested that growth substances, such as IAA or FC [8] stimulate the level of H^+-ATPase activity at cell membranes *via* up to now unknown processes, thereby increasing proton extrusion and finally cell wall loosening and cell elongation. In order to get more information about the characteristics and the modes of regulation of H^+ pumps we used membranes from coleoptiles, a plant organ, which exhibits a strong proton extrusion and elongation growth.

Using isolated microsomal membrane vesicles of low density from coleoptiles, Hager *et al.* [9] were the first to demonstrate the existance of ATP dependent H^+ pumps by means of a spectrophotometric method. These H^+ ATPase activities of isolated vesicles exhibited a high specificity for the substrate ATP. Surprisingly, the energy requiring H^+ transport into the vesicles was strongly dependent on a Cl^- co-transport, not influenced by vanadate, but completely inhibited in the presence of NO_3^- and to a minor degree by sulfate [10].

Subsequently, proton pumps exhibiting similar properties have been reported for (vacuolar) membranes from different plant species and organs (corn roots: [11, 12]; oat roots [13, 14]; *Hevea* latex [15, 16]; *Hevea* vacuoles: [17]; corn coleoptiles: [18]; pumpkin hypocotyls: [19]; tulip petals: [20]; red beet storage tissue: [21]; *Saccharomyces:* [22]; tobacco callus: [23]; pea stem: [24]; radish: [25]).

Furthermore, the inhibition by NO_3^- of the tonoplast located ATP-dependent H^+ transport has been recognized as an important feature to distinguish between tonoplast and plasmalemma H^+ ATPases [17, 26, 27]; in addition vanadate was found to specifically inhibit the plasmalemma H^+ ATPase *in vitro* [28, 29] and *in vivo* [30]. Another characteristic of vacuolar membranes was suggested to be a H^+/Ca^{2+} antiporter mechanism which is thought to function as a secondary active Ca^{2+} pump [31–33].

The aim of the present study was to get more information about properties and functions of ATPases and proton pumps belonging to different membrane species of the microsomal membrane fraction of coleoptiles and to characterize the co- and countertransport of ions accompanying the active H^+ transport. In addition, mechanisms which possibly regulate the H^+ pump activities of tonoplast and plasma membranes are reported and implications for metabolic processes, *e.g.* elongation growth, discussed.

Material and Methods

Plant cultivation

The hybrid corn variety "Infrafrüh", category 4b (Nungesser KG, Darmstadt) was irrigated with water for 5 h and germinated on moistened cellulose. The seedlings were cultivated over a period of 96 h at 26 °C, 90% humidity and red light for 2 h daily.

Preparation of microsomal vesicles

Deleaved coleoptiles (normally 10 g) were plasmolized (10 min) by vacuum infiltration in medium I (10 ml/g fr. weight) for 10 min, thereafter chopped with a razor blade in 3.0 ml medium II/g fresh weight. The homogenate was pressed through 4 layers of surgical gauze and made up to 35 ml with medium II. Then it was centrifuged for 20 min at $6000 \times g$ (Beckman, JA 20). The supernatant (microsomal membrane fraction) was centrifuged for another 30 min at $143\,000 \times g$ (Kontron TGA 65; 45 Ti), and the pellet suspended in 1.2 ml medium III/g fr. wt with a homogenizer (Potter-Elvehjem). Medium I: 450 mM sucrose, 10 mM HEPES/KOH, pH 7.5. Medium II: 300 mM sucrose; 75 mM HEPES/KOH, pH 7.5; 10 mM EGTA; 20 mM ascorbic acid; 2.5 mM DTT, 0.1% BSA (w:v). Medium III: 300 mM sucrose; 10 mM HEPES/KOH pH 7.5; 1 mM $MgSO_4$, 0.1% BSA. When the membrane preparations were assayed immediately after isolation, the addition of ascorbic acid and BSA was not essential. If required, the pH of the media was adjusted to 7.5 with LiOH, CsOH or TRIS instead of KOH.

Separation of membrane fractions by density gradient centrifugation

In this case coleoptiles were chopped in 1 ml medium II/g fresh weight, and medium II (pH 7.8) contained 1 mM EDTA.

Eleven ml of the $6000 \times g$-supernatant, layered onto a linear sucrose gradient (medium IV: 15–45% sucrose (w/w), 7.5 mM HEPES, pH 7.0 or 7.5, 2 mM EGTA, 1 mM DTT, 0.05% BSA) were centrifuged at $110\,000 \times g$ (Beckman, SW 27) for 15 h. The gradient was separated into 1.5 to 1.8 ml fractions (Isco density gradient fractometer 185), and the density monitored.

Demonstration of proton accumulation in microsomal vesicles

The experiments were performed according to Hager *et al.* [9], and Hager and Helmle [10]. In order to test fractions of the sucrose gradient, 450 µl aliquots were mixed with 50 µl of medium V (final concentration: 2 mM $MgSO_4$, 50 mM salt, 4 µm NR and other compounds tested). In variance to the normal method H^+ transport was measured in 2 min intervals after addition of 2 mM ATP as ΔA of neutral red at 490 minus 430 nm (medium pH 7.5) and 480 minus 430 nm (medium pH 7.0) respectively using a Hewlett Packard 8450 A spectrometer.

Assays of enzymes

Enzymes were assayed as follows: NADH-cytochrome-*c*-reductase (EC 1.6.99.3), antimycin A resistant [34]; cytochrome-*c*-oxidase (EC 1.9.3.1) [35]; acid phosphatase, 5 mM *p*-nitrophenylphosphate as substrate [36]; β-mannosidase (EC 3.2.1.25), 10 mM *p*-nitrophenyl-β-D-mannopyranoside as substrate [37]; glucan synthetase I and II (EC 2.4.1.12) and the latent IDPase [38]. ATPase (EC 3.6.1.3) was assayed by measuring the decrease of ATP *via* luminescence (LKB-Wallace-Luminometer 1250) or by determining the formation of P_i (39) after 45 min incubation at 35 °C (or after 25 min incubation, if the K_m was determined); latent inosine diphosphatase (EC 3.6.1.6) was assayed according to [38], in the presence or absence of 0.15 mg/ml digitonin, followed by the measurement of P_i as described for the ATPase-test. Protein was determined according to [40] using BSA as a standard.

Results

Isopycnic distribution of microsomal membranes in a linear sucrose gradient

Fig. 1 shows the distribution of microsomal membranes from maize coleoptiles, Fig. 11 those from oat coleoptiles, in a sucrose density gradient. Organelles and particles of the homogenate with higher density, such as cell wall fragments, nuclei, mitochondria, etioplasts, were removed by centrifugation ($6000 \times g$). The supernatant, containing "microsomal" membranes, was layered onto a density gradient without any further centrifugation step in order to avoid membrane sticking; for the same reason EDTA was present in the medium. The gradient was centrifuged for 15 h. In addition to soluble proteins (strong absorption at $\lambda_{280\,nm}$) small vacuoles occur at 18% sucrose. They band together with the vacuolar enzyme AP (36) and β-M, an enzyme bound to tonoplast membranes [37]. The AP activity with pNP or ATP as substrates was completely inhibited by molybdate (0.1 mM).

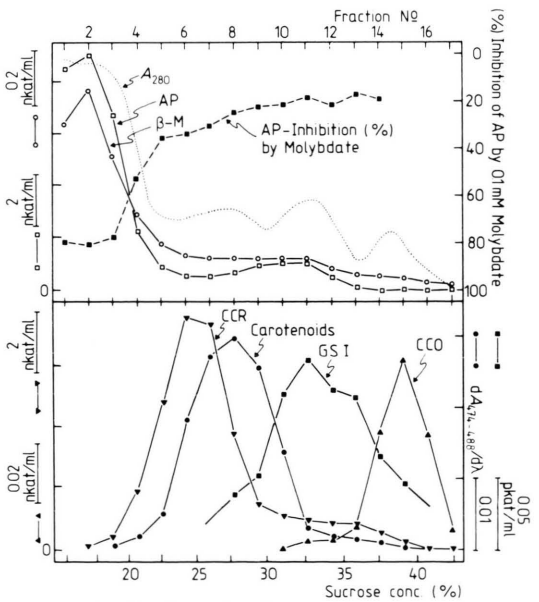


Fig. 1. Distribution of microsomal membranes ($6\,K \times g$ supernatant) from cells of the maize coleoptiles in a linear, isopycnic sucrose gradient. Characterization by protein content (A_{280}) and various marker enzymes; AP, molybdate sensitive (vacuolar enzyme); β-M (tonoplast membrane); CCR (ER); carotenoids (membranes of etioplasts); GS I (Golgi apparatus); CCO (residual mitochondrial membranes). β-M = β-mannosidase.

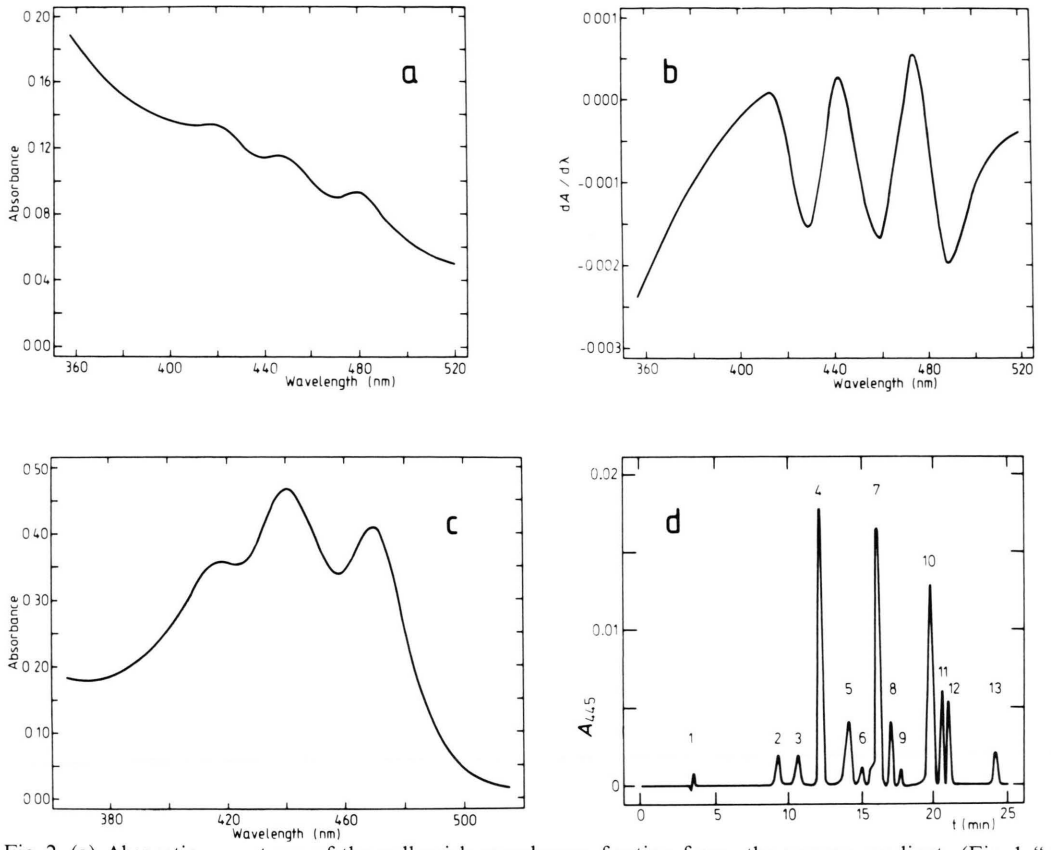


Fig. 2. (a) Absorption spectrum of the yellowish membrane fraction from the sucrose gradient (Fig. 1 "carotenoids"); (b) first derivative of the spectrum given in (a); (c) the extracted pigments in ethanol, and (d) separation of the pigments by HPLC (2, 3 = neoxanthin; 4 = violaxanthin; 5= antheraxanthin; 7 = lutein + zeaxanthin; 13 = β-carotene).

Between 20 and 30% sucrose several membrane species were localized. A narrow band around 24% sucrose contained ER (marker enzyme CCR). Around 28% sucrose, a very small amount of a carotenoid-containing membrane fraction was localized, possibly derived from etioplasts or carotenoid-containing envelopes of plastides [41] with a reported density of 1.113 [42, 43]. The carotenoids were determined spectroscopically and by HPLC analysis (Fig. 2). When using coleoptiles which were grown in the light to develope plastides in the bundle sheets, chlorophyll-containing membranes were recovered from the gradient at densities higher than those of carotenoid-containing membranes. At about 32% sucrose Golgi membranes banded which showed GSI (Fig. 1) and phosphatase activity expressed not only as IDPase activity (Fig. 4) but also as AP activity (with the substrate pNP or ATP). This phosphatase was, in contrast to the vacuolar AP, molybdate insensitive. When ATP was used as a substrate for the Golgi phosphatase, then its hydrolysis was also insensitive to molybdate and azide (Figs. 3 and 5). At 38% sucrose a residual CCO activity from submitochondrial particles was present which coincided with plasmalemma membrane markers. It should be noted, however, that the usually employed marker enzyme for plasmalemma, GS II, was inactive due to the presence of EDTA and EGTA in the homogenization medium. Thus, K^+-stimulated pH 6.5-ATPase was used as a marker (not shown).

Distribution of ATP hydrolyzing activities in the linear density gradient

Fig. 3 demonstrates the isopycnic distribution of ATPase activities measured at pH 7.5. Specific inhibitors were used to discriminate different ATPases.

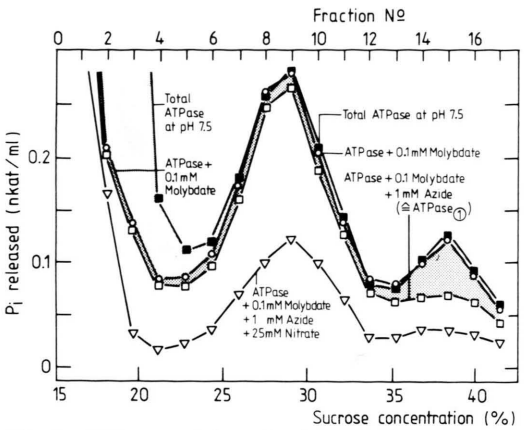


Fig. 3. ATPase activity of microsomal membranes on a sucrose gradient (as in Fig. 1) and effect of inhibitors; molybdate inhibits ATPase activity of AP, azide that of residual mitochondrial ATPases. In the presence of molybdate and azide a residual hydrolyzing activity is observed which is termed $ATPase_1$: it consists of the NO_3^- sensitive H^+ ATPase of the TO, the phosphatase of the Golgi apparatus (latent IDPase at about 30% sucrose) and the PL H^+ ATPase (around 39%); the latter shows very low activity at pH 7.5 which was used in this experiment (pH optimum: 6.5).

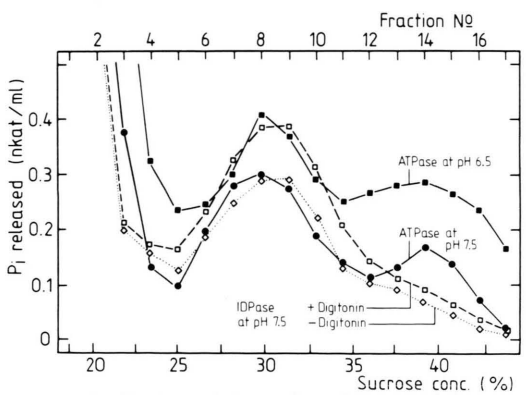


Fig. 4. Distribution of the entire ATPase activity on microsomal membranes in the sucrose gradient at pH 6.5 and 7.5 and the IDPase activity with and without digitonin (0.15 mg/ml); substrate concentrations: 1 mM ATP resp. 2.5 mM IDP.

At lower densities high levels of ATP-hydrolysis activity were recovered, a great part of which appeared to be due to the AP, localized within or set free from vacuoles. This ATPase activity was completely inhibited by molybdate.

In addition, the ATPase activity resulting from submitochondrial particles (38% sucrose) was completely inhibited by azide. The plasmalemma

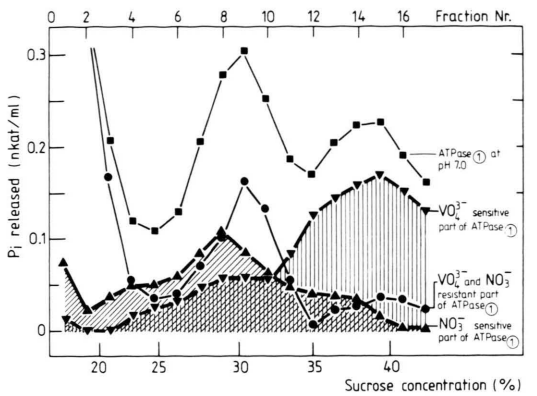


Fig. 5. Distribution of membrane-bound $ATPase_1$ activity over the sucrose gradient: part of the NO_3^--sensitive moiety is identical with the H^+ ATPase of the TO, the vanadate-sensitive part coincides with the PL H^+ ATPase, the vanadate and NO_3^--resistant ATPase with the Golgi apparatus.

H^+ ATPase, occurring in the same density range, was inactive at pH 7.5. After inhibition of AP by molybdate (50 µM) and mitochondrial ATPase by azide (500 µM) or oligomycin (20 µM) a residual ATPase activity could be observed which is named $ATPase_1$. It obviously represents (at pH 7.5) the tonoplast H^+ ATPase and the Golgi phosphatase. The latter is visible in Fig. 3 in the region between 25% and 32% sucrose, after inhibition of the tonoplast H^+ ATPase by NO_3^-, and coincides with the IDPase activity shown in Fig. 4 which is normally used as a marker for Golgi membranes. At pH 7 both the H^+ ATPases of tonoplast and of the plasmalemma are active; therefore the $ATPase_1$ hydrolizing activity can be referred to 3 different enzymes (Fig. 5): the H^+ ATPase of tonoplast vesicles ($Km_{(ATP)} = 0.28$ mM at pH 7.5), which can be inhibited by NO_3^-; the Golgi phosphatase, which correlates with GS I (Fig. 1) and IDPase activity (Fig. 4) and the ATPase activity in the dense region of the gradient, resulting from the vanadate sensitive H^+-ATPase of the plasmalemma ($Km_{(ATP)} = 0.39$ mM at pH 6.5).

The H^+-ATPase activities of TO and PL were stimulated by uncouplers (Fig. 6), indicating that they are electrogenic pumps and localized in membranes of sealed vesicles.

In this context it is interesting to know that most of PL vesicles are originally formed inside-in. Therefore the binding site for ATP at the H^+-

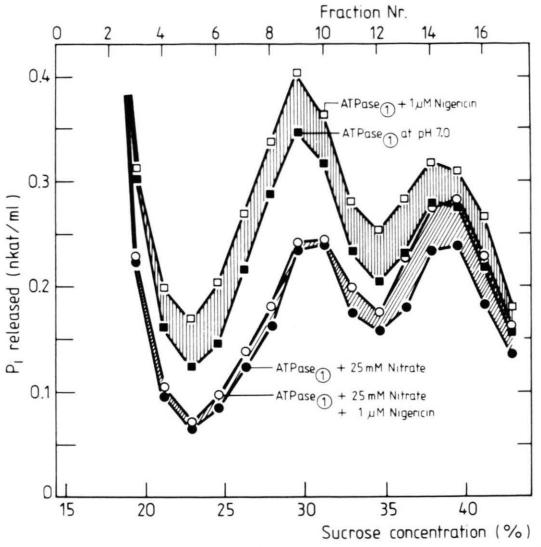


Fig. 6. $ATPase_I$ activity of microsomal membrane vesicles in the sucrose gradient. The ATP-hydrolysis associated with a H^+ transport into vesicles is stimulated by the uncoupler nigericin (1 µM). Stimulation is only observed in the low-density fractions containing the NO_3^--sensitive TO H^+ ATPase (20–30% sucrose) and in the dense fractions due to the NO_3^--resistant PL H^+ ATPase (30–43%), however not in the Golgi fraction.

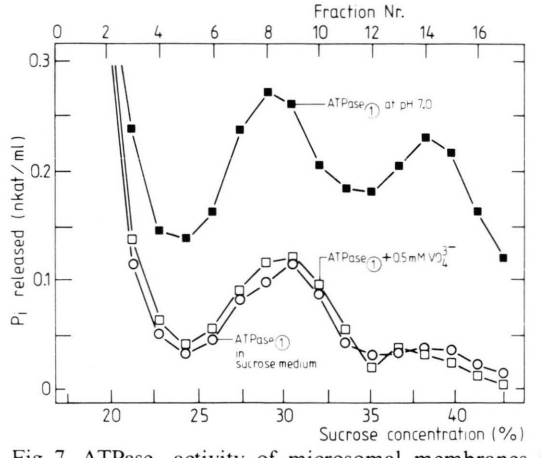


Fig. 7. $ATPase_I$ activity of microsomal membranes in a sucrose gradient. No ATPase activity can be shown in the high-density regions of the gradient (PL), since a dilution step (osmotic shock) during the ATPase test has been avoided. An indication that PL vesicles are in their original inside-in conformation.

ATPase is not accessible, leading to very low levels of ATP hydrolizing activity (Fig. 7). Only after an osmotic shock, *i.e.* osmotic lysis which usually occurs during the addition ATP, an ATP hydrolizing activity can be demonstrated.

ATP dependent transport of H^+ across TO and PL

Within the sucrose density gradient membrane vesicles occur, which exhibit an ATP dependent intravesicular acidification as shown by difference spectroscopy with NR [9, 10]. At pH 7.5 this activity is only found in membrane fractions with low density (isolated from maize: Fig. 8–10, and from oat: Fig. 11). Sometimes, two peaks of activity were found (Fig. 9 and 10). One peak was around 18% the other at 28% sucrose. The amount of the vesicle moiety changed alternatively in different experiments. The vesicles with the lowest density were obviously small vacuoles. Both these vesicle populations exhibited identical properties with respect to H^+ pumping and ion transport: the Cl^- dependent intravesicular acidification [10] was smaller in the presence of K^+ compared to Li^+ (Fig. 8). This effect is assumed to be caused by a H^+/K^+ antiporter mechanism at the TO vesicles leading to an influx of K^+ and an efflux of H^+. A similar mechanism has already been postulated for the PL [23]. In addition, the two TO vesicle moieties revealed the same anion dependency of ATP-fueled acidification: besides Cl^-, organic anions, such as fumarate or malate were also used (Fig. 9).

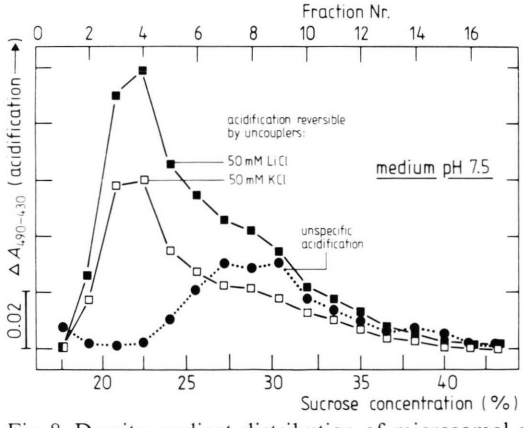


Fig. 8. Density gradient distribution of microsomal membrane vesicles exhibiting an ATP-fueled, Cl^--dependent acidification (TO vesicle, pH 7.5) Acidification (ΔA of NR) was measured 8 min after addition of ATP (2 mM) and 4 min after the further addition of gramicidin (10 µg/ml). The term "unspecific acidification" describes a decrease of the medium pH, which is caused by the phosphatase activity (IDPase) of Golgi membranes and which is not reversible by uncouplers. The lower rate of H^+ accumulation by the vesicles in the presence of K^+ and compared to Li^+ is a consequence of a H^+/K^+ antiporter mechanism at the TO membranes.

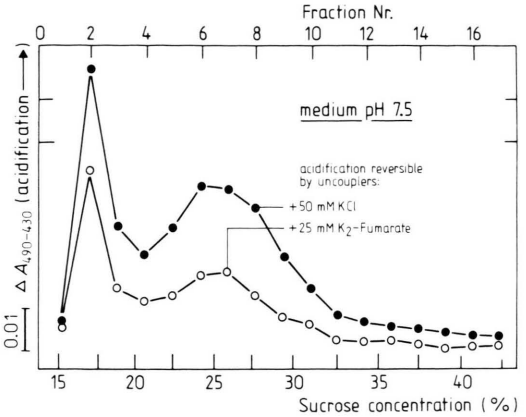


Fig. 9. Density gradient distribution of membrane vesicles showing an ATP-dependent intravesicular acidification (reversible by uncouplers, *e.g.* 0.5 µM nigericin) in the presence of 50 mM Cl^- or 25 mM fumarate^{2-}. Reaction time after addition of 2 mM ATP with KCl resp. K_2 fumarate was 8 min resp. 10 min. The co-transport of Cl^- or fumarate^{2-} with H^+ occurs at the same membrane species (TO). In most preparations the proton pump at 17% sucrose was not distinct (Fig. 10) or not detectable (Fig. 8) (see text).

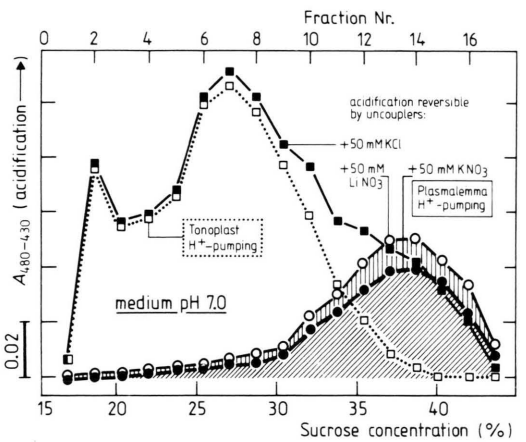


Fig. 10. Distribution of membrane vesicles in a density gradient exhibiting an ATP-fueled acidification (14 min after adding ATP; pH 7.0). The acidification is Cl^--dependent and completely inhibited by NO_3^- in the case of TO vesicles. The H^+ transport into PL vesicles cannot be inhibited by NO_3^- and is smaller in the presence of K^+ compared to Li^+.

The plasmalemma H^+-ATPases could be demonstrated together with that of the TO at pH 7.0 (though its optimal activity is at pH 6.5). After inhibition of the TO pump by NO_3^- the PL H^+-pumping activity became distinguishable. In respect

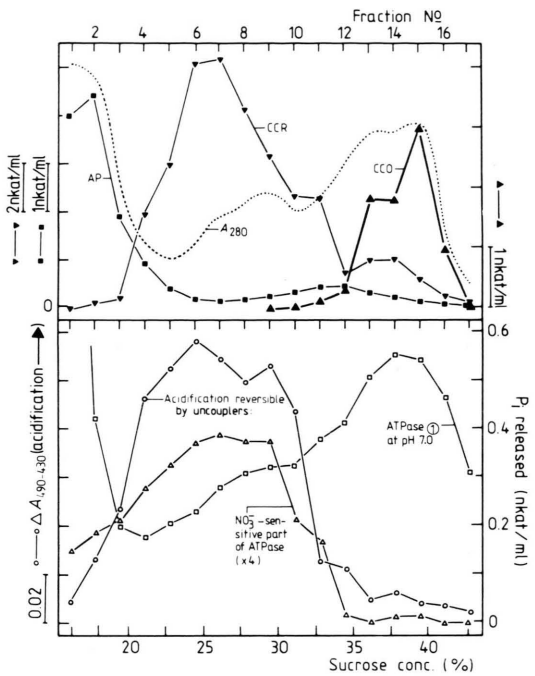


Fig. 11. Isopycnic distribution of microsomal membrane vesicles from cells of coleoptiles of *Avena sativa* over a linear sucrose gradient. Characterization (a) by protein (A_{280}) and marker enzymes, (b) by the ATP hydrolyzing activity (at pH 7.0), and the NO_3^--sensitive part of ATP_1 activity (at pH 7.5) which corresponds to ATP-dependent (NO_3^--sensitive) intravesicular acidification ($\Delta A_{490-430}$).

to the ATP hydrolysis activity of PL and TO H^+ pumps, the amount of acidification in PL vesicles is low compared with that in TO vesicles. This could implicate that PL vesicles are more leaky toward H^+ [44] or that part of the PL forms membrane sheets during the assay.

Similar to conditions at the TO, PL vesicles show a lower acidification in the presence of K^+ compared with Li^+ (Fig. 10). This could result from a K^+ channel, already postulated for the PL [53] or from a H^+/K^+ countertransport (Fig. 10). Interestingly, the NO_3^--insensitive proton pumping activity (PL) could only partially be inhibited by vanadate (Fig. 12; also shown by [21]) whereas there was nearly a complete inhibition of its considerably higher ATPase activity (see Discussion). Fig. 12 shows that the ATP dependent acidification of sealed PL vesicles like that of TO vesicles is also anion-dependent but that the anion transporter is very unspecific; acidification also occurs in the presence of NO_3^-.

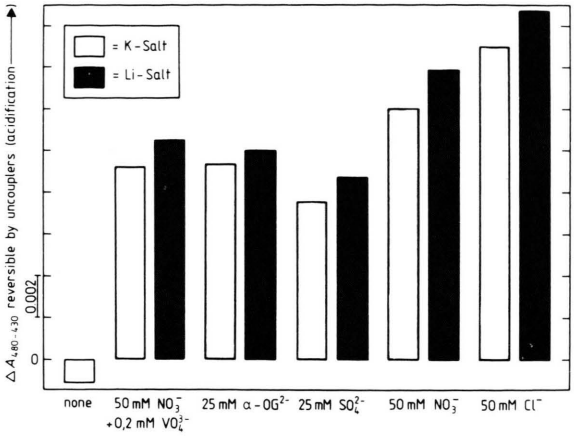


Fig. 12. Dependency of ATP-fueled acidification of sealed PL membranes (41% sucrose fraction of a density gradient as in Fig. 1 or 10) on anions and cations. In contrast to the ATP hydrolyzing activity (Fig. 5) the H^+-pumping activity is only partially inhibited by vanadate. Reaction time after addition of ATP was 14 min.

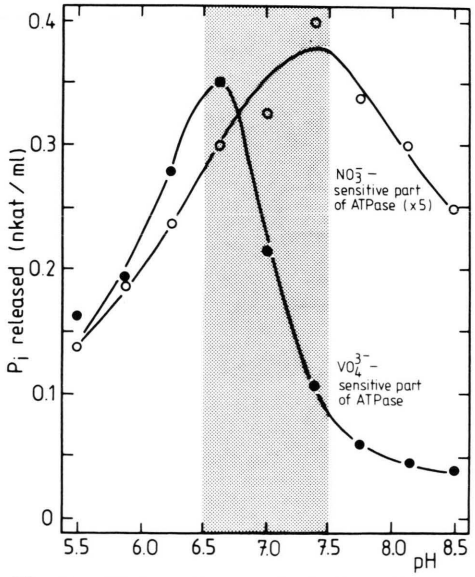


Fig. 13. pH-dependency of PL (vanadate-sensitive) and TO (nitrate-sensitive) H^+ ATPase. For determination of TO ATPase the 25% sucrose band, for that of the PL ATPase, the 41% sucrose band was used. The difference of P_i release in the presence or absence of 25 mM KNO_3 (TO) or 0.5 mM K_3VO_4 are plotted.

Fig. 15. Inhibition of ATP-dependent acidification of TO-vesicles by pHMB and its reversion by DTT.

Different sensitivity of PL and TO H^+ ATPases toward pH and sulfhydryl reagents

Fig. 13 demonstrates the very narrow pH optimum of PL H^+-ATPases, exhibiting highest levels of activity at pH 6.5 and a significant increase of activity between pH 7.5 and 6.5. It has been suggested [52] that *in vivo* a regulation of H^+ pump activity occurs by small changes of the cytoplasmic pH (see Discussion). In Fig. 14 data on the sensitivities of TO and PL associated H^+ pumps toward SH reagents is given. Only the TO H^+ pump was inhibited by SH-blocking compounds such as pHMB (Fig. 15) or NEM (Fig. 14, 16). Its inhibition by pHMB could be restored by DTT (or GSH and Cys; not shown). Furthermore, the TO H^+ pump

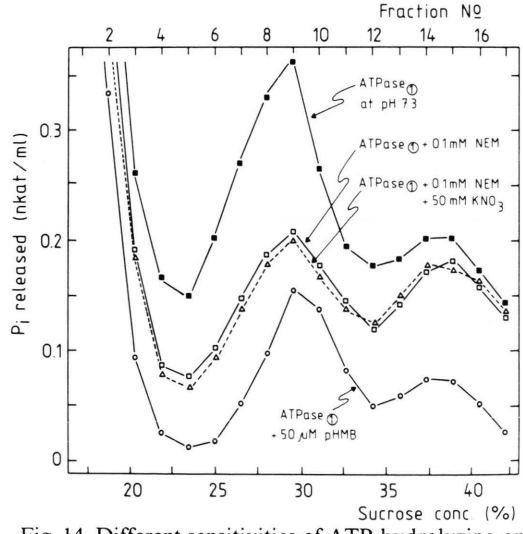
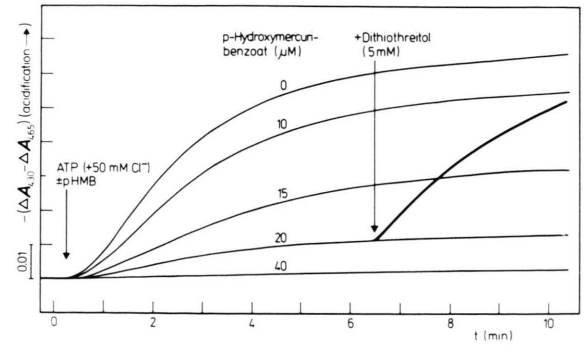


Fig. 14. Different sensitivities of ATP-hydrolyzing enzymes to SH-blockers. NEM (0.1 mM) inhibits the TO ATPase only, pHMB (50 µM) the TO ATPase and, partially, the PL ATPase; the Golgi-Phosphatase was not sensitive to these reagents.



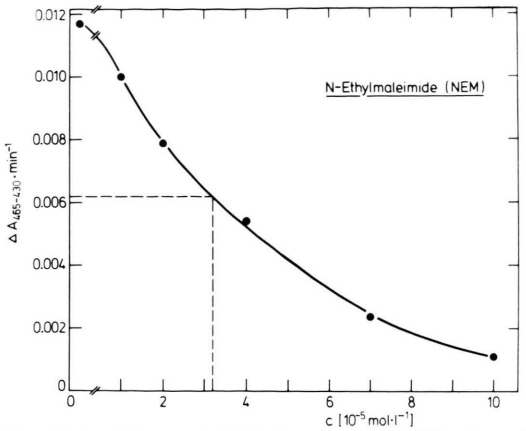


Fig. 16. Inhibition of ATP-dependent acidification of TO vesicles by various concentrations of NEM.

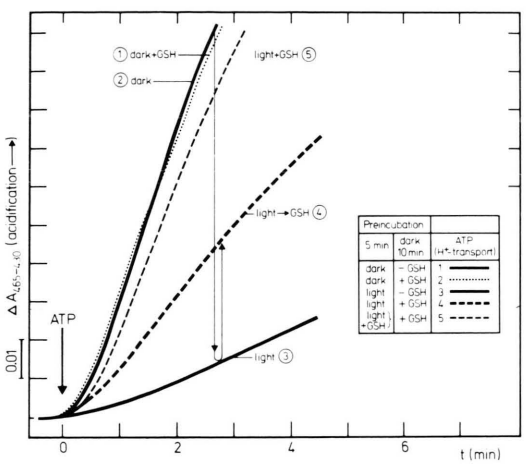


Fig. 17. Blue light dependent inhibition of the ATP-dependent acidification of TO-vesicles in the presence of FMN (20 μM). ③ After illumination (447 nm; 5.85 mW cm^{-2}; 5 min) the degree of intravesicular acidification is reduced. Q Illumination in the presence of GSH prevents the inhibition by light. ○ The inhibitory effect of light on H^+-pumping can be restored by GSH (5 mM) added immediately after illumination [51]. Instead of HEPES 10 mM phosphate buffer was used in this experiment.

was inactivated by light and in the presence of a flavin, obviously due to an oxidation of essential SH-groups of the pump to disulfides [51]. The activity was restored by GSH (Fig. 17).

Discussion

From a linear sucrose gradient two fractions of microsomal membrane vesicles from corn coleoptiles were recovered which exhibited ATP dependent H^+ pumping activity (Fig. 10). The activity in the low density region could be referred to small vacuoles and TO vesicles whereas that in the high density region coincided with PL membrane markers. TO vesicles were spread over a large density region also covering the very narrow band of ER membranes. Sometimes, in the lowest density region, TO vesicles formed a peak separated from the other TO vesicles moiety (Figs. 9, 10). This peak consisted of vacuoles which are believed to be native undamaged organelles, still containing AP. Similar results have been reported by [45]. One can assume that TO vesicles should be heavier than vacuoles due to there formation during homogenization, which is performed in a sucrose containing medium and is accompanied by an exchange of vacuolar content (AP is lost and sucrose is taken up). However, it is also possible that a different cleavage of membrane constituents could have led to two TO membrane moieties of different density.

The properties of the two TO derived vesicle population are identical with respect to the properties of H^+ ATPases and ion transport. The H^+ pumps of TO membranes have a high substrate specificity; ATP was shown to be the only nucleoside triphosphate [10, 12, 13, 19]. This specificity seems to be less expressed when ATP hydrolysis instead of H^+ pumping is measured, obviously due to some other contaminating phosphatases.

A TO associated H^+ pumping activity occurs only in the presence of certain anions, Cl^- being most effective [10, 11]. For the transport of Cl^- through the membrane a channel or carrier has been suggested (Fig. 18) similar to that found in animal membrane systems [10]. The uptake of Cl^- into the TO vesicles as a consequence of the active H^+ transport has also been shown by a filtration technique using $^{36}Cl^-$ [46, 47]. In addition to Cl^-, organic ions like C_4 and C_5 dicarboxylates (Fig. 9), such as malate, fumarate, succinate, α-oxoglutarate, oxaloacetate etc., can be cotransported with protons across the TO [48]. Their uptake has been shown using labelled compounds, such as [^{14}C]succinate [47]. In the presence of citrate no ATP dependent intravesicular acidification could be observed, which is in contrast to results obtained with TO vesicles from *Nitella* [49] or luteoids from *Hevea* [50].

The influx of K^+ into vacuoles and TO vesicles obviously takes place *via* a K^+ specific H^+/K^+ antiporter mechanism at the TO membranes (already

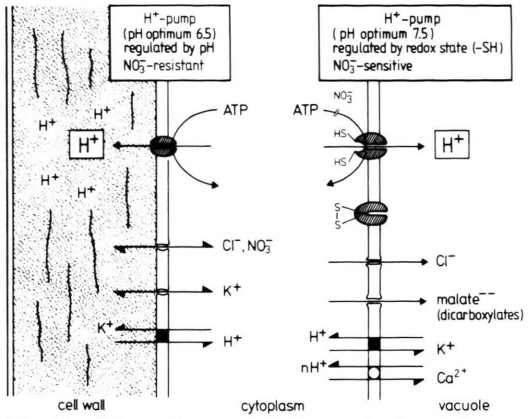


Fig. 18. Schematic presentation of the primary and secondary active ion transport mechanisms at the TO and PL, together with the properties suggested to be important for a regulation of H^+-pumping activity. The H^+/Ca^{2+} antiporter mechanism is described in [31].

proposed by [23] for the PL); as a consequence the intravesicular acidification is smaller in the presence of K^+ compared to Li^+ or Cs^+ (Fig. 8). The K^+ uptake into the vacuoles is a secondary energized mechanism, leading to an ATP dependent accumulation of KCl and K_2-malate (Fig. 18).

The activity of TO H^+ pumps reveal a broad pH optimum with a maximum around pH 7.5 (Fig. 13). At such a pH PL proton pumps are no longer active (Figs. 8–10). Consequently, within the entire unseparated microsomal membrane fraction, at pH 7.5 only the TO H^+ pumps are active and can be studied without further purification [10].

A regulation of TO H^+ pumps possibly proceeds *via* the redox state of specific SH-groups at the enzyme. When these sulfhydryls were blocked by pHMB (Fig. 15) or NEM (Fig. 16) an inhibition of the H^+ pumps resulted. In the case of pHMB this inactivation was abolished by SH-reagents, such as DTT, GSH or Cys (Fig. 15). In addition, the reactive SH-groups of the H^+ pumps were oxidized to disulfides by light in the presence of flavins (Fig. 17). The resulting inactivation can completely be restored by the above mentioned SH-reducing reagents [51]. This light-dependent mechanism of regulation of TO H^+ pumping (Fig. 18) could be an initial step in a series of blue light dependent processes in plants, though no experimental evidence exists till now.

Unlike TO H^+ pumps the PL pumps are insensitive to NO_3^- (Fig. 11). This property can be used to assay PL H^+ ATPases in the presence of TO pumps. As the results show, the isolated PL vesicles are obviously rather permeable to protons, an assumption already made by Perlin and Spanswick [44]. Compared to the ATP hydrolyzing activity the measurable ATP-dependent intravesicular acidification is small. The hydrolyzing activity became, however, only measurable when the vesicles were shocked by a dilution step. Obviously, PL vesicles are originally formed during homogenization mainly inside-in, and thus the hydrolytic site is inaccessible for ATP (Fig. 7). As a consequence of the osmotic shock they partially are inverted, but part of them also seems to form membrane sheets. Consequently, H^+ pump activity can be demonstrated only with sealed inside-out vesicles (Fig. 10). In contrast to the ATP-hydrolyzing activity of PL membranes (Fig. 5) the H^+ pumping activity is scarcely inhibited by vanadate (Fig. 12), an observation also reported by [21]. It is possible that binding of vanadate and ATP occurs at different and opposing sites of the enzyme and that in sealed vesicles, which only exhibit H^+ pumping activity, the vanadate binding site is inside.

Remarkable is the very narrow pH optimum of PL H^+ ATPases exhibiting highest levels of activity at pH 6.5 and with a pronounced increase between pH 7.5 and 6.5 (Fig. 13). *In vivo* this feature seems to be important for a regulation of PL H^+ pumps and the H^+ extrusion into the cell wall compartment; in this respect evidence has been given [52] that all conditions and compounds leading to a decrease of pH in the cytoplasm cause an enhanced rate of H^+ extrusion and elongation growth. Lipophilic esters of acetic acid, which are hydrolized within the cytoplasm, have been shown to be most effective.

Fig. 18 summarizes some properties of the investigated ATP-dependent PL and TO H^+ pumps and the suggested co- and countertransport mechanisms at these membranes.

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