ATP-dependent Proton Transport into Vesicles of Microsomal Membranes of *Zea mays* Coleoptiles

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ATP-dependent proton pumps were found in the vesicles of microsomal membrane fractions of maize coleoptiles. Two membrane fractions isolated by density gradient centrifugation were identified by the aid of marker enzymes and electron microscopic analysis. Membrane fraction A largely consisted of vesicles of smooth ER and of the Golgi complex, fraction B predominantly of vesicles of plasmalemma and rough ER.

The pH-indicator, neutral red, was used to measure changes in pH in the vesicles after ATP addition. Due to the binding of protonated neutral red molecules (NRH^+) to negative charges of the energized membrane, a strong metachromasy of NRH^+-absorption can be observed. Therefore, in order to accurately measure ΔpH a pH-dependent change in absorption of neutral red covering the whole NR-spectrum was set up as difference spectra. The commonly employed method of measuring ΔA of neutral red at just one wavelength (525 nm) leads to entirely incorrect results.

It could be demonstrated that the ATP-dependent translocation of H^+-ions into the interior of the vesicles was most efficient at pH 7. Acidification, which reaches its maximum 10–15 min after ATP addition, can be reverted by adding CCCP.

An ATP-dependent proton-translocation into the vesicles of fraction B was also observed, however, the proton translocation is less than that found in fraction A in relation to the amount of protein found in each.

The membrane fraction A displays a strong oxidation of NADH subsequently followed by an alkalization of the medium. This process cannot be reverted by adding CCCP. NADH oxidation at membranes of fraction A is consequently not an integral part of a redox-pump.

A possible significance of the ATP-dependent proton pump in membranes of the ER and Golgi fraction of coleoptiles is discussed in connection with auxin induced elongation growth.

Introduction

In order to explain many of the physiological processes in plants the activity of an energy dependent proton transport is required [1–4]. While the presence of an H^+-transport in the energy conserving membranes of chloroplasts, mitochondria and bacteria seems to be fact, the presence of an H^+-pump on the remaining membranes of the plant cell is presently debatable. There are, however, a number of facts indicating that ion transport [1, 5–8] as well as the transport of neutral substances such as sugars [9–12] and amino acids [13, 14] are linked with an H^+-movement across the membrane. Even phytochrome-dependent processes are seen in connection with an H^+-excretion [15, 16], light-dependent stomata movement as well [17].

Further it was postulated that elongation growth of plant cells is initiated by an auxin-regulated active excretion of H^+-ions into the cell wall compartment [18], whereby the plasticity of the cell wall is increased thus allowing elongation growth to take place [19–25]. However, it has not yet been demonstrated whether in the above mentioned cases the energy for the H^+-ion transport is supplied by ATP or redox energy.

The presence of ATP-cleaving enzymes on the plasmalemma [26, 27] and on the endoplasmatic reticulum and tonoplast [28] have been detected histochemically (but see also Van Steveninck [29]).

After isolation of cell membranes from various cell organs, mainly from roots, an ATPase activity can also be observed on the plasmalemma [30–35].

Even isolated vacuoles display an ATPase activity [36] and an ATP-dependent sugar uptake [37, 38]. Further, Bowman *et al.* [39] characterized an ATPase on the plasma membrane of *Neurospora* more thoroughly which possibly is connected with the out-

Abbreviations: BSA, bovine serum albumin (defatted); CCCP, carbonylcyanide-*m*-chlorophenylhydrazone; ER, Endoplasmic reticulum; HEPES, N-2-hydroxyethylpiperazine-N′-ethanesulfonic acid; MES, 2-(N-morpholino)ethane sulfonic acid; NR, neutral red; NRH^+, protonated NR.

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ward movement of H^+-ions. All these facts support the presence of an H^+-ATPase.

On the other hand the alternative means of an H^+-transport initiated by redox energy is not excluded [40]. The prerequisites are apparently given, since cytochrome b [41–43] as well as NADH-reductases [43] were found in the microsomal and plasmalemma enriched membrane fractions [41, 42]. O_2 could possibly function as terminal electron acceptor of this electron transport chain starting from NADH possibly functioning together with peroxidases which are found in the cell wall [44].

This present report tries to give an explanation to these questions on the basis of investigations performed with isolated membranes of maize coleoptiles. With the aid of the pH-indicator, neutral red, direct proof of an ATP-dependent proton transport tested on membrane vesicles of an ER- and plasmalemma-enriched membrane fraction is furnished.

Materials and Methods

Plant cultivation: The hybrid corn variety "Inrakorn" category 2a (Deutsche Saatgut AG) was washed overnight in running H_2O and set out on damp cellulose. Seedlings growth took place over a period of 96 h at 26 °C and 90% humidity in the presence of a green safety light. Coleoptile segments 2–3 cm long were used for the experiments after removal of the primary leaf. They were plasmolized by infiltration (0.4 M sucrose, 20 mM Hepes, pH 8). This effected a greater stability of cell organelles such as etioplasts and mitochondria during homogenization. The membrane fraction A (see below) which is usually yellow due to etioplast membrane fragments (carotenoid spectrum) thus stays colorless after plasmolysis.

Membrane isolation: 15–20 g of coleoptiles were placed in a mortar with homogenization medium (2 ml/g coleoptiles) at 2 °C and first cut with a razor blade into small pieces, then ground. The homogenate was pressed through four layers of cheesecloth.

*Homogenization medium: 250 m*M sucrose, 100 mM Hepes, 2 mM EDTA, 2.5 mM dithiothreitol, 1 mM $MgCl_2$, pH 7.5.

The *homogenate* was centrifuged for 15 min at $13000 \times g$ (10500 rpm in the Sorvall Rotor SS 34) in order to remove whole cells, cell wall fragments, nuclei and mitochondria. The supernatant (13 KS) was layered on a two-step sucrose gradient; the *gradient solution* consisted of 1.0 and 1.4 M sucrose, 20 mM Hepes and 1 mM $MgCl_2$ adjusted to pH 7.5 with HCl. The layers from bottom to top were 5 ml solution with 1.4 M sucrose, 10 ml with 1.0 M sucrose and 23 ml of the 13 KS supernatant; centrifugation was carried out for 90 min at $131000 \times g$ (= 27000 rpm, Beckman Rotor SW 27).

The bands A (top) and B (bottom) occurring in the border phases of the gradient solutions were lifted off and treated with 20 mM Hepes, 1 mM $MgCl_2$, 50 mM KCl, pH 7.0 and centrifuged for 30 min at $143000 \times g$ (35000 rpm Beckman-Rotor, typ 35). The pellets of fractions A and B were each taken up in 12 ml *BSA medium* (2% BSA, 1 mM $MgCl_2$, 50 mM KCl, 0.03 mM neutral red) or in *Hepes medium* (20 mM Hepes, 0.1% BSA, 1 mM $MgCl_2$, 50 mM KCl, 0.03 mM NR) per 20 g coleoptile fresh weight and suspended with a homogenizer (Potter-Elvehjen).

Fraction A contains an average of 0.27 mg protein/ml, fraction B an average of 0.1 mg protein/ml.

The *membrane fractions* were tested (Hager, Haschke, and Weigel, in preparation) for the usual marker enzymes [45]: NAD(P)H-cytochrome c reductase [46], carotenoids, cytochrome c oxidase [47], succinate dehydrogenase and malate dehydrogenase [48], glucan synthetase I and II [49, 50], latent IDPase [51], K^+-ATPase [33, 52].

The membranes of fraction A (Fig. 1 A) are primarily smooth ER and Golgi vesicles, those of fraction B (Fig. 1 B) are plasmalemma and rough ER. No cytochrome c oxidase activity could be detected.

Difference spectra: The absorption and difference spectra were obtained in a double beam/difference/dual wavelength recording spectrophotometer UV-300 made by the Shimadzu Company. The solutions in the cuvettes (3 ml, optical path 1 cm) could be stirred with additionally installed magnetic stirrers. The membrane suspension and neutral red concentrations in the sample cuvette and the reference cuvette were identical when obtaining difference spectra. The reaction was initiated with the simultaneous addition of ATP to the sample cuvette and ADP or AMP to the reference cuvette and ended with the simultaneous addition of CCCP.

Electron microscopy: Glutaraldehyd/OsO_4 was used for fixation of the membranes.

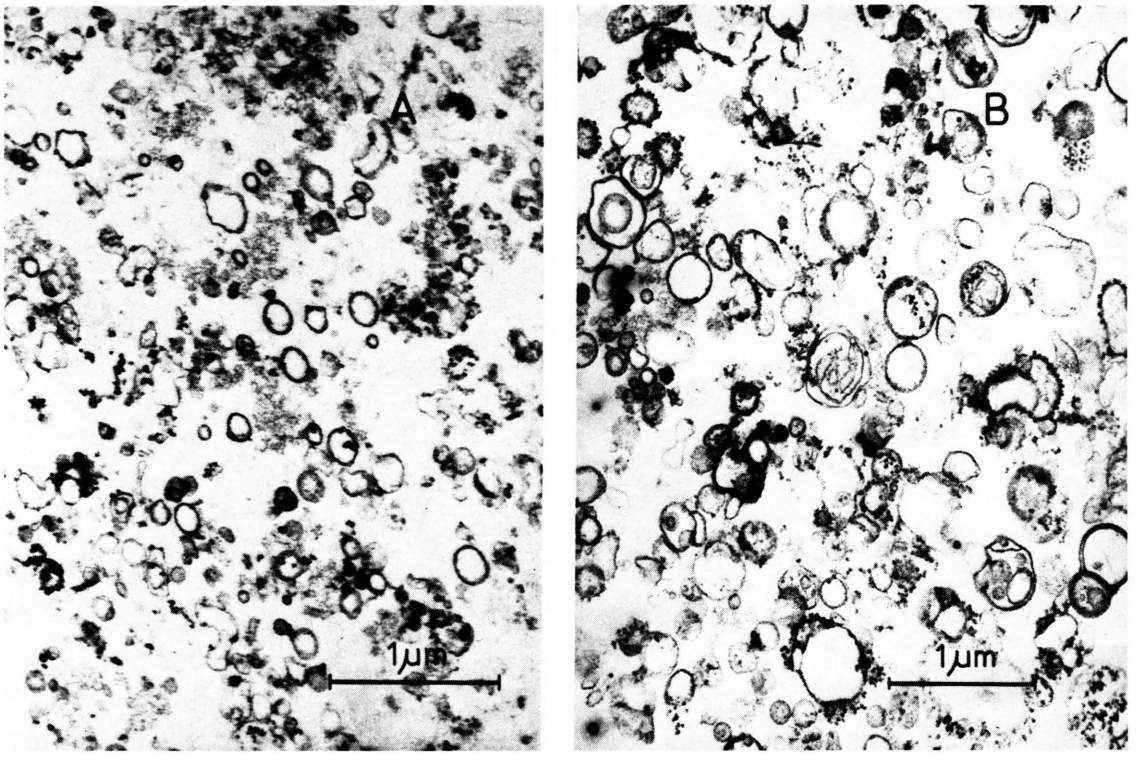

Fig. 1. Electron microscopy of membrane fraction A and B.

Results

Change in the absorption of the pH-indicator NR due to the concentration, pH-value and negative charge

In order to measure a change in pH in the compartments of plant organelles and restituted membrane vesicles neutral red is commonly used [53–59, 67].

Neutral red or toluylene red (=3-amino-7-dimethylamino-2-methylphenazine) is a weak acid with an apparent pK of 6.8. According to the pH-value the steady state between the protonized and non-protonized form in watery solution follows the law of mass and action (Fig. 2). When using difference spectra (point of reference pH 7.5) a change in the concentration of the protonized (λ 525 nm) and the non-protonized (450 nm) NR molecules due to a change in pH of the solution becomes very apparent (Fig. 3).

In the presence of agar as an example of a polyanion the maximum point of absorption of protonized NR shifts from 525 nm to 490 nm (Fig. 4). Even though no change in pH has taken place the light absorption of NR at 525 nm decreases nearly 50%. Since an increase in negative charge of the membrane can be observed when energized through light or ATP [55] a simple binding of NRH^+ can mislead to assuming a change in pH.

The metachromasy illustrated in Fig. 4 is observable in the curve of the difference spectrum (Fig. 5) as often observed in experiments with membrane vesicles (Fig. 10). Contrarily, the absorption (in the wavelength range around 425 nm) of non-protonized NR-molecules is not influenced by negative charge.

A comparison between the binding capacity of non-protonized and protonized NR-molecules to microsomal membranes (Fig. 6) shows that the non-protonized NR-molecules (at pH 8) are inspite of their higher lipophility less strongly bound to the membrane than the chiefly charged NR-molecules at pH 6 (in this experiment, the membrane suspension of fraction A was centrifuged at 143000×*g* for 30 min after addition of 0.03 mM NR and subsequently the concentration of NR in the supernatant measured).

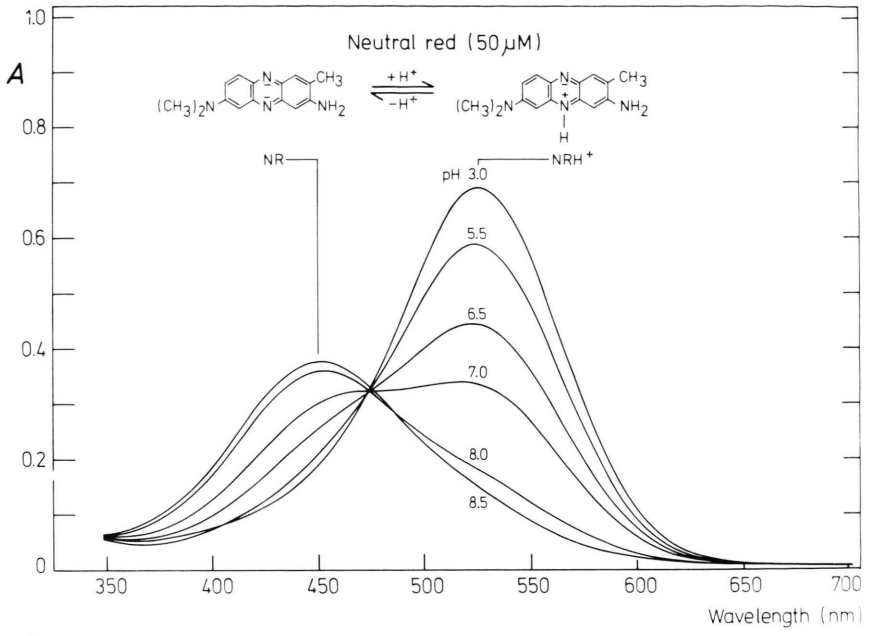

Fig. 2. Absorption spectra of neutral red solutions with different pH. d = 1 cm.

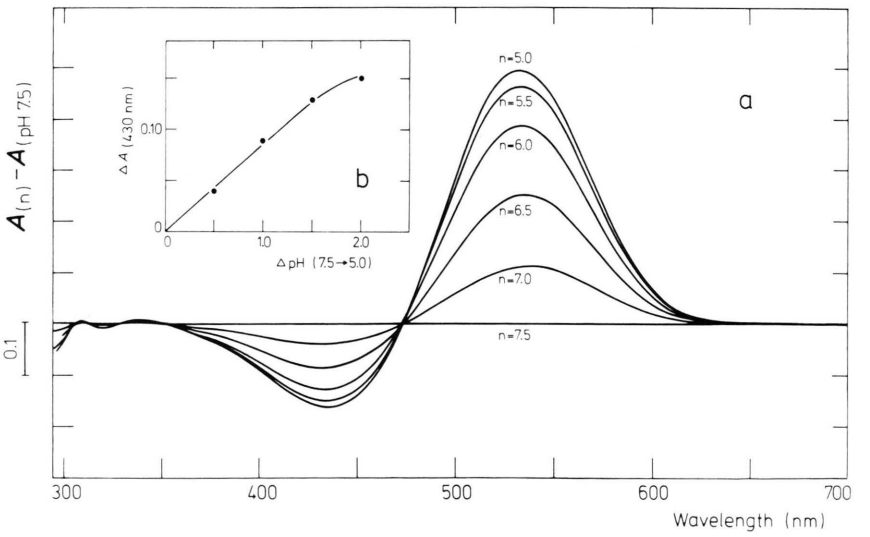

Fig. 3. a) Difference spectra of neutral red solutions (0.03 mM NR, 0.1% BSA, 10 mM Hepes) with different pH against a solution with pH 7.5 (reference). b) Relationship between the change in absorption of the difference spectra at 430 nm ($\Delta A_{430\,nm}$) and the pH-value of the NR-solutions.

From the above mentioned observations it becomes apparent that a fairly accurate evaluation of pH change with the help of NR can only be assured if the whole neutral red spectrum is used as comparative basis. Under certain circumstances a change in absorption (between 420 and 450 nm) of non-protonized neutral red molecules can be used to indicate a change in pH. However, at this wavelength the absorption of CCCP disturbs ($\lambda < 450$ nm), given that it is added in the experiment.

ATP-dependent proton transport into the membrane vesicles of membrane fraction A of maize coleoptiles; proof with the aid of the pH-indicator NR

In analogy to the findings in chloroplasts and mitochondria only on the outer side of a closed membrane vesicle can an ATP hydrolysis lead to an increase of the proton concentration in the interior of the vesicle. Likewise the same is to be expected if NADH functioning as primary electron donator of a

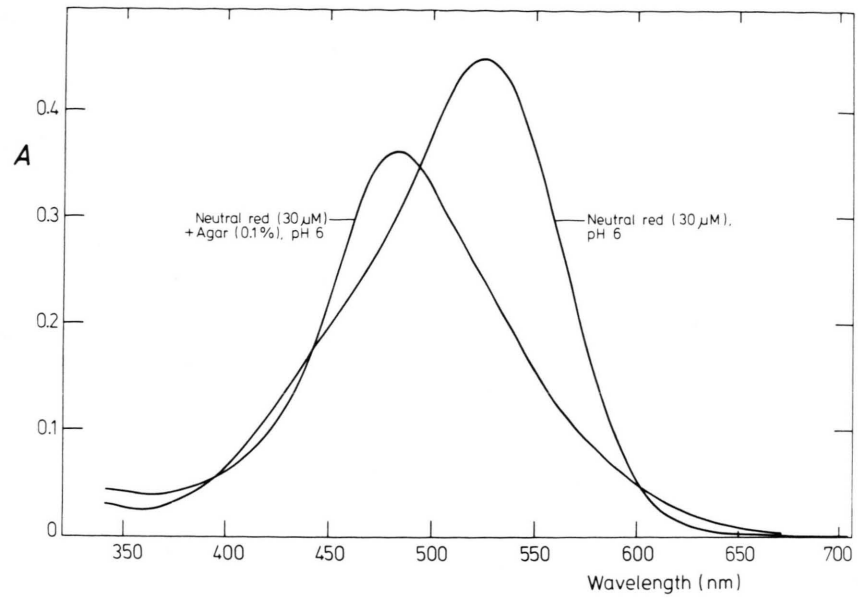

Fig. 4. Metachromatic shift of the absorption maximum of NR with pH 6 due to polyanions (0.1% Agar). The decrease in absorption at 525 nm simulates an alkalization of the medium.

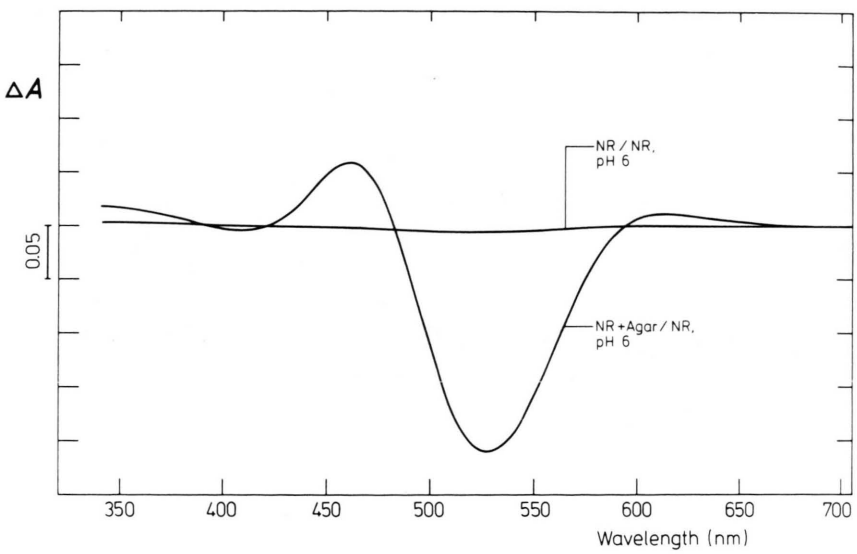

Fig. 5. Difference spectrum of the absorption of NR (10 mM MES, pH 6) in the presence and absence of 0.1% Agar.

redox pump is oxidized on the outer side of a vesicle.

If vesicles are present, in which hydrolysis of ATP or NADH can only take place on the inner side of the membrane, then no reaction is observed, since these substrates normally cannot cross membranes or if in the presence of translocators the complimentary substrates are missing.

In order to measure a change in pH one must further take into consideration that an acidification of the membrane suspension caused by the activity of ATPases in open membrane fragments can be accounted for on the basis of the following reaction:

$$ATP^{4-} + H_2O \rightleftharpoons ADP^{3-} + P_i^{2-} + H^+.$$

A prerequisite for proving an active proton secretion into the interior of the membrane vesicle is thus to intercept acidification outside of the vesicle. Unfortunately the membranes are partially permeable for nearly all eligible buffers in question [54]; thus it

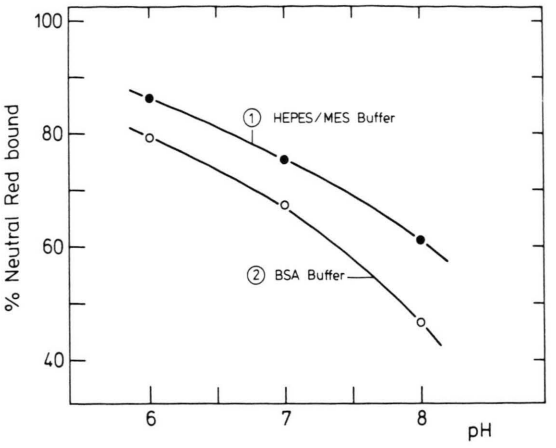

Fig. 6. pH-dependence of the binding of NR (0.03 mM) to membranes of fraction A. 1) 20 mM HEPES/MES, 1 mM $MgCl_2$, 50 mM KCl; 2) 2% BSA, 1 mM $MgCl_2$, 50 mM KCl.

could lead to a partial masking of a change in pH in the interior of the membranous vesicles caused by an active proton pump. Therefore, BSA was additionally used as non-permeable buffer in the experiments [53, 54]. Because of the low buffer capacity of BSA small changes in pH outside of the vesicles have to be put up with.

In Figs. 7 to 10 the change in pH in the membrane vesicles of fraction A (consisting mainly of smooth ER and Golgi-vesicles) is illustrated as a function of a change in absorption of the pH-indicator NR. The indicator, which can cross the membrane as an uncharged, lipophil molecule, is protonized in the vesicle if acidification caused by an active proton pump takes place.

When the membrane vesicles are kept in a buffer at pH 8 (Fig. 7) and ATP is added, an increase in the absorption at 525 nm (increase of NRH^+) in the difference spectrum can be noticed in comparison with the ADP control and also a decrease at 450 nm (decrease of NR). If the pH of the membrane suspension is initially lower than 8, *e.g.* 7.5 (Fig. 8) or 6.8 (Fig. 9), then the number of charged NRH^+ molecules is higher from the start (pK = 6.8); an increase in number caused by the activity of a proton pump inside the vesicles, also increases the portion of bound NRH^+ molecules. This leads to metachromasy and can even be observed in the difference spectrum in which a new absorption peak at 490 nm (Fig. 9) emerges.

After addition of CCCP the ATP-induced change in pH in the vesicles is reversed. The outflow of H^+ from the vesicles increases the pH-value there, NRH^+ molecules are deprotonized and thus become lipophil. They flow out of the vesicle until a new steady state is established; the concentration within the vesicles and thus also the number of NRH^+-bound to the membrane decreases; the absorption peak shifts from 480 nm back to the range around 525 nm and the amplitude decreases as well (Fig. 9).

The peak at 525 nm usually does not disappear completely; this signifies that in the cuvette containing ATP a slight acidification has taken place, the ADP control cuvette being point of reference; this slight acidification is due to H^+-ions released

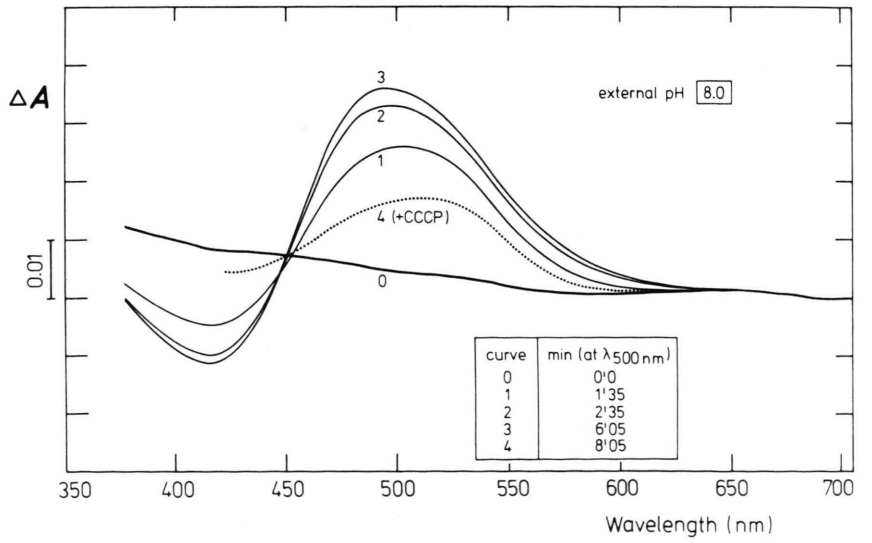

Fig. 7. ATP-dependent decrease in pH in membrane vesicles of fraction A, in an environmental pH of 8. Curves 0–4: Difference spectra of neutral red absorption. Curve 0: Sample and reference cuvettes with equal contents (membranes, 0.03 mM NR, HEPES medium; curve 1: 2 min after addition of 1 mM ATP to the sample and 1 mM ADP to the reference cuvette; curve 4: addition of CCCP (50 μM) 8 min after the beginning of the reaction.

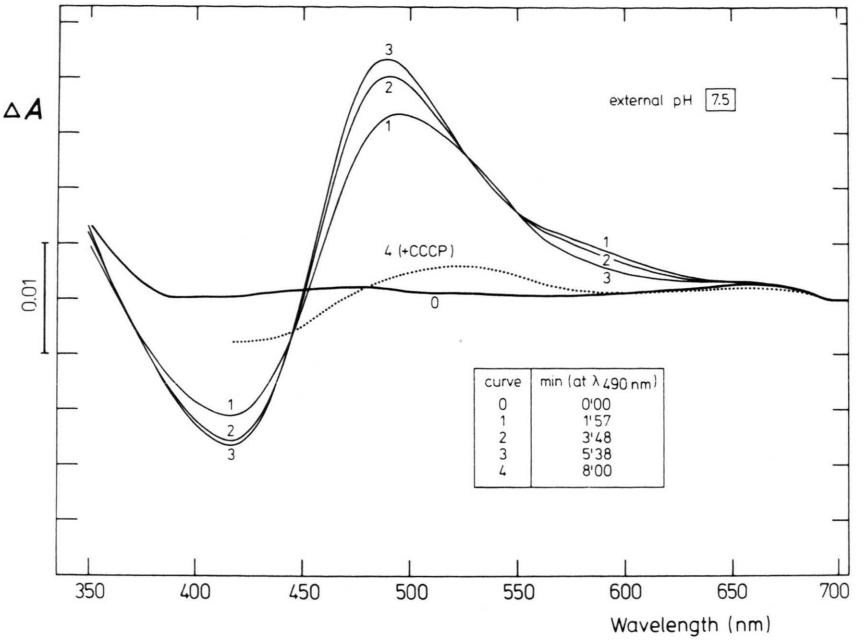

Fig. 8. ATP-dependent decrease in pH in membrane vesicles of fraction A in an surrounding pH of 7.5. Curves 0–4: Difference spectra of neutral red absorption. Curve 0: sample and reference cuvettes equal (membranes, 0.03 mM NR, HEPES medium); curve 1: 1 min, 57 sec after addition of 1 mM ATP to the sample and 1 mM ADP to the reference cuvette. Curve 4: addition of 50 µM CCCP 8 min after the beginning of the reaction. At this environmental pH a metachromatic shift of the NRH^+ absorption from 525 nm to 490 nm due to the binding of NRH^+ to the membranes is visible.

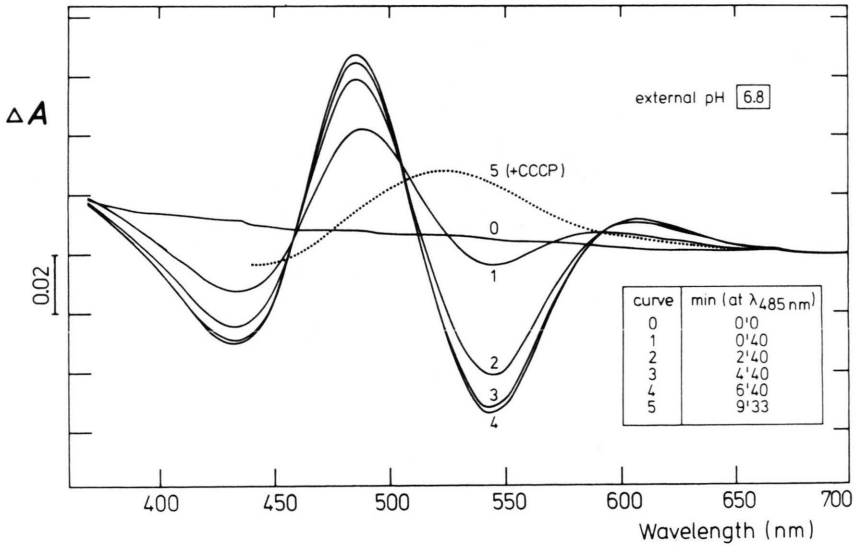

Fig. 9. ATP-dependent decrease in pH in membrane vesicles of fraction A in an environmental pH of 6.8. Curves 0–5: Difference spectra of neutral red absorption. Curve 0: sample and reference cuvettes equal (membranes 0.03 mM NR, HEPES medium). Curve 1: 40 sec after addition of 1 mM ATP to the sample and 1 mM ADP to the reference cuvette. Curve 5: addition of 50 µM CCCP to both cuvettes 9 min 33 sec after the beginning of the reaction. The absorption maxima at 480 nm show a strong binding of NRH^+ to negative charges of the membranes.

when ATP-hydrolysis takes place and which cannot be counter balanced by the low buffer capacity.

These experiments prove that asserting a change in pH by only measuring a single wavelength (ΔA 525 nm of the neutral red spectrum) as normally done, leads to entirely faulty results due to the strong metachromasy in this wavelength range. The most reliable means of measuring a change in pH is to use a change in absorption at 420 nm (non protonized NR-molecule). Because of the intrinsic absorption of CCCP, a protonophoric substance ($\lambda < 450$ nm) which is added to reverse acidification in the interior of the vesicles, it is necessary to check the absorption above 450 nm.

Fig. 11 illustrates the decrease in pH (ΔA 420 nm) in the vesicles after addition of ATP at different

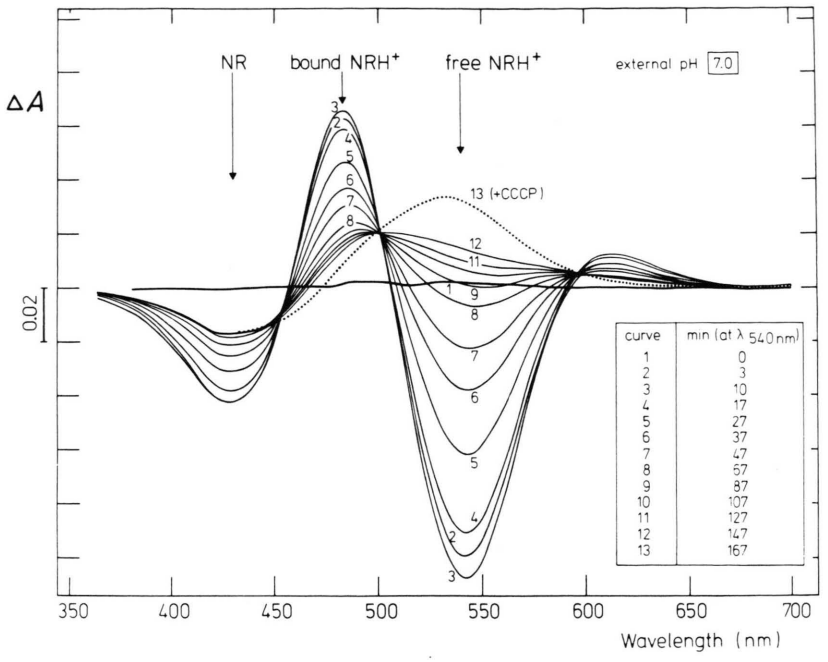

Fig. 10. ATP dependent change in pH in membrane vesicles of fraction A in an environmental pH 7.0 in relation to time. Curves 1–13: Difference spectra of NR absorption. Curve 1: sample and reference cuvettes equal (membrane vesicles 0.03 mM NR, BSA medium); curve 2: 3 min after addition of 1 mM ATP to the sample and 1 mM ADP to the reference cuvettes; curve 3: 10 min after; maximum acidification in the vesicles; curve 4 and following curves: destabilization of the vesicles and regression of acidification; curve 13: addition of CCCP (50 μM), 167 min after the beginning of the reaction.

environmental pH-values. The best pumping efficiency of H^+-ATPases is present at an environmental pH around 7. At pH-values under 7 the vesicles seem to become increasingly unstable, even though the pH-optimum of ATPase activity in microsomal fractions lies between 6.0 and 6.5 [34]. From pH-values won by these means one cannot, however, make any inferences as to the efficiency of the H^+-pump or the actual acidification *in vivo*, since the buffer capacity in the interior of the vesicle is unknown as well as what portion of the whole membrane fraction can be said to be vesicles.

Further it is necessary to point out that it is very plausible that due to the strong ATP-induced acidification in the interior of the vesicles the membranes become instable and burst after about 10–15 min. The pH-induced changes in the NR absorption regress (Fig. 10). Therefore, in the experiments the reaction was stopped earlier by addition of CCCP.

Proof of oxidation of NADH on membranes of fraction A of maize coleoptiles using the pH-indicator NR

The presence of redox substances (cytochrome b) and NADH oxidases in microsomal fractions of plants (see introduction) leads to postulating that an active H^+-transport could possibly be driven by redox energy (NADH). It should be possible to prove the presence of such redox pumps by similar means as for detecting the presence of ATP-dependent pumps on membrane vesicles. The difference spectra of NR absorption (Fig. 12) demonstrate

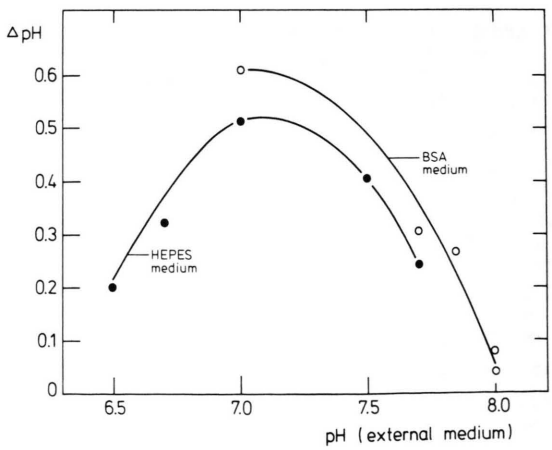

Fig. 11. Dependence of the ATP-caused pH decrease in the membrane vesicles of fraction A on the environmental pH. ΔpH was calculated from difference spectra (see Fig. 4). 1: Membranes in BSA medium; 2: membranes in HEPES medium.

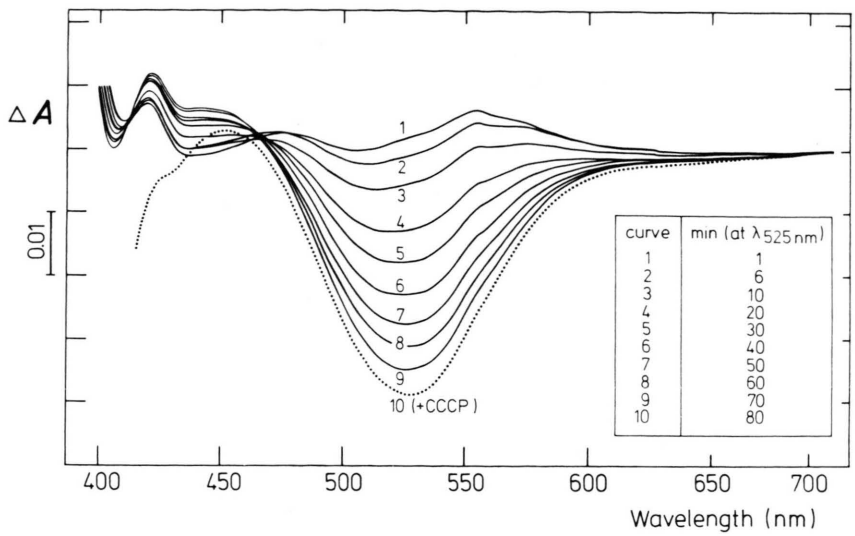

Fig. 12. Time dependent NADH induced pH increase in membrane fraction A. Curves 1–10: Difference spectra of NR absorption. Curve 1: Sample and reference cuvettes equal (membrane vesicles, 0.03 mM NR, BSA medium); curve 2: 6 min after addition of 1 mM NADH to the sample and 1 mM NAD to the reference cuvette; curve 3: 10 min after; curve 10: addition of 90 μM CCCP to both cuvettes 80 min after the beginning of the reaction.

that an alkalization of the medium takes place after addition of NADH to the membrane fraction A (reference cuvette: NAD). In this case it cannot be the result of an H^+-transport into the vesicles, since the increase in pH is not reversed after addition of CCCP, instead it even increases. The alkalization can be ascribed to an oxidation of NADH and transfer of hydrogen to an acceptor (A) on the surface of the membrane:

$$NADH + H^+ + A \rightarrow NAD^+ + AH_2.$$

These results do not support the thesis that a proton pump driven by redox energy is present on the vesicles of the membrane fraction A.

The question concerning the function of this NADH-oxidizing enzyme remains open. It is plausible, however, that it has something to do with mixed-function oxidases (mono-oxygenases) or dioxygenases engaged in the hydroxylation or cleavage of metabolites [60].

Discussion

The presence of ATP-dependent proton pumps in vesicles of an ER-enriched membrane fraction raises the question as to their function. To begin with it is plausible that the H^+-ATPases are of importance in the transport of substances into the ER and the ER-derived vesicles; according to general opinion [61] cell wall precursors are brought into the cell wall compartment by way of vesicle transport. Furthermore the H^+-ions accumulated in such vesicles could effect an increase in plasticity of the cell wall and thereby initiate elongation growth [18, 62, 63]. These vesicles would thus have a double function: acidification and the resulting increase in cell wall plasticity, and the supply of fresh cell wall material (Ray developed similar ideas [64]).

In such a case one would of course have to require that the activity of these proton pumps be controllable with auxin. It is remarkable in this context that the auxine receptor with the highest affinity was found on the ER [64–66].

If one assumes that auxin controls ATP-dependent acidification of vesicles, and subsequent transport to the plasmalemma, then several phenomena become explicable:

a) The lag phase of about 10 min, at 30 °C, in the effect of auxin, could be due to the duration of vesicle transport to the plasmalemma.

b) The fusion of the vesicle membrane with the plasmalemma increases the surface area of the latter; this would explain the simultaneous growth of the plasmalemma during elongation growth; acid-induced elongation growth, on the other hand, which only lasts 1–2 h, would be limited by a plasmalemma no longer expandible and capable of area increase.

c) The dependence of elongation growth on protein synthesis, *i. e.* its inhibition by cycloheximid [20, 21] would become understandable, since constant

synthesis of new membrane material is necessary for vesicle formation (even a constant synthesis and decomposition of the auxin receptor would also explain the sensitivity of growth processes to cycloheximid).

The auxin-dependent proton pumps postulated in 1971 [18] would thus be located on the ER (or on Golgi vesicles). However, an ATP-dependent proton transport is also found in membrane fraction B even if it is less than that in fraction A relative to protein content. This could mean, that the measurable pumping effect of an ATP-dependent plasmalemma proton pump is impeded by less prolific or incomplete vesicle formation.

Acknowledgements

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