# A New Method to Prepare Membrane Fractions Containing Ionophore-Stimulated ATPase from Pumpkin Hypocotyls (*Cucurbita maxima*, L.)

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In membrane fractions from pumpkin hypocotyls ATPase activity was stimulated by a combination of CCCP (carbonyl cyanide *m*-chlorophenylhydrazone), a protonophore, and valinomycin, a $K^+$-ionophore. Singly, these ionophores stimulated ATPase activity much less. Nigericin, an $H^+/K^+$-antiporter, and nystatin, a cation pore, had similar effects as the combination of CCCP and valinomycin. The results suggest the presence of a cation-translocating ATPase which is stimulated by ionophores by dissipating cation gradients formed in the vesicles. A major part of the ionophore-stimulated ATPase activity correlated with marker enzymes for plasma membranes but part of it could be located in other compartments.

## Introduction

*In vitro* evidence for a proton-translocating ATPase in higher plants has been only recently presented [1–4]. Hager *et al.* [1980] demonstrated ATP-dependent $H^+$-transport in microsomes from corn coleoptiles with a pH indicating dye and Sze [1980] showed that CCCP and valinomycin singly stimulated $K^+$-ATPase only slightly but the combination of both ionophores stimulated $K^+$-ATPase significantly. Both groups postulate an $K^+$-ATPase which, in principle, might be related to the *in vitro* hormone-sensitive ATPase [5, 6]. It is demonstrated here that membranes prepared with a totally different method contain an ionophore-stimulated ATPase and therefore must at least partially consist of ion-impermeable vesicles. In addition to this, a correlation with marker enzymes was conducted.

## Materials and Methods

*Plant material.* Pumpkin seeds ("Gelber Zentner") were surface sterilized and grown for four days at 30 °C on moist cotton in the dark. About 1 cm long hypocotyl hooks were harvested.

*Chemicals.* Chemicals were of the highest grade commercially available. Nupercaine was a gift from Hoffmann-La Roche.

*Membrane preparation.* Membranes were prepared as described [6]. After separation of membranes from soluble proteins by Sepharose 2B-CL chromatography and overnight centrifugation on a linear sucrose gradient, the fractions were chromatographed on small (1 × 30 cm) Sephadex G50 columns in 10 mM 2-(N-morpholino)ethanesulfonic acid (=MES)/piperidine pH 6.5 containing 20 mM KCl. These fractions were tested for ATPase and marker enzyme activities. All enzyme activity calculations were based on protein content prior to Sephadex chromatography. Marker enzyme tests were done with the modifications described in [6]. In particular the methods were: Glucan synthetase I and II [7]; NADH-cytochrome c reductase (antimycin A-insensitive) and cytochrome c oxidase [8]; UDPG-sterol-glucosyltransferase [9]; α-mannosidase [10]. Quantitative determinations were done for carotenoids in the total lipid extract [11] and protein [12].

*ATPase assay.* ATPase activity was assayed for 30 min at 30 °C in a total volume of 0.25 ml of 30 mM MES/piperidine pH 6.0 or MOPS/piperidine pH 8.0, 3 mM ATP (piperidine salt), 3 mM $MgCl_2$, 20 mM or 50 mM KCl and ionophores at a final ethanol concentration of 0.4%. Ionophore-stimulated ATPase activity was determined as the difference between ATPase activity in the presence and absence of ionophore. Inorganic phosphate was determined as described [5].

## Results and Discussion

The stimulation of ATPase activity in the presence of 20 mM KCl by ionophores is shown in Fig. 1B. Valinomycin and CCCP alone stimulated ATPase activity only in some fractions whereas the combination of both ionophores caused a marked stimulation in all fractions. Since the ionophores could dissipate cation gradients originating from the action of an ATP-driven ion pump in partially ion-impermeable vesicles, these results suggest the presence of an ATPase-mediated, coupled movement of $H^+$ and $K^+$ ions. This view is further supported by the very similar stimulations obtained with nigericin, an $H^+/K^+$-exchange ionophore, and with nystatin, a pore for cations.

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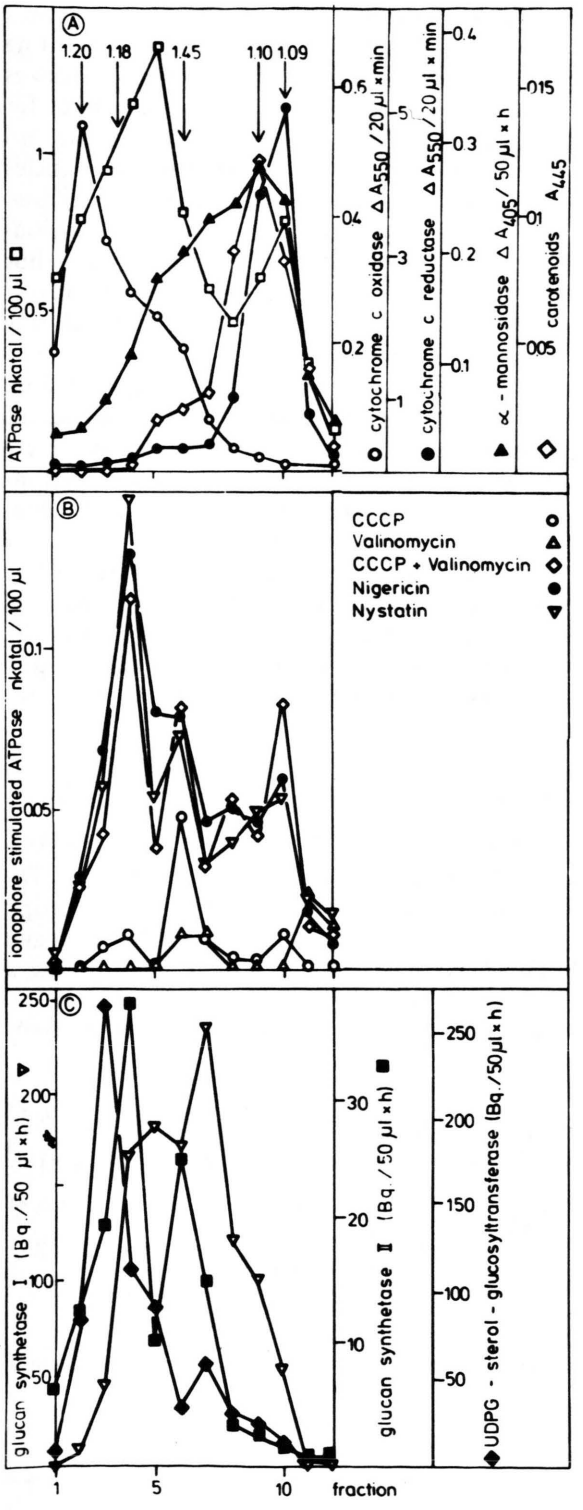


The stimulation of about 10% in this experiment represent typical values for ionophore stimulation which ranged from 5% to 20% (in a single instance 30%) in many experiments. The duplicate values for background ATPase activity and ATPase activity in the presence of ionophores (*e.g.* nigericin) were clearly separated. The basal ATPase activity is likely the sum of several enzyme activities. Nonspecific acid phosphatase activity was 90% removed by gel-chromatography and the remaining 10% was closely correlated with the activity profile for α-mannosidase. Other ATP hydrolysing enzymes, for example apyrase, have been found in plant membranes [13] and may as well contribute to basal ATPase activity.

The great advantage of membrane preparation in choline/ethanolamine buffers [6] is that the ionophore-stimulated ATPase activity can be readily correlated with marker enzymes. The rather selective enrichment procedure for ion-impermeable vesicles employed by Sze [3] yields one fraction which has not been characterized except for the absence of mitochondrial markers. The bulk of the ionophore-stimulated ATPase was found at the density of glucan synthetase II and UDPG-sterol-glucosyl-transferase, both markers for plasma membranes (Fig. 1B and 1C). Clearly, the profile for cytochrome c oxidase, a mitochondrial marker, did not correlate with the ionophore-stimulated ATPase activity in agreement with other observations [3].

This was further substantiated by the experiment shown in Fig. 2 where the inhibition of ATPase of mitochondrial origin by oligomycin was compared to the profile of cytochrome c oxidase and to the profile of nigericin-stimulated ATPase. With a shorter centrifugation time in this experiment it be-

Fig. 1. Gradient profile of the ionophore-stimulated ATPase and of marker enzymes.
(A) □ $Mg^{2+}$-ATPase; ○ cytochrome c oxidase (mitochondria); ● antimycin A-insensitive NADH-cytochrome c reductase (ER); ▲ α-mannosidase (presumptive for vacuoles); ◇ carotenoids (broken etioplasts); arrows: density ($g \times cm^{-3}$).
(B) ○ 2 µM CCCP; △ 10 µM valinomycin; ◇ 2 µM CCCP + 10 µM valinomycin; ● 2 µM nigericin; △ 156 µg/ml nystatin.
(C) ▷ glucan synthetase I (Golgi membranes); ■ glucan synthetase II (plasma membranes); ◆ UDPG-sterol-glucosyltransferase (plasma membranes).
The values for the ATPase activity are an average of duplicate determinations except for ATPase activity in the presence of nystatin (single assay). With the exception of fraction 1 the duplicate values did not overlap.

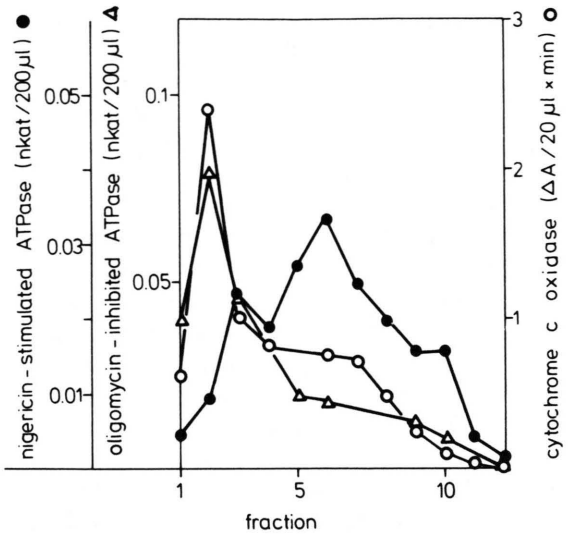


Fig. 2. Gradient profile of oligomycin-inhibited ATPase and nigericin-stimulated ATPase after 5 h centrifugation. Cytochrome c oxidase ○ (mitochondria); oligomycin-inhibited ATPase △ (measured at pH 8.0 as the difference of the control ATPase activity minus the ATPase activity in the presence of 5 µg/ml oligomycin); nigericin-stimulated ATPase ● (measured as ATPase activity in the presence of 10 µM nigericin minus the control ATPase activity at pH 6.0). The gradient profiles of basal ATPase activity were measured in the presence of 50 mM KCl at both pH 8.0 and 6.0 and were not identical. At pH 8.0 nigericin alone had no effect, and oligomycin plus nigericin had the same effect as oligomycin alone (not shown).

came more evident that the oligomycin effect was correlated with the mitochondrial marker but not with the nigericin-stimulated ATPase.

Fractions of lower density, however, still possessed a substantial amount of ionophore-stimulated ATPase activity which cannot be attributed to plasma membranes. An electrogenic, cation-translocating ATPase has been demonstrated in isolated vacuoles [14, 15] and α-mannosidase, a possible marker for vacuolar vesicles [10], had a high activity in fractions of low density (Fig. 1A) so that vacuolar vesicles might have an ionophore-stimulated ATPase. But, other compartments cannot be excluded as contributors to ionophore-stimulated ATPase activity. Thus it seems premature to assign ionophore-stimulated ATPase to plasma membranes alone [3] which was also questioned by others [1, 2].

A crucial aspect of the experiments reported here is the synergistic stimulation of the combination of valinomycin and CCCP as compared to the singly applied ionophores (Fig. 1B). Even though one cannot postulate a mechanism for the apparently coupled, ATP-driven movement of $H^+$- and $K^+$-ions, it is difficult to deny the possibility that a part of the ATPase activity is an $H^+$-ATPase regardless of mechanism or stoichiometry. The properties of the ionophore-stimulated ATPase and its relationship to the auxin-stimulated ATPase are currently being investigated.

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