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# Effect of detergents on the $H^+$-ATPase activity of inside-out and right-side-out plant plasma membrane vesicles

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**In search for a detergent to be used to assess the sidedness of plant plasma membrane vesicles by enzyme latency we tested the effect of 42 detergents on the ATPase activity of right-side-out and inside-out plasma membrane vesicles from sugar beet leaves. Most of the detergents seemed to inactivate the ATPase in addition to disrupting the permeability barrier to ATP. There were two main exceptions, namely long chain polyoxyethylene acyl ethers, such as detergents of the Brij series and Lubrol WX, and long chain lysophospholipids. These two types of detergents permeabilized the membranes at low concentrations and did not inhibit the ATPase at higher concentrations. Unmasking of latent active sites seemed to explain the activation of the plasma membrane $H^+$-ATPase produced by long chain polyoxyethylene acyl ethers. These detergents should therefore be ideal for determination of vesicle orientation based on ATPase latency. By contrast, long chain lysophospholipids were found to be highly specific activators of the enzyme. In addition, long chain fatty acids were found to strongly inhibit ATP-dependent proton accumulation in the vesicles without inhibiting ATP hydrolysis. This uncoupling effect of the fatty acids could be abolished by the addition of fatty acid-free bovine serum albumin (BSA). Similarly, the proton transport capacity of ageing vesicles could be restored by addition of BSA. The latter findings may explain why isolated plasma membranes so often exhibit increased permeability to protons on ageing.**

## Introduction

Due to the impermeability of membrane vesicles to hydrophilic substrates, many membrane-bound enzymes fail to display activity in vitro as long as the membrane surrounding them is intact. The degree of inaccessability for the enzyme towards its substrate, the latency of the enzyme, is used as a measure of the sidedness of the membrane vesicles. In order to expose all substrate binding sites in the membrane preparation, detergents were introduced early as a mean to disrupt the permability barrier to the substrate [1]. The usual interpretation of the effects of detergents on membrane enzymes consider only this unmasking of latent active sites. However, detergents may also have other effects, such as stimulating or inhibiting the activity of the enzyme studied [2].

The latency of the plant plasma membrane $H^+$-ATPase can be used to assess plasma membrane vesicle sidedness since the active site of the enzyme is located on the cytoplasmic surface of the membrane [3]. Commonly used detergents in such latency studies are Triton X-100 [4] and lyso-PC [5]. However, we have recently shown that lyso-PC functions as an activator of the $H^+$-ATPase [6,7], and Triton X-100 may act as an inhibitor of the enzyme in addition to opening the vesicles [4,5].

It has now become possible to isolate inside-out plasma membrane vesicles from plants [8]. In such a latency free system the stimulatory or inhibitory effects of detergents are more easily tested. We first screened the effect of several detergents on the plasma membrane $H^+$-ATPase activity and found that long chain lysophospolipids were highly specific activators of this enzyme in agreement with earlier results [6,7]. All other detergents inhibited the ATPase activity in addition to

Abbreviations: BSA, bovine serum albumin; BTP, 1,3-bis[tris(hydroxymethyl)methylamino]propane; lyso-PC, lysophosphatidylcholine; lyso-PA, lysophosphatidic acid; Mops, 4-morpholinepropanesulphonic acid.

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revealing latent activity; the only exception were long chain polyoxyethylene acyl ethers, such as detergents in the Brij series. The latter detergents seem to be very suitable for latency studies of the plant plasma membrane $H^+$-ATPase, since they permeabilized the membrane at low concentrations and did not inhibit the ATPase activity at high concentrations. Nor did they seem to stimulate the ATPase by any other mechanism than to permeabilize the vesicles. Thus, these detergents should be ideal for determination of vesicle orientation.

## Materials and Methods

*Chemicals.* Labelled L-lyso-PC, 1-[1-$^{14}$C]palmitoyl was from Amersham. Triton X-100, lactate dehydrogenase (127 221, solution in glycerol), pyruvate kinase (109 045, solution in glycerol), NADH, and phospho*enol*pyruvate were from Boehringer. Bovine serum albumin (BSA, proteinase-free), BSA (essentially fatty acid and globulin-free), casein (enzymatic hydrolysate, type I), fatty acids, and lysophospholipids were from Sigma. Zwittergents were obtained from Calbiochem, and detergents of the Brij series were from Aldrich and Sigma. All other chemicals were of highest commercially available grade. Detergents were dissolved in 48% (w/v) ethanol, 10 mM Mops (4-morpholinepropanesulphonic acid)-BTP (1,3-bis[tris(hydroxymethyl)methylamino]propane) (pH 7.0), 1 mM dithiothreitol, 1 mM EDTA-BTP, and were stored at −20°C. In the case of fatty acids and lyso-PA, additional 15 mM BTP was added. Prior to use, detergents were diluted with 10 mM Mops-BTP (pH 7.0), 1 mM dithiothreitol, 1 mM EDTA-BTP, and were added as 50 μl.

*Plant material.* Sugar beet plants (*Beta vulgaris* L.) were kindly supplied by Hilleshög AB, Sweden.

*Right-side-out plasma membrane vesicles.* Plasma membranes consisting predominantly of right-side-out vesicles were purified from a microsomal fraction (10 000–50 000 × *g* pellet) of sugar beet leaves in an aqueous polymer two-phase system [9,10] with minor modifications. The homogenization medium was essentially as in [7] and contained 50 mM Mops-BTP (pH 7.5), 330 mM sucrose, 5 mM EDTA-BTP, 5 mM dithiothreitol, 1 mM phenylmethylsulphonyl fluoride, 0.2% (w/v) BSA (proteinase-free), 0.2% (w/v) casein (boiled for 10 min). The membranes were diluted in 5 volumes of 330 mM sucrose, 10 mM Mops-BTP (pH 7.0), 2 mM dithiothreitol, 5 mM EDTA-BTP, and were pelleted and resuspended to about 15 mg/ml protein in a buffer of the same composition. The membranes were used immediately for assays.

*Inside-out plasma membrane vesicles.* The highly pure plasma membrane preparation obtained by two-phase partitioning was subfractionated essentially as described in Ref. 8. In brief, the plasma membranes were diluted in 330 mM sucrose, 50 mM KCl, 2 mM dithiothreitol, 0.1 mM EDTA-BTP, 5 mM potassium phosphate (pH 7.8) to about 15 mg/ml protein. After freezing in liquid $N_2$ the membranes were allowed to thaw in a waterbath at room temperature. Freezing and thawing was repeated three times and the plasma membranes were added as 2.4 ml to a 21.6 g phase mixture to give a 24.0 g phase system with a final composition of 6.2% (w/w) Dextran T500, 6.2% (w/w) polyethylene glycol 3350, 330 mM sucrose, 5 mM KCl, 0.1 mM EDTA-BTP, 1 mM dithiothreitol, 5 mM potassium phosphate (pH 7.8). The phase system was shaken and spun for 5 min at 1500 × *g* (swinging bucket rotor) to facilitate phase separation. About 90% of the upper phase was removed and discarded without disturbing the interface. Fresh upper phase was added to the tube containing the lower phase and the interface. Mixing and centrifugation was repeated, and the upper phase removed. This washing of the lower phase + interface was repeated twice more to produce a tube containing lower phase + interface enriched in inside-out plasma membrane vesicles. The plasma membranes were pelleted and resuspended as for right-side-out vesicles. The membranes were used immediately for assays.

*$H^+$-ATPase assay.* ATPase activity was determined simultaneously with proton pumping using an $H^+$-ATPase assay essentially as described previously [7]. The assay medium consisted of 2 mM ATP-BTP, 4 mM $MgCl_2$, 10 mM Mops-BTP (pH 7.0), 140 mM KCl, 1 mM EDTA-BTP, 1 mM dithiothreitol, 20 μM Acridine orange, 0.25 mM NADH, 1 mM phospho*enol*pyruvate, 15 μg/ml lactate dehydrogenase, 30 μg/ml pyruvate kinase, and 50 μg/ml plasma membrane protein in a total volume of 1 ml. Detergents were added to the reaction mixture after addition of the membranes. After 4 min preincubation at 20°C, the reaction was initiated by addition of $MgCl_2$. In this assay ATP hydrolysis is coupled to oxidation of NADH measured at 340 nm, and proton uptake into the vesicles is monitored as the absorbance decrease at 495 nm of the ΔpH probe Acridine orange. ATPase activity and proton pumping were thus monitored simultaneously in the same cuvette by plotting the absorbance at 340 and 495 nm, respectively, versus time.

*Lysophospholipase.* Endogenous hydrolytic activity against lyso-PC was determined using labelled lyso-PC. Lyso-PC [$^{14}$C]palmitoyl (148 MBq) was dried under $N_2$, dissolved in $H_2O$ and added to 1 ml of the $H^+$-ATPase assay mixture to give a final concentration of 40 μM. After 0.25, 2.5, 10, 20 and 40 min samples of 140 μl were removed and immediately mixed with 260 μl chloroform/methanol (72 : 28, v/v). After freezing and thawing, 300 μl methanol was added to produce a one phase system and the samples were subsequently analyzed by thin-layer chromatography as described previously [6].

*Protein.* Protein was measured essentially as in Ref. 11, with BSA as standard.

## Results

*Effect of detergents on right-side-out vesicles*

In search for a suitable detergent to be used in latency studies of the plant plasma membrane $H^+$-ATPase we screened the effect of several detergents on the ATPase activity over a wide concentration range using right-side-out plasma membrane vesicles isolated from sugar beet leaves (Fig. 1). In the absence of any detergent very little ATP hydrolytic activity was present. Addition of detergents at low concentrations caused the rate of ATP hydrolysis to increase several-fold.

However, most of the detergents seemed to inactivate the ATPase in addition to disrupting the permeability barrier to ATP. In general detergents showed maximal effect around their critical micelle concentration (CMC), e.g. for the zwittergent series (d, e, and f in Fig. 1A), and those detergents used in concentrations below their CMC (such as CHAPS, CHAPSO, and octyl glycoside) had no effect on enzyme activity. This suggests a correlation between inhibition of ATPase activity and initial solubilization of the ATPase, which would presumably take place close to the CMC of the detergent used. Therefore, the enzyme activity measured in the presence of optimal detergent concentration is probably already partly inhibited with some detergents. There were two exceptions, namely long chain polyoxyethylene acyl ethers, such as detergents of the Brij series ($C_xE_y$) and Lubrol WX ($C_{16\ \&\ 18}E_{\langle 16.7\rangle}$) (Fig. 1B), and long chain lyso-PC (Fig. 1A). These two types of detergents permeabilized the membranes at low concentrations and did not inhibit the ATPase activity at higher concentrations. The ATPase activity in the presence of long chain polyoxyethylene acyl ethers approximated those measured at the optimal concentrations of strong detergents (e.g., zwittergent 3-14, zwittergent 3-16, and sodium dodecyl sulphate). Unmasking of latent active sites therefore seem to explain the activation of plant plasma membrane $H^+$-ATPase by these detergents. However, the effect of lyso-PC was different. In the presence of lyso-PC the activity was 3-times as high as with any of the other detergents (Fig. 1).

*Effect of detergents on inside-out vesicles*

To be able to determine more accurately whether a detergent inhibited or activated the ATPase we tested the effect of 42 detergents on inside-out plasma membrane vesicles. Since the active site of the ATPase is freely accessible with these vesicles no latent activity is expected, which otherwise may be confused with stimulation or conceal an inhibition. Of non-ionic detergents we tried Brij 35 ($C_{12}E_{\langle 23\rangle}$), Brij 58 ($C_{16}E_{\langle 20\rangle}$), Brij 78 ($C_{18}E_{\langle 20\rangle}$), Brij 96 ($C_{18:1}E_{\langle 10\rangle}$), Brij 99 ($C_{18:1}E_{\langle 20\rangle}$), Lubrol WX ($C_{16\ \&\ 18}E_{\langle 16.7\rangle}$), Triton X-100 (*tert*-$C_8\Phi E_{\langle 9.6\rangle}$), Nonidet P-40 (*tert*-$C_8\Phi E_{\langle 9\rangle}$), Tween 20 ($C_{12}SE_{\langle 20\rangle}$), octyl glycoside ($C_8G$), and digitonin. The ionic detergents tested were sodium deoxycholate, sodium dodecyl (12 : 0) sulphate, oleoyl (18 : 1) lyso-PA,

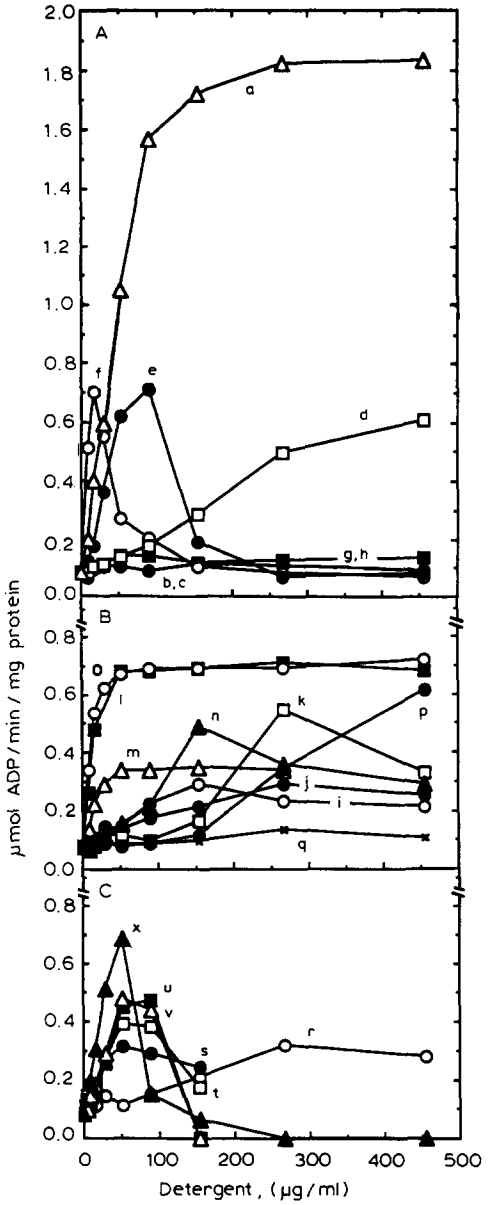


Fig. 1. Effect of detergents on the ATPase activity of right-side-out plasma membrane vesicles (50 µg protein/ml). Detergent titrations were made with zwitterionic detergents (A), nonionic detergents (B) and ionic detergents (C). (A) *a*(△), egg lyso-PC (Sigma type I); *b*(●), zwittergent (8 : 0) 3–8; *c*(●), zwittergent (10 : 0) 3–10; *d*(□), zwittergent (12 : 0) 3–12; *e*(●), zwittergent (14 : 0) 3–14; *f* (○), zwittergent (16 : 0) 3–16; *g*(■), CHAPS; *h* (■), CHAPSO. (B) *i*(○), Nonidet P-40 (*tert*-$C_8\Phi E_{\langle 9\rangle}$); *j* (●), Tween 20 ($C_{12}SE_{\langle 20\rangle}$); *k*(□), *N*-lauroyl (12 : 0)sarcosine; *l* (■), Lubrol WX ($C_{16\&18}E_{\langle 16.7\rangle}$); *m* (△), digitonin; *n*(▲), Triton X-100 (*tert*-$C_8\Phi E_{\langle 9.6\rangle}$); *o* (○), Brij 58 ($C_{16}E_{\langle 20\rangle}$); *p* (●), Brij 35 ($C_{12}E_{\langle 23\rangle}$); *q* (×), octyl glucoside ($C_8G$). (C) *r* (○), sodium deoxycholate; *s* (●), oleic (18 : 1) acid; *t* (□), linoleic (18 : 2) acid; *u* (■), linolenic (18 : 3) acid; *v* (△), arachidonic (20 : 4) acid; *x* (▲), sodium dodecyl (12 : 0) sulphate. Observe that concentrations of detergents are given in µg/ml whereas in other figures they are given in µM.

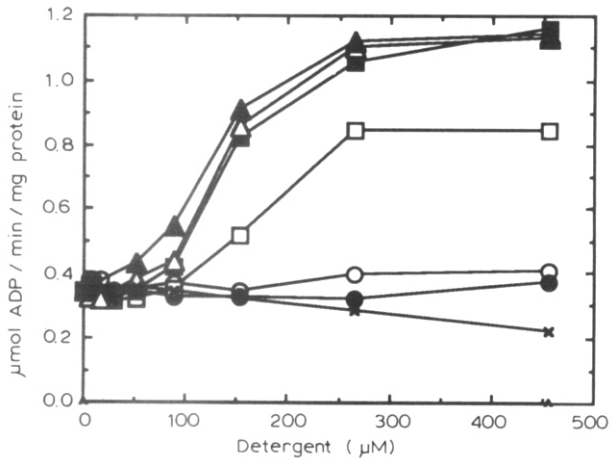


Fig. 2. Effect of lyso-PC on the ATPase activity of inside-out plasma membrane vesicles (50 µg protein/ml). Detergent titrations were made with lyso-PC of increasing chain lengths. ×, Triton X-100 (*tert*-$C_8\Phi E_{\langle 9.6\rangle}$); ●, decanoyl (10:0) lyso-PC; ○, lauroyl (12:0) lyso-PC; □, myristoyl (14:0) lyso-PC; ■, palmitoyl (16:0) lyso-PC; △, stearoyl (18:0) lyso-PC; ▲, oleoyl (18:1) lyso-PC.

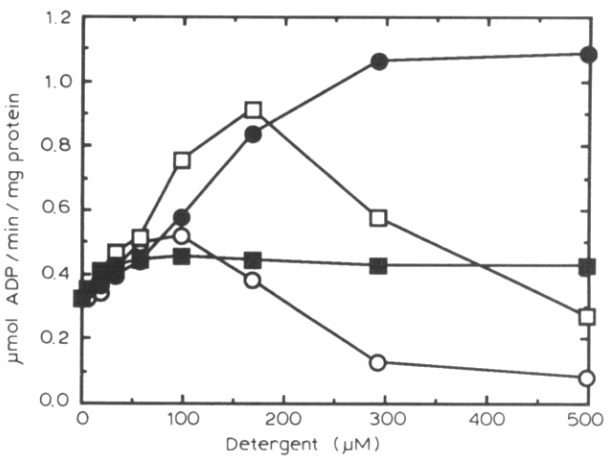


Fig. 3. Effect of oleoyl (18:1) detergents on the ATPase activity of inside-out plasma membrane vesicles (50 µg protein/ml). ●, oleoyl lyso-PC; □, oleoyl lyso-PA; ○, oleic acid; ■, polyoxyethylene oleoyl ether (Brij 96, $C_{18:1}E_{\langle 10\rangle}$).

decanoic (10:0) acid, lauric (12:0) acid, myristic (14:0) acid, palmitic (16:0) acid, palmitoleic (16:1) acid, stearic (18:0) acid, oleic (18:1) acid, linoleic (18:2) acid, linolenic (18:3) acid, arachidonic (20:4) acid, *N*-lauroyl (12:0) sarcosine, and cetyl(16:0)trimethylammonium bromide. Zwitterionic detergents were decanoyl (10:0) lyso-PC, lauroyl (12:0) lyso-PC, myristoyl (14:0) lyso-PC, palmitoyl (16:0) lyso-PC, oleoyl (18:1) lyso-PC, zwittergent (8:0) 3-8, zwittergent (10:0) 3-10, zwittergent (12:0) 3-12, zwittergent (14:0) 3-14, zwittergent (16:0) 3-16, CHAPS, and CHAPSO.

Long chain lyso-PC stimulated the ATPase activity of inside-out vesicles 2–3-fold (Fig. 2). Lyso-PC is characterized by having a long hydrophobic hydrocarbon chain in combination with a zwitterionic polar headgroup. To examine the importance of the hydrocarbon chain, detergent titrations were made with increasing acyl chain lengths of the lyso-PC used (Fig. 2). Ten or twelve carbons in the chain resulted in an increase in ATPase activity of about 20%. The palmitoyl (16:0), stearoyl (18:0), and oleoyl (18:1) lyso-PC gave a 2–3-fold increase in activity. Myristoyl (14:0) lyso-PC had an intermediary effect. The less pronounced effect of stearoyl lyso-PC previously reported [6,7] could have been due to problems of dissolving this hydrophobic compound. The importance of the zwitterionic headgroup was tested by comparing the effect of a variety of oleoyl (18:1) detergents (Fig. 3). Oleoyl lyso-PC was the most effective compound in stimulating the ATPase, but oleoyl lyso-PA, a strong anionic detergent, was also very effective, although it inhibited the ATPase at higher concentrations. Oleic acid, another anionic detergent, also stimulated the ATPase, but to a lower degree. Polyoxyethylene oleoyl ether (Brij 96, $C_{18:1}E_{\langle 10\rangle}$), a nonionic detergent, did not produce more activation than expected from permeabilizing contaminating right-side-out vesicles.

All unsaturated fatty acids investigated stimulated the plasma membrane $H^+$-ATPase activity under the assay conditions used (Fig. 4). The degree of stimulation increased as a function of both the number of hydrocarbon atoms in the acyl chain and the number of double bonds, to a maximum with arachidonic (20:4) acid. The lack of stimulation by long chain saturated fatty acids could be due to their relative insolubility in water. Above a fatty acid/protein ratio of 3:1 (w/w) fatty acids were strongly inhibitory.

Brij 96 ($C_{18:1}E_{\langle 10\rangle}$) (Fig. 3), Brij 58 ($C_{16}E_{\langle 20\rangle}$) (Fig. 4), Brij 78 ($C_{18}E_{\langle 20\rangle}$), Brij 99 ($C_{18:1}E_{\langle 20\rangle}$), Lubrol WX

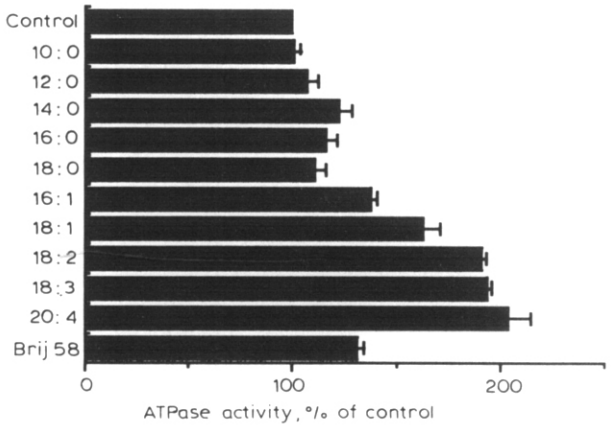


Fig. 4. Effect of fatty acids (150 µM) on the ATPase activity of inside-out plasma membrane vesicles (50 µg protein/ml). 10:0, decanoic acid; 12:0, lauric acid; 14:0, myristic acid; 16:0, palmitic acid; 18:0, stearic acid; 16:1, palmitoleic acid; 18:1, oleic acid; 18:2, linoleic acid; 18:3, linolenic acid; 20:4, arachidonic acid; Brij 58, polyoxyethylene palmitoyl ether ($C_{16}E_{\langle 20\rangle}$). One hundred per cent corresponds to 0.4 µmol ADP/min per mg protein.

($C_{16\,\&\,18}E_{\langle 16.7\rangle}$), and Brij 35 ($C_{12}E_{\langle 23\rangle}$) (the latter only at high concentrations) (data not shown) stimulated the activity about 20%, which we interpret as being due to contamination by 20% right-side-out vesicles.

The other detergents tested produced a maximal activation of 20% (consistent with a contamination of 20% right-side-out vesicles) before inhibiting the ATPase activity or had no effect at all at the concentrations used.

*Effect of detergents on the permeability toward protons*

ATPase activity and proton pumping were measured simultaneously in all cases using an $H^+$-ATPase assay [7]. None of the tested detergents stimulated proton pumping. We have previously shown that lysophospholipids at 30 $\mu$M markedly increase (nearly 2-fold) the rate of MgATP-dependent proton accumulation in oat root plasma membrane vesicles, while the passive permeability of $H^+$ is unchanged [7]. However, with inside-out plasma membrane vesicles from sugar beet leaves, such an effect of lysophospholipids could not be demonstrated. Even at low concentrations oleoyl lyso-PC abolished the proton pumping capacity of sugar beet plasma membrane vesicles, with 50% inhibition at about 15 $\mu$M (Fig. 5). For comparison, more than 60 $\mu$M of Triton X-100 was required to reduce the proton accumulation rate to half maximum. The strongest agents in eliminating the build-up of any proton gradient were unsaturated fatty acids, which made the membranes totally permeable to protons at very low concentrations with half-maximal inhibition at 5 $\mu$M or less. The inability of lyso-PC to stimulate proton pumping with plasma membranes from sugar beet leaves could therefore be due to the presence of lyso-PC degrading enzymes, e.g. lysophospholipase that releases fatty acids from lyso-PC.

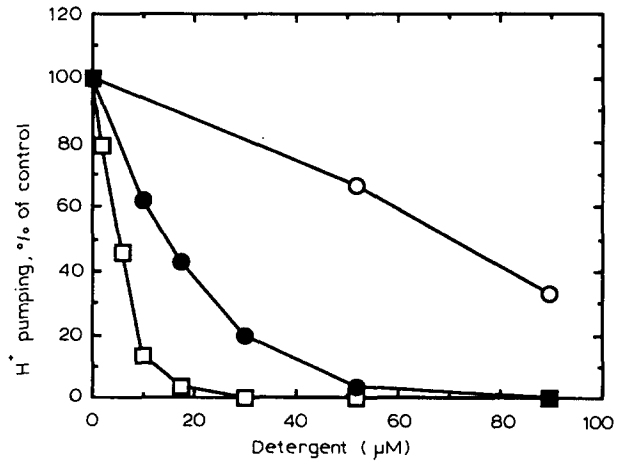


Fig. 5. Effect of Triton X- 100 (○), oleoyl lyso-PC (●), and oleic acid (□) on the proton pumping capacity of inside-out plasma membrane vesicles (50 $\mu$g protein/ml). One hundred per cent corresponds to 0.3 $\Delta A_{495}$/min per mg protein.

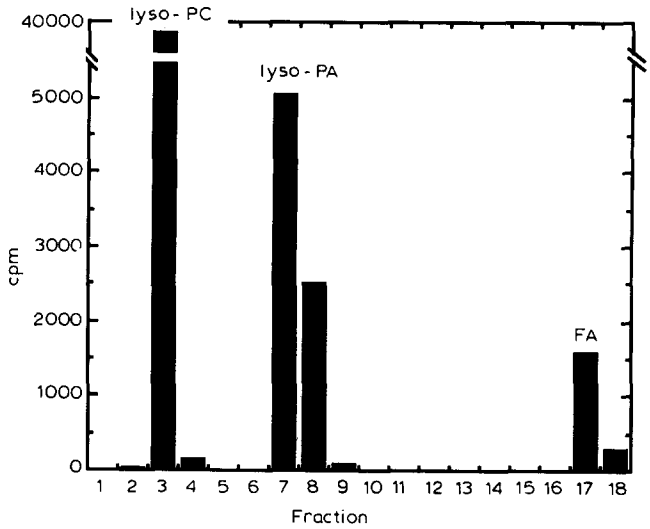


Fig. 6. Lyso-PC hydrolytic activity of inside-out plasma membrane vesicles. Plasma membranes (50 $\mu$g protein/ml) were incubated for 2.5 min at 20°C with [$^{14}$C]palmitoyl lyso-PC (final concentration 40 $\mu$M). Lipid extracts were separated by thin-layer chromatography. The lane on the plate was cut into equal-sized zones from 1 (the application point) to 18 (the front). FA, fatty acid (palmitic acid).

*Identification of lyso-PC hydrolytic activity in the plasma membranes*

When the inside-out plasma membrane vesicles were incubated with [$^{14}$C]palmitoyl lyso-PC for 2.5 min at the assay conditions used for the detergent treatments, the reaction mixture generated two radioactive products which co-chromatographed with palmitoyl lyso-PA and palmitic acid, respectively (Fig. 6). In controls without plasma membranes no lyso-PA or palmitic acid were observed. The initial rates of product formation were very fast, but levelled off after the first 15 s (Fig. 7). This indicates a possible product inhibition of the enzymes involved in the breakdown of lyso-PC. The degradation of lyso-PC could be the result of the activity of at least two enzymes. A lysophospholipase producing fatty acid, and a phospholipase D generating lyso-PA. Lysophospholipase activity was previously identified in oat root plasma membrane vesicles but only under very different assay conditions [6].

*Effect of BSA on the proton pumping capacity*

Upon prolonged storage of the plasma membrane vesicles on ice they lost their proton pumping capacity while their ATP hydrolytic capacity was unchanged (data not shown). This indicates that some uncoupler was produced in the membrane preparation and rendered the membranes leaky to protons. Since fatty acids were found to be very effective in collapsing the proton gradient, it was reasonable to assume that the decrease in proton pumping capacity with time was due to endogenous lipolytic activity. By adding fatty acid-free BSA to the plasma membranes the rate of MgATP-de-

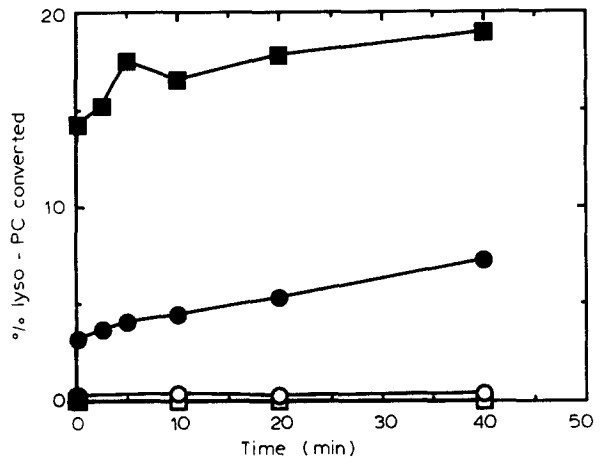


Fig. 7. Time dependent production of palmitoyl lyso-PA (○, ●) and palmitic acid (□, ■). Plasma membranes (50 μg protein/ml) were incubated with [$^{14}$C]palmitoyl lyso-PC (final concentration 40 μM). Samples were withdrawn at indicated times and analyzed by thin-layer chromatography. ○, lyso-PA; ●, lyso-PA (control without plasma membranes); □, palmitic acid; ■, palmitic acid (control without plasma membranes).

pendent intravesicular acidification indeed increased about 100%, whereas the rate of ATP hydrolysis was only slightly increased (Fig. 8). By adding BSA to plasma membranes isolated from old leaves, which showed very low proton pumping capacity, the rate of proton accumulation in the vesicles could be increased more than 5-fold (data not shown). This suggests that BSA exerted its effect by binding fatty acids, and that removal of fatty acids made the membranes more sealed to protons. BSA also reversed the inhibiting effect of exogenously added oleic acid and lyso-PC (Fig. 9).

## Discussion

By screening the effect of a wide range of detergents on the ATPase activity of both right-side-out and in-

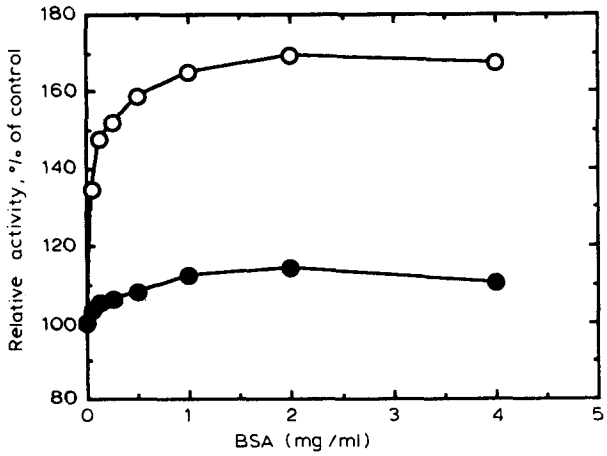


Fig. 8. Effect of fatty acid-free BSA on the proton pumping capacity (○) and ATP hydrolytic activity (●) of inside-out plasma membrane vesicles (50 μg protein/ml). One hundred per cent corresponds to $0.3\Delta A_{495}$/min per mg protein (○) and 0.45 μmol ADP/min per mg protein (●).

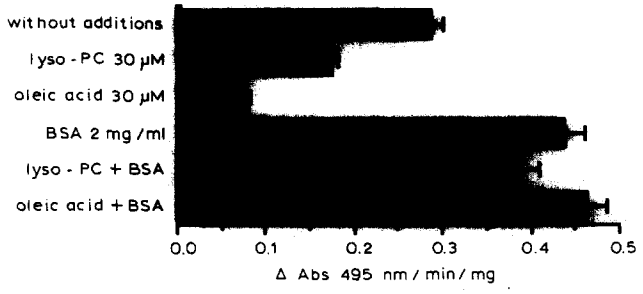


Fig. 9. Reversal by fatty acid-free BSA (2 mg/ml) of the inhibitory effect of exogenously added lyso-PC and oleic acid on the rate of proton accumulation in inside-out plasma membrane vesicles (100 μg protein/ml).

side-out plasma membrane vesicles long chain polyoxyethylene acyl ethers were identified as detergents that neiher inhibited nor activated the plasma membrane $H^+$-ATPase (Figs. 1–4). These detergents therefore seem to be ideal for determination of vesicle orientation by determining latent ATPase activity. We choose Brij 58 ($C_{16}E_{\langle 20\rangle}$) as a suitable detergent for latency studies because it contains a saturated acyl chain, has a low c.m.c., is cheap, and is very soluble in water. A concentration of 0.02% (w/v) can be used routinely since the permeabilizing effect of Brij 58 at this concentration is independent of the amount of protein used in standard assays (less than 50 μg/ml). The use of polyoxyethylene acyl ethers in the study of the plasma membrane $H^+$-ATPase might also have wider implications since Brij 58 ($C_{16}E_{\langle 20\rangle}$), Brij 96 ($C_{18}E_{\langle 10\rangle}$), and Brij 99 ($C_{18:1}E_{\langle 20\rangle}$), at high concentrations (10 mg detergent/mg protein) solubilized the plasma membranes (as judged from clarification) without inhibiting the ATPase activity (data not shown). Use of these detergents for solubilization would be preferable since they do not not directly modulate the activity of the protein as in the case of the hitherto used detergent lyso-PC (Figs. 1–3). Polyoxyethylene acyl ethers with intermediary and longer chain lengths have earlier been shown to be suitable detergents for solubilization, long time storage, and subsequent crystallization of the sarcoplasmic reticulum $Ca^{2+}$-ATPase [12,13]. The related $C_{12}E_8$ with an intermediate (12 : 0) chain length, which has been shown to be very suitable for solubilization of $Na^+/K^+$-ATPase and $Ca^{2+}$-ATPase, solubilizes the plant plasma membrane $H^+$-ATPase concomitant with a total loss of activity [14].

A plausible explanation for the inability of long chain polyoxyethylene acyl ethers to inactivate the plasma membrane $H^+$-ATPase is that these detergents fail to remove essential lipids bound to the hydrophobic region of the $H^+$-ATPase. In a recent study [15] addition of long chain detergents with polyoxyethylene headgroups to delipidated sarcoplasmic reticulum $Ca^{2+}$-ATPase led to rapid inactivation of the ATPase. By contrast, $Ca^{2+}$-ATPase prepared by gel chromatog-

raphy in the presence of such detergents shows long-term stability. This stability is due to the retention of a layer of endogenous lipids around the hydrophobic region of the $Ca^{2+}$-ATPase, and even a large excess of long chain polyoxyethylene ethers fails to delipidate the $Ca^{2+}$-ATPase completely [16]. The authors argue, that due to steric restrictions caused by the bulky head group of these detergents they are unable to establish adequate contact with the hydrophobic region of the ATPase and hence interact with the surrounding lipids of the ATPase instead. We therefore suggest that the efficiency of these detergents in latency studies with the plant plasma membrane $H^+$-ATPase, is coupled to their inefficiency in completely delipidating this enzyme.

Long chain lyso-PC was found to stimulate the ATPase activity of inside-out vesicles with a high degree of specificity. Substitution of the long (more than 14 carbon) acyl chain with a 10 or 12 carbon chain resulted in a total loss of activating capacity (Fig. 2). Alteration of the zwitterionic glycerophosphocholine moiety to a nonionic polyoxyethylene group also resulted in a total loss of activating capacity (Fig. 3). A few strong anionic detergents with long hydrocarbon tails, namely lyso-PA (Fig. 3) and to a minor degree unsaturated fatty acids (Fig. 4), stimulated the ATPase in the latency free system. These acidic lipids inhibited the ATPase at higher concentrations, whereas lyso-PC did not show any such inhibitory effect. We therefore conclude that both hydrophobic and polar interactions are important for the lyso-PC effect. Yeast plasma membrane $H^+$-ATPase, solubilized with lyso-PC, aggregates during purification into inactive oligomers containing lyso-PC as the main lipid [17]. Activity can be restored by adding more lyso-PC with the same degree of chain specificity as observed in the present study, but the ATPase can also be reactivated by several lecithins, albeit to different extent and efficiencies, and reactivation by exogenous lipid is suggested to be nonspecific [17]. The effect of exogenously added lipids in that study is due to their ability to dissociate the oligomeric structure of the purified ATPase, but this property of lyso-PC does not seem to be related with the effects observed in the present study.

The activating effect of lyso-PC on plasma membrane ATPase activity in a latency-free membrane system (sealed, inside-out vesicles; Fig. 2) confirms earlier results with oat root plasma membranes where a latency-free system was produced by fragmentation of the membrane vesicles [7]. Using yeast plasma membrane vesicles it has also been shown in lyso-PA produces 150% more activation of the ATPase than expected from the mere exposure of active sites associated with right-side-out vesicles [2].

It has recently been shown that the plasma membrane $H^+$-ATPase is a substrate for endogenous protein kinase(s) [18,19], and it has been suggested that phosphorylation stimulates ATPase activity [18]. By adding lyso-PC to tonoplasts and plasma membranes purified by gradient centrifugation the phosphorylation of several polypeptides increased [20]. However, in the latter study no 100 kDa polypeptide, the molecular weight of the $H^+$-ATPase, was phosphorylated, and the increased phosphorylation of other polypeptides could be due to the detergent effect of lyso-PC, thus making latent phosphorylation sites accessible for protein kinases.

The activating effect of lyso-PC may also be explained by assuming that the ATPase contains structural elements which inhibit the activity of the enzyme but are released by lyso-PC. The C-terminal domain and maybe part of the N-terminal domain of the erythrocyte plasma membrane $Ca^{2+}$-ATPase seems to function as a built-in inhibitor of the enzyme, which can be removed by certain lipids and other modulators involved in the regulation of the enzyme [21]. The activating effect of lyso-PA on the yeast plasma membrane ATPase has also been suggested to be exerted by such a mechanism [2].

Lysophospholipids, as well as all other detergents, did not stimulate ATP-dependent proton accumulation in the sugar beet plasma membrane vesicles (Fig. 5). In fact they rendered the membranes permeable to protons already at very low concentrations. This is in contrast to our previous results using oat root plasma membranes [7]. However, lyso-PC was partly metabolized by the sugar beet plasma membranes, releasing fatty acids as one of the products (Fig. 6). Fatty acids were found to eliminate the proton gradient across the vesicle membrane at very low concentrations (Fig. 5). Long chain fatty acids are very hydrophobic substances that tend to aggregate or stick to the inner surface of test tubes or the water air interface when added to aqueous solutions. The amount of fatty acids added to the samples that was actually absorbed by the membrane vesicles was not measured. Thus, it is possible that the measured potency of fatty acids in collapsing the proton gradient is an underestimate, and that even lower amounts of fatty acids in the membranes can abolish their proton accumulating capacity. Fatty acids probably act as uncouplers, as is well established with other membrane systems, e.g. in mitochondria [22–24].

The inhibitory effect of fatty acids was reversed by adding fatty acid-free BSA to the membranes (Fig. 9), but BSA could not be used in combination with lyso-PC, since also lyso-PC was bound to the BSA. The effect of lyso-PC in a fatty acid-free system could therefore not be tested with the sugar beet plasma membranes. BSA furthermore increased the proton pumping capacity of plasma membranes not treated with lyso-PC, indicating that some fatty acids were already present in the membrane preparation (Fig. 8). The proton pumping capacity of the membrane vesicles decreased with time and the effect was reversed by adding BSA (data not shown).

Loss of proton transport activity on storage has previously been suggested to be primarily associated with the degradation of membrane phospholipids [25]. It is therefore possible that the often observed 'leakiness' of plasma membrane vesicles (particularly on ageing) is due to phospholipase mediated release of fatty acids. This problem of 'leakiness' is partly solved by adding fatty acid-free BSA (1–2 mg/ml) to the assay medium (Figs. 8 and 9). Regarding the physiological function of fatty acids it is important to note that lyso-PC and fatty acids in equimolar amounts (as produced by phospholipase $A_2$) form a bilayer, even though the individual components form micelles when dispersed in an aqueous phase [26].

In conclusion, we have shown that the plasma membrane $H^+$-ATPase is extremely sensitive to changes in its lipid environment as induced by detergents. Most detergents inactivate the ATPase activity already at low concentrations possibly because an unfavorable hydrophobic environment for the ATPase is produced when essential lipids surrounding the enzyme are substituted with detergent. The exceptions are long chain polyoxyethylene acyl ethers and long chain lyso-PC. The former should be ideal for determination of vesicle orientation based on ATPase latency, since they permeabilize the plasma membrane to ATP without inhibiting or activating the ATPase. Whether the activating effect of lyso-PC on the $H^+$-ATPase is of physiological relevance remains to be established. Particularly, it has to be shown that physiological factors which regulate ATPase activity and growth also regulate the production of lysophospholipids in the plasma membrane.

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