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ABSCISIC ACID INCREASES MEMBRANE PERMEABILITY BY INTERACTING WITH PHOSPHATIDYLETHANOLAMINE

WILLIAM STILLWELL* and PAUL HESTER

Purdue University School of Science, Biology Department, Indianapolis, Indiana 46223, U S A

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Key Word Index—Abscisic acid, liposomes, permeability, phosphatidylethanolamine

Abstract—Abscisic acid is shown to enhance the permeability of crude egg lecithin and asolectin bilayers to water, urea and erythritol although it exhibits no effect on pure synthetic (phosphatidylethanolamine-free) dimyristoylphosphatidylcholine bilayers Addition of dipalmitoylphosphatidylethanolamine to dimyristoylphosphatidylcholine bilayers at 10 or 20 membrane mole percent makes the membrane permeability responsive to abscisic acid An abscisic acid–phosphatidylethanolamine interaction is also described for liposome aggregation Both abscisic acid-induced permeability and aggregation changes are pH dependent with the undissociated form of the hormone exhibiting a greater effect than the dissociated, charged form Enhancement of erythritol permeability is greater with the physiologically active *cis-trans* ABA isomer than with the inactive *trans-trans* isomer

INTRODUCTION

Although many gross physiological processes are known to be altered by plant hormones, a precise mode of action at the molecular level has not been described for any of them [1] Abscisic acid (ABA) is involved in a poorly understood fashion with stomatal regulation, breakage of bud and seed dormancy, leaf abscission and protection from water stress [2, 3] While these observations on gross physiological processes do not suggest a mode of action at the molecular level, it has been proposed that a probable initial site of action of ABA is at the membrane where the hormone may alter permeability [4, 5] Numerous, often conflicting studies have demonstrated ABA-induced changes in ion or water permeability of guard cells, coleoptiles, seeds, roots and other tissues [5, 14] Observed changes in guard cell permeability to K^+, for example, have been attributed to an ABA effect on a membrane-bound H^+/K^+ exchange pump [15] The alternative possibility—that ABA may interact directly with a lipid component of the membrane, altering permeability—has not received much attention Even with the simple, protein-free model bilayer systems, conflicts exist over the effect of ABA on permeability While Lea and Collins [16] reported that ABA induced transient channels in egg lecithin planar bimolecular lipid membranes, Hipkins and Hillman [17] could detect no increase in proton permeability with liposomes made from similar lipids At the present time the nature of membrane–ABA interactions remains unclear and controversial Here we report bilayer membrane permeability changes caused by specific ABA–phosphatidylethanolamine interactions

*To whom correspondence should be addressed

Abbreviations ABA, abscisic acid, DMPC, dimyristoylphosphatidylcholine, DPPE, dipalmitoylphosphatidylethanolamine, DLPE, dilauroylphosphatidylethanolamine

RESULTS

In Fig 1 abscisic acid is shown to increase the permeability rates of crude egg lecithin bilayers when added to the bathing solution from 0 to 3 8 mM Permeability is solute size dependent with water > urea > erythritol The egg lecithin lipid mixture contained phosphatidylcholine (71%) and phosphatidylethanolamine (29%) Permeability of erythritol to membranes made from a crude plant phospholipid mixture, asolectin, (40% phosphatidylcholine, 33% phosphatidylethanolamine) is also shown to be enhanced by ABA (Fig 2) at 0–2 8 mM However, erythritol permeability to phosphatidylethanolamine-free bilayers made from pure synthetic dimyristoylphosphatidylcholine (DMPC) is not altered by ABA (Fig 2) Since these results imply a possible phosphatidylethanolamine–ABA interaction, we next tested the permeability of water, urea and erythritol on a series of pure synthetic DMPC bilayers to which dipalmitoylphosphatidylethanolamine (DPPE) was incorporated in increasing amounts Figures 3 (water), 4 (urea) and 5 (erythritol) present the results for 0, 10 and 20 membrane mole percent DPPE in DMPC bilayers Permeability rates for all three solutes is dependent on both the ABA content (from 0 to 3 8 mM) as well as the phosphatidylethanolamine content (from 0 to 20 membrane mole percent) Additional experiments with 30 and 40 membrane mole percent phosphatidylethanolamine however showed no further ABA effect above the 20% phosphatidylethanolamine membrane and so these results are not reported

Since weak organic acids are known to cross lipid bilayers almost exclusively in the protonated, uncharged form [18], one would anticipate that ABA might be more effective at altering membrane permeability below its pK_a (4 85 [17]) than above it We therefore tested the permeability of crude egg lecithin bilayers to urea and erythritol (Fig 6) as a function of pH The results are expressed as the difference in swelling rates with 3 8 mM

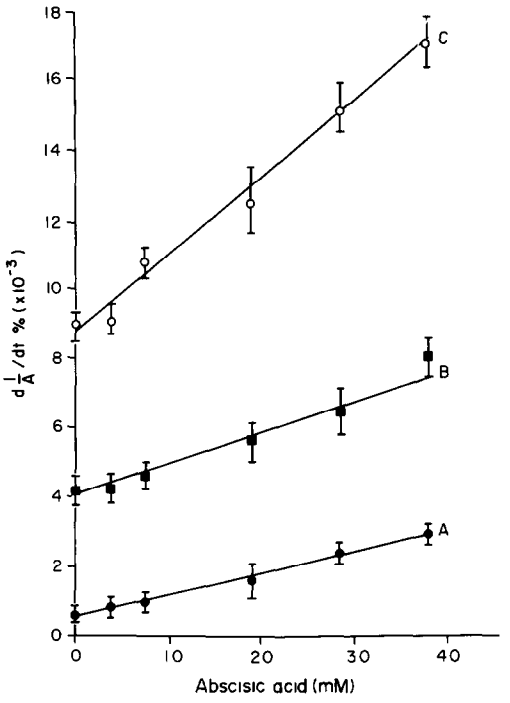

Fig 1 Effect of ABA on permeability of egg lecithin liposomes to erythritol (A), urea (B) and water (C) Permeability is followed spectrophotometrically by liposome swelling (dl/A/dt %)

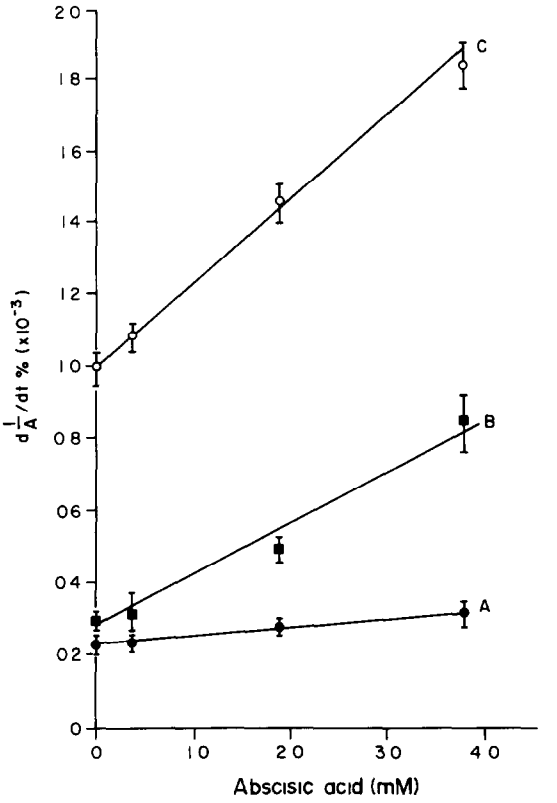

Fig 3 Effect of ABA on the permeability of water to lipid bilayers made from 100% DMPC (A), 90% DMPC–10% DPPE (B), and 80% DMPC–20% DPPE (C) Water permeability is followed spectrophotometrically as liposome swelling (dl/A/dt %)

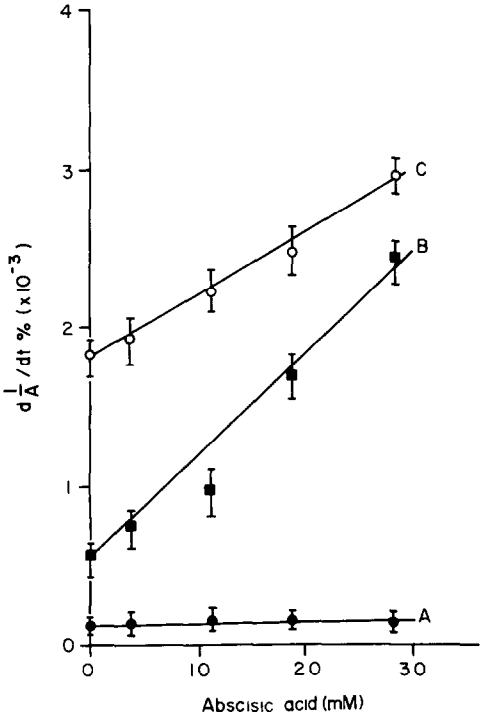

Fig 2 Effect of ABA on the permeability of erythritol to lipid bilayers made from DMPC (A), crude egg lecithin (B) and crude asolectin (C) Erythritol permeability is followed spectrophotometrically as liposome swelling (dl/A/dt %)

ABA compared to the ABA-free controls With both solutes, ABA enhanced membrane permeability much more in its protonated form (low pH) than it did in the ionized, charged form (high pH)

Previously, we have reported aggregation of sonicated small unilamellar liposomes (SUV) measured spectrophotometrically as a method of following hormone-induced bilayer changes [19] In Fig 7, we show the effect of ABA on liposome aggregation (A_{350nm}) for sonicated DMPC liposomes containing 0, 10 and 20 membrane mole percent DPPE at either pH 3 0 or 7 0 ABA-induced aggregation is clearly phosphatidylethanolamine and pH dependent Non-dissociated ABA interacting with phosphatidylethanolamine appears to be responsible for the measured aggregation

A comparison was made between the effect of pure, physiologically active *cis-trans* ABA and a mixture of ABA isomers (24% *cis-trans* and 76% inactive *trans-trans*) on the permeability of erythritol to bilayers composed of 80% DMPC and 20% DLPE The results presented in Table 1 clearly indicate that the physiologically active *cis-trans* isomer is also the more effective form at enhancing permeability

DISCUSSION

In 1944, Veldstra [20] proposed that plant hormones may exert their initial effects on membranes by altering

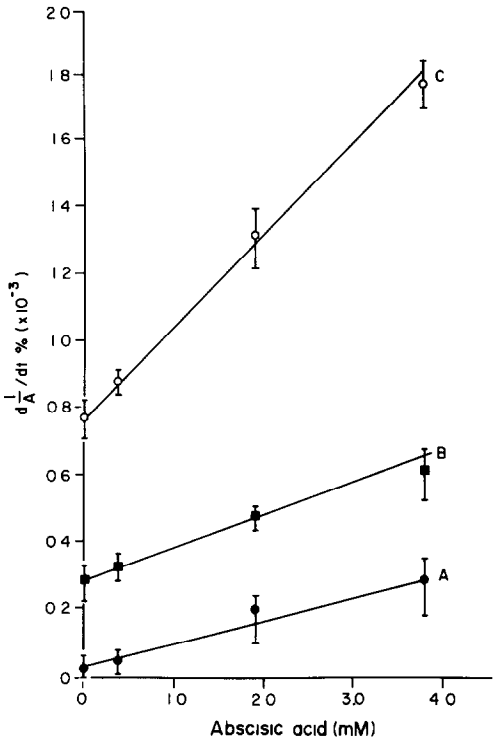

Fig 4 Effect of ABA on the permeability of urea to lipid bilayers made from 100% DMPC (A), 90% DMPC–10% DPPE (B), and 80% DMPC–20% DPPE (C) Urea permeability is followed spectrophotometrically as liposome swelling (dl/A/dt %)

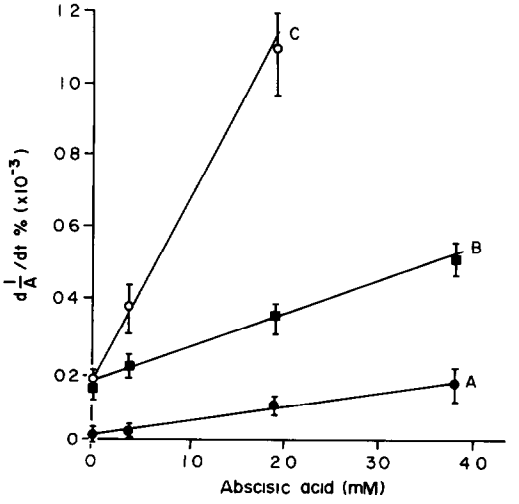

Fig 5 Effect of ABA on the permeability of erythritol to lipid bilayers made from 100% DMPC (A), 90% DMPC–10% DPPE (B), and 80% DMPC–20% DPPE (C) Erythritol permeability is followed spectrophotometrically as liposome swelling (dl/A/dt %)

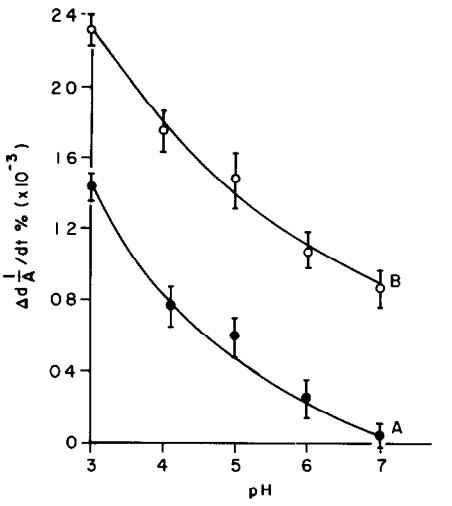

Fig 6 Effect of pH on the ABA-induced increase in crude egg lecithin bilayer permeability to erythritol (A) and urea (B) Liposome swelling rate differences (Δdl/A/dt %) between 3 8 mM ABA and 0 mM ABA are reported for identical liposome population at each pH

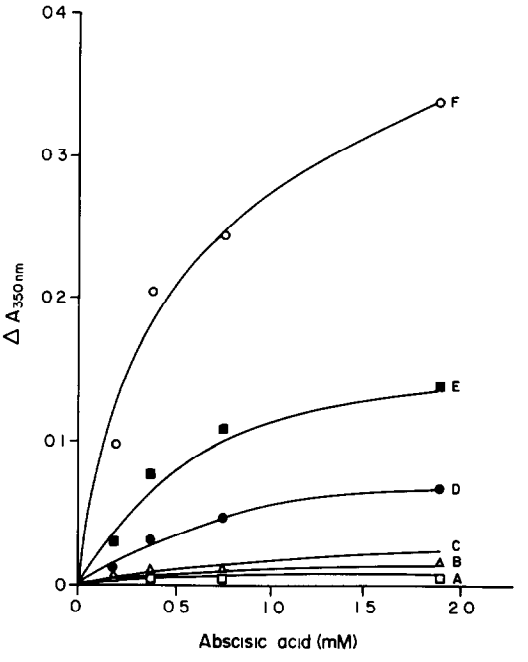

Fig 7 ABA-induced aggregation of DMPC liposomes (reported as absorbance charges at 350 nm) as a function of pH and phosphatidylethanolamine content (A) 100% DMPC, pH 7 0, (B) 90% DMPC–10% DPPE, pH 7 0, (C) 80% DMPC–20% DPPE, pH 7 0, (D) 100% DMPC, pH 3 0, (E) 90% DMPC–10% DPPE, pH 3 0, and (F) 80% DMPC–20% DPPE, pH 3 0

permeability Since then many reports have linked the growth substances with increases or decreases in membrane permeability for various ions, solutes and water These reports have either concerned whole tissues, cells or organelles, avoiding molecular questions, or, if done with

Table 1 Enhancement of erythritol permeability (expressed as liposome swelling, dl/A/dt %) to bilayers composed of 80% dimyristoylphosphatidylcholine and 20% dilauroylphosphatidylethanolamine by different ABA isomers

Abscisic acid (mM)	Liposome swelling (dl/A/dt %) A	B
0	0 35	0 35
0 32	0 47	0 51
0 63	0 56	0 67
1 26	0 59	0 83
2 52	0 84	2 19

A, 24% *cis-trans* ABA, 76% *trans-trans* ABA, B, 100% *cis-trans* ABA

well-defined membranes or artificial bilayers, have produced difficult to interpret or conflicting results Since its discovery, abscisic acid has often been proposed to be a membrane permeability altering agent While it is agreed that ABA causes stomatal closure by decreasing K^+ influx into guard cells [21, 22], the nature of this effect is by no means clear ABA may be altering an H^+/K^+ exchange pump [15] or else may be directly effecting the membrane bilayer [9, 16]

To further complicate the picture, conflicting reports have appeared concerning ABA's effect on ion and water permeability with other plant tissues, particularly roots [5–14] While some suggest ABA enhances permeability [10, 11, 23] others, often working with the same tissues, are equally convinced that the growth substance decreases permeability [5, 9, 12] Using the protein-free planar bimolecular lipid membrane system, Lea and Collins [16] proposed that ABA may effect all membranes by providing transient, transmembrane channels However, Hipkins and Hillman [17] using the spherical bimolecular lipid membrane system (liposome) composed of similar egg lecithin to that reported by Lea and Collins [16], could detect no ABA-induced proton flux Since they also could not detect any ABA-dependent permeability change with chloroplasts or chromophores, the existence of membrane channels remains in doubt It has also been suggested that ABA may effect membrane permeability not by forming channels but rather by increasing hydrocarbon chain mobility [9] Regardless of which possibility is correct, ABA can cause membrane depolarization [23] In this regard, Mansfield [24] has proposed that ABA may even serve as an uncoupler of oxidative phosphorylation Many uncouplers are known to specifically increase ion permeability or else can decrease membrane bilayer integrity resulting in generally 'leaky' membranes [25] Either would alter membrane polarization Despite many different approaches to the ABA-membrane problem, at the present time a realistic account of the nature of the interaction has not been formulated

Because natural membranes are such a complex mixture of lipids and proteins, determination of precise molecular interactions have not been easily obtained As a result, the protein-free artificial bimolecular lipid membrane systems, the planar bimolecular lipid membranes and liposomes, have been used to investigate the nature of bilayer interactions free of protein complications [26, 27] Despite the fact that these bilayers have proven to be excellent models and are often highly analogous to the natural membrane systems [28], they have only infrequently been employed in the study of the mode of action of plant hormones Besides the conflicting reports on the effect of ABA on the permeability of planar bimolecular lipid membrane and liposomes of Lea and Collins [16], and Hipkins and Hillman [17], Paleg and co-workers [29–32] have reported gibberellic acid–phosphatidylcholine and auxin–phosphatidylcholine interactions by NMR and changes in bilayer permeability to chromate, glucose and sucrose Gutnecht and Walter [18] measured the permeability of auxin to planar lipid bilayers and Lelkes [33] reported an auxin-dependent decrease in the phase transition temperature of dimyristoylphosphatidylcholine bilayers Recently we have reported cytokinin enhancement of water, urea and erythritol permeability with various phosphatidylcholine liposomes [19] The effect of kinetin was shown to be dependent on the degree of unsaturation of the fatty acid chains Prior artificial membrane experiments, done mostly with phosphatidylcholine bilayers, have shown that in high enough concentrations, the four classes of plant hormones—auxins, gibberellins, cytokinins and abscisic acid—can all enhance membrane permeability [34], however, specific interactions with other membrane lipid components have not been reported

In the experiments described here we used permeability to water, urea and erythritol as a method of following ABA-induced changes in bilayer structural integrity Isomolar liposome swelling in urea and erythritol has proven to be a good method for following general membrane permeability changes [35–37] and is particularly well-suited to spectrophotometers, such as the Beckman DU-8, which can accurately detect small absorbance changes in very turbid solutions With crude egg lecithin liposomes (Fig 1), ABA is shown to enhance the permeability to water, urea and erythritol As expected, the permeability rates are dependent on the size of the diffusing species with water > urea > erythritol

Although ABA clearly enhanced permeability to crude egg lecithin (29% phosphatidylethanolamine) and crude asolectin (33% phosphatidylethanolamine) bilayers, there was no measurable enhancement with phosphatidylethanolamine-free bilayers composed of synthetic DMPC (Fig 2) By incorporating pure synthetic DPPE at 0, 10 or 20% into DMPC bilayers we were able to demonstrate that for water, urea and erythritol, ABA increased permeability in proportion to its phosphatidylethanolamine content (Figs 3–5) Perhaps this requirement for phosphatidylethanolamine can explain the failure of Hipkins and Hillman [17] to detect any ABA-induced permeability to H^+ with pure egg lecithin bilayers (free of phosphatidylethanolamine) or to detect ABA enhancement of permeability to chloroplasts which contain only traces of phosphatidylethanolamine [38]

Auxin is a moderately lipophilic weak acid which has some physical properties similar to ABA Both growth substances, for example, have similar pK_as (4 7 for auxin [18] and 4 85 for ABA [17]) Auxin is known to penetrate lipid bilayers mainly in its uncharged, protonated form at pH less than 5 0 [18] Permeability rates vs pH curves however, are complicated by the dissociated form of auxin

assisting in transport of the undissociated form across unstirred aqueous layers immediately adjacent to the bilayer [18] On the basis of auxin studies, Gutnecht and Walter [18] predicted that ABA should exhibit similar behavior Also, Heilmann *et al* [39] demonstrated that only the uncharged, protonated form of ABA can penetrate the chloroplast envelope We therefore tested the ability of ABA to enhance permeability to urea and erythritol in its protonated (pH 3 0) and dissociated (pH 7 0) form Figure 6 shows that for both solutes uncharged ABA enhances permeability of crude egg lecithin membranes more than charged ABA

We have previously used aggregation of sonicated small unilamellar liposomes as a method of following bilayer perturbations [19] In this paper, ABA is shown to enhance the aggregation of DMPC sonicated liposomes as a function of both pH and phosphatidylethanolamine content (Fig 7) Aggregation, reported as ABA-induced increases in absorbance at 350 nm, is more efficient at pH 3 0 than 7 0 for all concentrations of phosphatidylethanolamine At pH 7 0 the dissociated form of ABA has little effect on membrane aggregation even at high phosphatidylethanolamine content However, at pH 3 0, the undissociated form of ABA clearly enhances aggregation in a phosphatidylethanolamine-dependent manner From these experiments we conclude that it is the undissociated form of ABA that interacts with phosphatidylethanolamine

The observed PE–ABA interactions (a) may be directly linked to physiological responses such as stomatal closure, (b) may be indirectly linked to such responses through subsequent lipid–lipid or lipid–protein interactions, or (c) may be totally unrelated to any physiological process at all If the permeability changes reported here have any physiological meaning, there should be a significant difference between the effect of physiologically active *cis-trans* ABA and its inactive isomer, *trans-trans* ABA [40] on permeability of PE-containing bilayers This was tested by comparing the effect of pure *cis-trans* ABA with a mixed isomer containing 24% active *cis-trans* and 76% inactive *trans-trans* ABA on the permeability of erythritol to liposomes made of 80% DMPC and 20% DLPE The results presented in Table 1 clearly indicate that the enhancement in erythritol permeability was directly related to the amount of *cis-trans* ABA present in the solutions Although these results by no means establish the physiological significance of PE–ABA interactions, it does strongly suggest that such interactions should be more carefully examined

Plant hormones exhibit both rapid time and slow time effects [41, 42] While the slow effects may be due to alterations in transcriptional or translational processes, the rapid effects are likely related to unresolved membrane responses Although it is generally believed that the growth substances probably interact initially with a specific (protein) receptor [43], the possibility of them interacting with a specific lipid component which in turn might effect bilayer structure, altering permeability or else effecting the activity of a crucial membrane protein, has not been investigated This paper defines a specific plant hormone–lipid interaction, that of undissociated ABA interacting with phosphatidylethanolamine It represents a first attempt to look at a biologically important process (permeability) which is altered by a plant growth substance interacting with a lipid other than phosphatidylcholine Since plant membranes contain such a wealth of different phospholipids, the possible combination of plant hormone–phospholipid interactions is vast and this potentially fruitful area of research is just beginning

EXPERIMENTAL

Phospholipids were purchased from Sigma Chemical Co , St Louis, MO [crude egg lecithin, Type IX-E, dimyristoylphosphatidylcholine (DMPC), dipalmitoylphosphatidylethanolamine (DPPE) and dilauroylphosphatidylethanolamine (DLPE)] and Associated Concentrates, Woodside, NY (asolectin, a mixture of phosphatidylcholine, 40%, phosphatidylethanolamine, 33%, phosphatidylinositol, 14%, lysophosphatidylcholine, 5%, and cardiolipin, 4% [44], which was further purified by the method of Kagawa and Racker [45]). Abscisic acid (mixed isomers) also purchased from Sigma, was used for most experiments, however, a pure *cis-trans* isomer was used for the experiments reported in Table 1 The mixed isomer was determined to contain 24% *cis-trans* and 76% *trans-trans* ABA by thin layer chromatography and UV spectroscopy [46]

Large multilayered vesicles (MLV), made by the method of Bangham [47], were used for the water, urea and erythritol permeability studies Liposomes were made above the phase transition temp (room temp for the crude egg lecithin and asolectin experiments and 60° for the DMPC or DMPC–DPPE, DMPC–DLPE experiments), in 40 mM glucose plus 10 mM Tris, pH 7 0 The pH experiments were buffered at pH 3 0–7 0 with 10 mM KPi and 40 mM glucose After removing large non-liposomal particles by a brief, 5 min centrifugation at 10000 *g*, the liposomes were pre-incubated for 1 hr with appropriate amounts of ABA Small aliquots of the lipids (generally 150 μl) were then rapidly mixed in cuvettes with 2 ml of the swelling buffer· 10 mM Tris for H_2O permeability, 10 mM Tris or KPi plus 40 mM urea for urea permeability and 10 mM Tris or KPi plus 40 mM erythritol for erythritol permeability The final lipid concentration in the cuvette was 0 5–2 0 mM Liposome swelling was followed spectrophotometrically by the method of de Gier *et al* [35] at 350 nm on a Beckman DU-8 Computing Spectrophotometer temperature controlled at 25° (±0 1°) The initial absorbance change rate, $\Delta A/\Delta T$, is proportional to the liposome vol change and was determined for each experiment [36, 37] The initial swelling velocity, $dl/A/dt$%, which has previously been shown to be proportional to permeability [36, 37], was then calculated Each experiment was repeated 3–5 times and the average initial swelling velocities are reported with standard errors

Liposomes for the aggregation experiments were prepared in 40 mM glucose, 10 mM KPi, pH 3 0 or 7 0 The multilayered vesicles, composed of DMPC and DPPE were sonicated on ice for 5 min (30 sec on, 30 sec off) at the no 7 position of a Heat Systems Model W-220 F Cell Disruptor The resulting small, unilamellar vesicles (SUV) were preincubated with appropriate amounts of ABA and then diluted with the same liposome-forming soln (40 mM glucose, 10 mM KPi, pH 3 0 or 7 0) Isotonic liposome aggregation was followed at 350 nm on a Beckman DU-8 Computing Spectrophotometer at 25° (±0 1°)

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