Biochimica et Biophysica Acta 862 (1986) 165–177
Elsevier

BBA 73157

Plasma membrane isolated with a defined orientation used to investigate protein topography

Howard D. Grimes, Nancy M. Watanabe and R. William Breidenbach

Plant Growth Laboratory and the Department of Agronomy & Range Science, University of California, Davis, CA 95616 (U.S.A.)

(Received 14 May 1986)
(Revised manuscript received 21 July 1986)

Key words: Plasma membrane; Protein topography; Silica microbead; (Tomato)

An investigation into the protein topography of tomato plasma membrane proteins was undertaken. Plasma membrane was isolated by phase partitioning to expose the extracellular leaflet, and by coating the protoplasts with silica microbeads to expose the cytosolic surface. Marker enzyme analysis indicated that both methods yielded relatively pure plasma membrane. Orientation of these plasma membrane fractions was established by investigating the latency of H^+-ATPase activity. Triton X-100 stimulated H^+-ATPase activity by 6-fold in the phase-partitioned plasma membrane fraction but did not stimulate this enzyme in the silica microbead-isolated plasma membrane. The impermeant photoactivable probes, 3-azido-(2,7)-naphthalene disulfonate and 5-azido-1-naphthalene monosulfonate, were used to probe the hydrophilic and hydrophobic regions of the plasma membrane, respectively. Using 5-azido-1-naphthalene monosulfonate, six proteins were labeled from the cytosolic leaflet of the plasma membrane and five proteins were labeled from the extracellular leaflet. Only two proteins were labeled by 3-azido-(2,7)-naphthalene disulfonate, and these were from the cytosolic-facing leaflet. The results indicate that these photoactive probes can be used in conjunction with aqueous two-phase partitioning and silica microbeads for transmembrane mapping of plasma membrane proteins.

Introduction

The plasma membrane delimits the cytosol from its environment and, in plant cells, is usually in close contact with the extracellular cell wall. The plasma membrane is therefore likely to have many important functions in relation to the interaction between the protoplast and its surroundings. These include the transport of ions and other solutes into or out of the cell [1], the synthesis and assembly of cell wall components [2], and the response to hormonal and environmental signals [3,4]. Furthermore, it is becoming increasingly apparent that we must view the plasma membrane as an asymmetric organelle with two distinct physical surfaces and biochemical interfaces. In plants, the external leaflet may be expected to interact with cell wall components, while the interior leaflet may be linked to the cytosol through the cytoskeleton [5,6].

To investigate the biochemical parameters of each leaflet of the plasma membrane, it is first necessary to isolate plasma membrane with a defined orientation. Gradients, especially sucrose,

Abbreviations: ANDS, 3-azido-(2,7)-naphthalene disulfonate; ANMS, 5-azido-1-naphthalene sulfonate.

Correspondence: Dr. H.D. Grimes, Plant Growth Laboratory, University of California, Davis, CA 95616, U.S.A.

are most commonly used to isolate the plasma membrane [7–11]. Since gradient separations rely on density, the membranes isolated are usually of mixed orientation. Hence, other methods must be explored. To isolate the plasma membrane with the cytosolic leaflet exposed, Chaney et al. [12] and Schmidt et al. [13] have used silica microbeads. This technique also has been used to isolate red beet plasma membrane [14]. In this procedure, protoplasts are coated with a layer of cationic silica microbeads that bind tenaciously to the plasma membrane. Upon protoplast lysis, the beads + plasma membrane sheets are centrifuged through a discontinuous sucrose gradient and, because of their high density (2.5 g/cm^3), form a pellet. Cytoplasmic debris and other organelles collect at various zones along the sucrose gradient. Since the beads were originally bound to the exterior of the protoplast, the cytosolic face of the plasma membrane is exposed after purification.

Another technique, the aqueous two-phase partition method, has recently become quite popular for isolation of the plant plasma membrane [15–18]. This method takes advantage of the different surface charge properties of membrane vesicles rather than density. Since the cytosolic- and exterior-facing leaflets of the plasma membrane have a different surface charge, it is possible to use phase-partitioning to isolate a vesicle population that is composed predominantly of right-side-out vesicles. Indeed, Larsson et al. [19] and Kormer et al. [20] have demonstrated that plasma membrane isolated via phase partitioning had a right-side-out orientation.

To further investigate the biochemical functions or interactions of the proteins associated with each leaflet of the plasma membrane, it is necessary to establish which proteins are associated with each side of this membrane. These topographic analyses have probably been most extensively studied in the human erythrocyte membrane. Reagents used to label surface components of the red blood cell membrane include lactoperoxidase-catalyzed iodination [21,22], pyridoxal phosphate [23], disulfonate stilbene derivatives [24], diazotized sulfonates [25,26], and others [27,28]. All of these reagents react with functional groups of surface components. While many of these have been tried on plant plasma membrane, only a few have been moderately successful [29,30]. Docktor [31] has synthesized a series of azidonaphthalene sulfonates that, upon photolysis, form fluorescent intermediates. By altering the position and number of sulfonic acid groups, these photoactivable probes can be used to probe either the hydrophilic or hydrophobic regions of the membrane.

The objectives of this study were (1) to isolate and characterize plasma membrane from tomato protoplasts by two different methods – the silica microbead procedure and aqueous two-phase partitioning, (2) to establish that these methods exposed different surfaces, or leaflets, of the plasma membrane, and (3) to label specific proteins associated with, or extruding from, specific leaflets of the plasma membrane.

Materials and Methods

Tomato cells. Tomato cell line No. 741505-45, an interspecific hybrid of *Lycopersicon esculentum* and *Lycopersicon peruvianum*, was obtained from D. Pratt (Department of Microbiology, University of California, Davis). This was kept as a suspension culture on a Linsmaier and Skoog based medium [32] with 2 mg/liter 2,4-dichlorophenoxyacetic acid and 1 mg/liter 2-isopentenyl adenine. A 1 : 10 dilution was performed when the culture was in late log (5 days after the previous dilution) and the cells were harvested for experiments in mid-log or 4 days after dilution.

To isolate protoplasts, cells were collected via filtration on a Buchner funnel with Whatman No. 1 filter paper and washed with 10–20 ml of protoplast digestion buffer (buffer 1: 400 mM mannitol, 4.3 g Murashige and Skoog salts (Gibco) per liter, 3 mM Mes, 7 mM $CaNO_3$, 0.1% bovine serum albumin; 0.1% gelatin). Approx. 5 g (fresh weight) cells were suspended in 25 ml buffer 1 containing 0.295 U pectinase and 2500 U cellulase (Cooper Biomedicals). These are high-grade enzymes with low amounts of contaminating proteinases. After approx. 3 h, the protoplast suspension was filtered through four layers of cheesecloth and centrifuged at $40 \times g$ for 5 min. The protoplast pellet was washed twice by gentle resuspension in buffer 1.

Two-phase plasma membrane isolation. Isolated

protoplasts were diluted (1 : 3 packed cell volume to buffer) with ice-cold tomato homogenization buffer (buffer 2: 0.46 M sucrose, 3 mM EDTA, 3 mM dithiothreitol. 25 mM Tris/Mes (pH 7.2)) and insoluble polyvinyl polypyrollidine was added to 0.5% (w/v). All subsequent procedures were done on ice or in a cold room. Tissue was homogenized via a polytron for about 10 s at low speed. The brie was centrifuged at $4300 \times g$ for 12 min. Supernatant was collected and centrifuged at $120\,000 \times g$ for 30 min. The resulting microsomal pellet (usually about 5 mg protein/5 ml packed cell volume of protoplasts) was resuspended in 4 ml of 250 mM sucrose/5 mM potassium phosphate (pH 7.8). A 2 ml volume of the resuspended microsome was added to a pre-made 8.0 g two-phase system. The two-phase system was prepared according to Hodges and Mills [10] and consisted of 6.5% (w/w) Dextran T500 (Pharmacia), 6.5% (w/w) poly(ethylene glycol) 3350 (Sigma), 0.125 M sucrose, 0.5 mM KCl, and 3.3 mM potassium phosphate (pH 7.8). Final weight of phase mixture was brought to 8.0 g with cold deionized H_2O. The phase system was then thoroughly mixed by harshly inverting the tube 50 times. Phase separation was accomplished by centrifugation at $2400 \times g$ for 10 min. The upper phase was collected, diluted 10-fold with buffer 2, and centrifuged at $120\,000 \times g$ for 30 min. This membrane pellet was resuspended in 0.46 M sucrose/5 mM Mes (pH 7.5), and either used immediately for experiments or frozen in liquid N_2 and kept for up to 4 weeks at $-80°C$.

Silica bead isolation. Microbead preparation. Colloidal silica microbeads, 11–16 and 17–25 nm (Nalco Chemical Co., Oak Brook, IL), were chemically converted to a positively charged form as described in Chaney and Jacobson [12]. Prior to each experiment, each bead size was diluted by suspending 0.25 ml beads in 3 ml of coating buffer (800 mM sorbitol/20 mM KCl/10 mM $MgCl_2$/25 mM sodium acetate (pH 5.0)). The microbead solutions were centrifuged at $1500 \times g$ for 10 min to remove any large aggregates.

Plasma membrane isolation was performed as described by Wasserman [14], with some modifications. Briefly, protoplasts were resuspended in coating buffer, adjusting the protoplast density to $(1–3) \cdot 10^6$ protoplasts/ml. In a 50 ml centrifuge tube, 1.5 ml 11–16 nm diluted beads plus 0.5 ml 17–25 nm diluted beads were mixed and diluted with an additional 1 ml volume of coating buffer. Protoplasts (3 ml) were added to diluted silica microbeads (3 ml) and gently mixed. Bead-coated protoplasts were mixed with 6 ml of overcoating buffer (10 mg/ml polyacrylic acid in coating buffer (pH 6.0)). Protoplasts were pelleted by centrifugation ($50 \times g$; 2 min), and supernatant was removed. This was repeated once, and the protoplasts were washed in wash buffer (900 mM sorbitol/5 mM Na_2EDTA/5 mM dithioerythritol/4 mM Tris-HCl (pH 7.5)). Protoplasts were lysed in lysis buffer (5 mM Na_2EDTA/5 mM dithioerythritol/4 mM Tris-HCl/0.1% bovine serum albumin (pH 7.5)).

This lysate was layered onto a discontinuous sucrose gradient (30, 40, and 50% sucrose) and was centrifuged at $6000 \times g$, 15 min in a Sorvall HS4 rotor. The pellet was resuspended in a resuspension buffer (15% sucrose/25 mM Tris (pH 6.5)) and centrifuged at $2000 \times g$ for 5 min. The final pellet was collected and resuspended in resuspension buffer.

Enzyme assays. Protein was measured using a modification of the method of Bradford [33] in which 60 μl of 0.2% Triton X-100 detergent was included in each assay tube to solubilize membrane proteins. Silica microbeads did not interfere with the linear response of this assay, up to 50 μg/ml protein (data not shown). Cytochrome *c* oxidase and antimycin A-insensitive NADH-cytochrome *c* reductase were assayed according to Hodges and Leonard [8]. H^+-ATPase activity was measured according to Hodges and Leonard [8] except that 0.1 mM molybdate and 1 mM sodium azide were included (except where noted). Latent IDPase activity was measured in the presence of 0.05% triton X-100. Other parameters were identical to those of Hodges and Leonard [8].

Trypsin digest. Purified plasma membrane fractions (150 μg protein/treatment) were resuspended in 900 μl of 25 mM Tris-HCl (pH 6.5). Trypsin was added in 100 μl of the same buffer, but the amount was varied between 10 μg and 125 μg. The digestion was done at 25°C and terminated by adding a 3-fold excess of soybean trypsin inhibitor. The membranes were diluted with ice-cold 0.46 M sucrose/5 mM Mes (pH 7.5),

pelleted, and prepared for electrophoresis.

Production of polyclonal antibodies. Plasma membrane was obtained by either the aqueous 2-phase method or silica microbeads. Plasma membrane (150 μg protein) was resuspended in phosphate-buffered saline and emulsified 1 : 1 with Freund's complete adjuvant and injected into the neck region of the rabbit. Subsequent biweekly booster injections consisted of 150 μg plasma membrane protein suspended in phosphate-buffered saline. The rabbit was bled from the ear vein 10 days after the third booster injection to determine the titer of serum antibodies. The rabbit was killed 7 days after the fourth or fifth booster injection and terminally bled by heart puncture.

ANDS and ANMS labeling of membrane proteins. The impermeant, photoactivable probes, ANDS and ANMS, were purchased from Molecular Probes and dissolved in labeling buffer (100 mM sodium phosphate/0.5 mM sodium azide (pH 7.4)) immediately before each experiment.

Plasma membrane was diluted in labeling buffer to a 1 mg/ml protein concentration. Aliquots of 150 μl were pipetted onto parafilm, forming small beads. ANDS or ANMS was added to a final concentration of 1 mM, unless otherwise stated. Long-wave (366 nm) ultraviolet light was administered by a hand held lamp (Blak-Ray, UVL No. 56) for 5 min at a distance of 1–2 cm. The samples were gently stirred twice during photolysis. After photolysis, samples were collected, transferred to tubes, diluted with labeling buffer, and pelleted. This was repeated two times to remove unbound label. Final pellets were prepared for electrophoresis. All operations, including electrophoresis, were done in the dark, under a red safelight, or in subdued room light. After electrophoresis, the gel was placed in deionized H_2O, as acid will quench the fluorescence. Labelled proteins were visualized on an ultraviolet (302 nm) lamp box.

Electrophoresis. Membrane fractions were resuspended in a Laemmli sample buffer [34] supplemented with 2.5 M urea. Insoluble material was removed by centrifugation prior to gel loading. Polyacrylamide gels were run according to Laemmli [34], with the following modifications. Acrylamide gradients of 5 to 15%, accompanied by a 5 to 15% glycerol gradient, were set up in 16 cm slabs with either 0.75 mm or 1.5 mm thickness spacers. Constant current at 20 mA (0.75 mm) or 40 mA (1.5 mm) was applied for approx. 3.5 h.

Gels were analyzed by several different means. Silver staining was performed as per Oakley [35]. Transblotting of gels was performed in 150 mM glycine/20 mM Tris-HCl/0.01% SDS/20% methanol. Transfer of proteins to nitrocellulose sheets was performed by applying a current of 1 A for 2 h or 100 mA overnight. Affinoblotting (or glycoprotein visualization) was performed as described by Faye and Chrispeels [36]. Briefly, blots were blocked in 3% gelatin in Tris-buffered saline for 60 min, incubated in 25 μg/ml concanavalin A and then washed with Tris-buffered saline, followed by Tris-buffered saline containing Tween-20 (0.1%). The blots were incubated in 50 μg/ml horseradish peroxidase (Sigma; Type III) for 60 min, followed by washing. Color was developed by immersing the blots in 60 mg 4-chloro-1-naphthol (Bio-Rad) in 20 ml cold methanol plus 60 μl H_2O_2 in 100 ml Tris-buffered saline. Color development was terminated by transferring nitrocellulose to deionized H_2O. Immunoblotting was performed as per Towbin [37] with peroxidase-conjugated goat-anti-rabbit IgG (Tago).

Results

Purity and initial characterization of plasma membrane isolated by two-phase partitioning

The purity of the plasma membrane fractions was determined via marker enzyme analysis. Typical results from the aqueous two-phase partitioning method are shown in Table I. The marker used most commonly for the plant plasma membrane is the K^+-stimulated vanadate-sensitive H^+-ATPase [8,10,38]. This marker activity was greatly increased in the upper phase, while only a small amount of activity was recovered in the lower phase. This distribution indicated that tomato plasma membrane was predominantly partitioned into the upper phase. Furthermore, potassium was shown to stimulate upper phase ATPase activity over 100% (Table III), and there was a pH optimum of 6.5 (data not shown). Antimycin A-insensitive NADH cytochrome *c* reductase, cytochrome *c* oxidase, and latent IDPase activities were markedly reduced in the upper phase when com-

TABLE I

MARKER ENZYME ASSESSMENT OF PLASMA MEMBRANE PURITY AFTER AQUEOUS TWO-PHASE PARTITIONING

n.d., not determined.

	Microsome	Upper Phase Expt. 1	Upper Phase Expt. 2	Lower Phase Expt. 2
Vanadate-sensitive ATPase [a]				
μmol P_i/h	0.147	0.276	0.304	0.03
μmol P_i/h per mg protein	3.92	8.35	9.42	0.938
Latent IDPase [a]				
μmol P_i/h	0.20	0.053	0.047	0.127
μmol P_i/h per mg protein	15.3	4.8	4.5	10.6
NADH cytochrome *c* reductase				
μmol/h	0.205	0.0008	0.001	0.150
μmol/h per mg protein	10.25	0.047	0.036	7.50
Cytochrome *c* oxidase				
μmol/h	0.049	0.0002	0.0001	n.d.
μmol/min per mg protein	0.565	0.008	0.004	n.d.

[a] Assay was performed in the presence of 0.1% Triton X-100.

pared to the microsomal activity. The distribution of marker enzymes for the endoplasmic reticulum and mitochondria demonstrated that virtually all of these membranes were removed by phase partitioning. Golgi apparatus also was significantly reduced, but represents the major contaminant in this plasma membrane fraction. Further partitioning of the upper phase increased the ratio of Golgi apparatus to plasma membrane (data not shown). Hence, only one phase separation step was used. This single phase partitioning, however, results in a plasma membrane fraction of high purity.

Further characterization of this plasma membrane fraction focused on ATPase activity. Early experiments showed no enrichment of ATPase activity in the upper phase compared to microsomal activity (data not shown). Since it is believed that the site of ATP hydrolysis is on the cytosolic side of the plasma membrane, this lack of enrichment of the ATPase could be due to having only the extracellular leaflet exposed. This possibility was tested by evaluating the effect of 0.1% Triton X-100 on the ATPase activity. It was found that Triton X-100 stimulated the upper phase ATPase activity 6–9-fold (Table III), while stimulating the microsomal ATPase activity by only about 1.5-fold (data not shown).

Since the Golgi apparatus seemed to constitute the major contaminating membrane system, we decided to compare the amount of Golgi activity found on a linear sucrose gradient between the commonly used starting material, a microsomal fraction, and the upper phase fraction, following phase partitioning. The results of this comparison are shown in Fig. 1. It can be seen that the upper phase membrane contained a negligible amount of Golgi contamination compared to a comparable plasma membrane 'cut' on a linear sucrose gradient.

To further characterize the plasma membrane vesicles isolated via phase partitioning, the behavior of these vesicles was investigated in a linear sucrose gradient. Plasma membrane isolated via two-phase partitioning tended to distribute in a peak in a linear sucrose gradient (Fig. 2). Equilibrium density of these plasma membrane vesicles

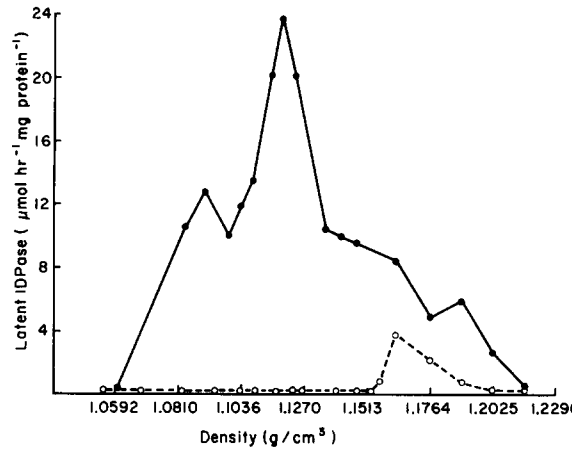

Fig. 1. Latent IDPase activity compared in a microsomal fraction (●) and the upper phase fraction after phase partitioning (○). Two linear sucrose gradients (10–50%) were run. On the first, a microsomal fraction was layered and on the second, resuspended upper phase vesicles. After equilibration, both gradients were fractionated and each fraction was assayed for latent IDPase activity and sucrose content.

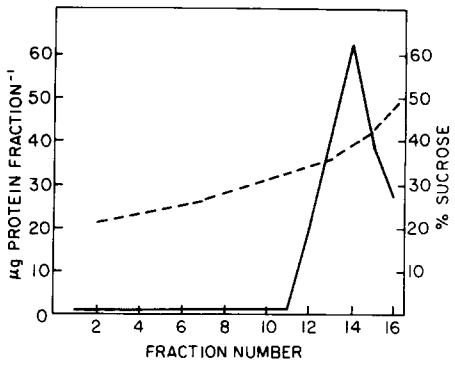

Fig. 2. Distribution of upper plasma membrane protein (solid line) on a 20–50% linear sucrose gradient. Phase partitioning was used to obtain a purified plasma membrane fraction which was layered on top of a linear sucrose gradient (% sucrose indicated by dashed line).

ranged from 1.1366 to 1.2186 g/cm^3, with a strong peak at 1.1764 g/cm^3.

Isolation of plasma membrane with silica microbeads

An assessment of plasma membrane purity following silica microbead isolation is shown in Table II. A 2- to 4-fold enrichment of vanadate-sensitive ATPase activity was observed in the plasma membrane fraction. Evaluation of markers for the endoplasmic reticulum, Golgi apparatus, and mitochondria demonstrated that each of these activities was significantly reduced following silica microbead isolation. Approx. 15–25% of original total cell protein co-purified with the plasma membrane in the silica microbead procedure (data not shown).

Although the ATPase activity associated with this plasma membrane fraction was quite sensitive to vanadate, K^+ stimulation was only 30% (Table III). An assessment of the substrate specificity indicated that while ATP was the favored substrate, this plasma membrane fraction could also utilize ADP, ITP, UTP, UDP, and *p*-nitrophenol phosphate to a lesser extent (Table IV). Activity measured with *p*-nitrophenol phosphate as a substrate demonstrated a 2.6-fold increase in the absence of molybdate, suggesting the presence of a non-specific phosphatase in the plasma membrane fraction. A pH curve of ATPase activity showed a strong peak at pH 6.5 (Fig. 3), with a second, smaller, peak at pH 7.5. This indicates that tonoplast was a minor contaminant in this plasma membrane fraction.

TABLE II

MARKER ENZYME ASSESSMENT OF PLASMA MEMBRANE PURITY FOLLOWING SILICA MICROBEAD ISOLATION

	Microsome [a]	Expt. 1	Expt. 2
Vanadate sensitive ATPase			
μmol P_i/h	0.018	0.074	0.106
μmol P_i/h per mg protein	1.29	3.7	4.61
Latent IDPase			
μmol P_i/h	0.451	0.191	0.12
μmol P_i/h per mg protein	10.5	4.7	2.6
NADH cytochrome *c* reductase			
μmol/min	0.038	0.013	0.002
μmol/min per mg protein	0.955	0.156	0.078
Cytochrome *c* oxidase			
μmol/min	0.008	0.001	None detected
μmol/min per mg protein	0.3	0.035	None detected

[a] The microsome was isolated from an aliquot of the cells used in Expt. 2 for plasma membrane purification.

TABLE III

K^+ AND TRITON X-100 EFFECT ON VANADATE-SENSITIVE ATPase SPECIFIC ACTIVITY IN SILICA MICROBEAD VS. PHASE-PARTITIONED PLASMA MEMBRANE

Membrane origin	Specific activity (μmol P_i/h per mg protein)		
	−K	+K	% K^+ stimulation
Silica microbead			
− Triton	3.38	4.82	29
+ Triton [a]	3.46	5.13	48
% Triton stimulation	2	6	
Phase partitioned			
− Triton	0.27	0.41	52
+ Triton [a]	1.74	3.52	102
% Triton stimulation	544	758	

[a] 0.1% Triton X-100.

TABLE IV

SUBSTRATE SPECIFICITY OF SILICA MICROBEAD ISOLATED ATPase

Silica bead preparations were assayed in the presence of 3 mM nucleotide, 3mM $MgSO_4$, 50 mM KCl, 1 mM NaN_3, 0.1 mM molybdate and 30 mM Tris/Mes (pH 6.5).

Substrate	% Control
ATP	100
ADP	43
ITP	26
GTP	23
UTP	15
UDP	11
PNP	28 [a]

[a] There was a 2.6-fold increase in activity in the absence of molybdate.

The silica microbeads were always present in the ATPase reaction along with the attached plasma membrane. A large number of ATPase experiments were examined to determine if there were any unexpected effects that may be due to the presence of the silica microbeads. Indeed, Fig. 4A demonstrates that ATPase specific activity was inversely proportional to increasing amounts of membrane protein (between 5 and 50 μg; a range that is considered standard for the ATPase assay), however, total activity was shown to increase with increasing amounts of membrane protein (Fig. 4B). This result suggested that there was some source of ATPase inhibition in this plasma membrane fraction. To establish whether this inhibition was due to the silica microbeads, an experiment was performed measuring apparent ATPase activity with a constant amount of membrane protein while silica microbeads were added in increasing amounts to the reaction mix. Increasing the silica microbead : membrane protein ratio resulted in an inhibition of apparent ATPase activity (Table V). Polyacrylic acid had no effect on ATPase activity (Table V).

Three possible explanations exist for the inhibition by silica microbeads on apparent ATPase. First, the silica microbeads may be binding inorganic phosphate and making it unavailable for detection in the assay. This possibility was refuted by adding silica microbeads to the phosphate standards. No deviation from the standard curve was seen (data not shown). The other two possibilities include (1) a direct inhibition of the enzyme or (2) removal of the ATP substrate or some other factor in the reaction mix that would result in an apparent inhibition of ATPase. This latter possibility was tested by adding silica microbeads to

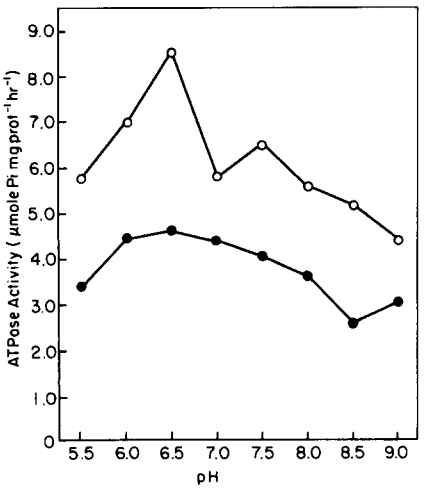

Fig. 3. ATPase activity in silica-bead-isolated plasma membrane as a function of pH. Activity was determined in the absence (○) and presence (●) of 500 μM vanadate. Assay samples contained 20 μg protein attached to silica microbeads.

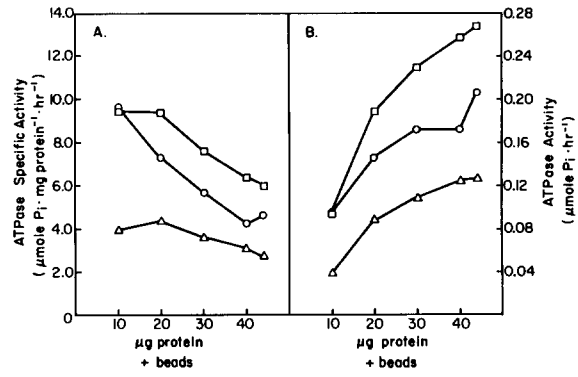

Fig. 4. (A) The effect of silica microbeads/plasma membrane on ATPase specific activity. The ATPase reaction was terminated after 22 min (□), 40 min (○) and 60 min (△). μg protein + beads represent the fact that the plasma membrane is bound to the silica microbeads. Hence, the numbers indicate μg membrane protein. As more membrane protein is added, more silica microbeads are added. (B) The effect of silica microbeads/plasma membrane on ATPase activity. Symbols as are in (A).

TABLE V

THE EFFECT OF SILICA MICROBEADS AND POLYACRYLIC ACID (PA) ON APPARENT ATPase ACTIVITY

S = simultaneous; silica microbeads or polyacrylic acid were added with the membrane preparation. Subsequently, the standard ATPase protocol was followed. P/R = pelleted and removed; silica microbeads were added to the ATPase reaction cocktail (containing 3 mM ATP, 3 mM $MgSO_4$, 50 mM KCl, 1 mM Na_3, 0.1 mM molybdate and 30 mM Tris/Mes (pH 6.5)) and incubated for 10 min. Then, the cocktail was centrifuged at 2500 × *g* to pellet the silica microbeads. The supernatant was collected and used for an ATPase assay. PA = polyacrylic acid. The amount of membrane protein was in all cases 10 μg.

Added beads	Volume of beads (μl)	Added PA	Volume of PA (μl)	Activity (μmol P_i/h)	% Control
0	0	0	0	0.104	100
S	10	0	0	0.086	83
S	20	0	0	0.048	46
S	30	0	0	0.016	15
S	40	0	0	0.020	19
P/R	10	0	0	0.033	32
P/R	30	0	0	0.000	0
0	0	S	10	0.115	111
0	0	S	20	0.117	113
0	0	S	30	0.110	106
0	0	S	40	0.098	94
0	0	S	50	0.096	92

TABLE VI

ESTIMATION OF MOLECULAR WEIGHT OF ANDS- OR ANMS-LABELED PROTEINS

SM, silica microbeads; PP, phase partitioned.

Spot	Membrane origin	Label	R_F	M_r
A	SM	ANDS	0.117	167000
B	SM	ANDS	0.596	39000
C	SM	ANMS	0.117	167000
D	SM	ANMS	0.430	64000
E	SM	ANMS	0.559	41000
F	SM	ANMS	0.641	33600
G	SM	ANMS	0.797	21000
H	SM	ANMS	0.875	16500
I	PP	ANMS	0.43	64000
J	PP	ANMS	0.58	41000
K	PP	ANMS	0.63	34000
L	PP	ANMS	0.72	26400
M	PP	ANMS	0.78	22000

the reaction mixture, and incubating for 5 min. Silica microbeads were then pelleted. The supernatant was removed and an ATPase assay was started by adding membrane protein. Table V demonstrates that when this supernatant was used, apparent ATPase activity was strongly inhibited. This datum suggests that the silica microbeads remove a critical component of the reaction mixture. Potassium, magnesium, ATP, K^+ + ATP, and Mg^{2+} + ATP were added to the supernatant to determine what the silica microbeads were removing. Only the Mg^{2+} + ATP would restore activity, indicating that silica microbeads bind both Mg^{2+} and ATP.

Establishment of external and internal orientation of the two-phase and the silica-microbead isolated plasma membrane

The fact that Triton X-100 stimulated ATPase activity in two-phase isolated plasma membrane (Table III) is strong evidence that these vesicles are tight and right-side-out (extracellular leaflet exposed). Plasma membrane isolated with silica microbeads exhibited no such stimulation upon

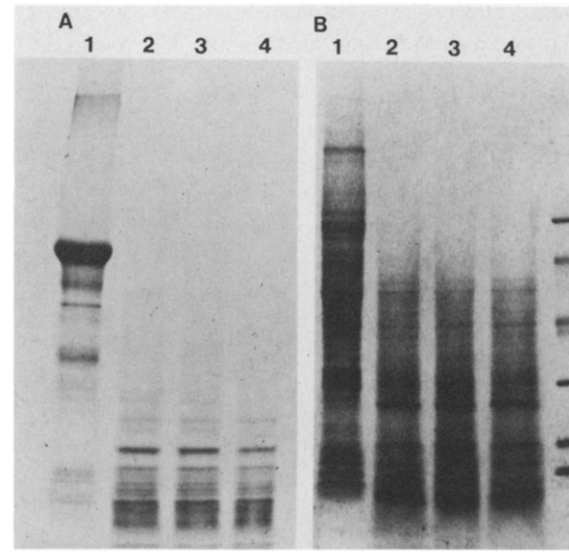

Fig. 5. SDS-polyacrylamide gel electrophoresis protein patterns following trypsin digestion of (A) silica microbead plasma membrane with 10 μg of trypsin and (B) two-phase plasma membrane with 100 μg of trypsin. Lanes 1, 2, 3 and 4 represent zero, 30 s, 2 min and 5 min incubation with trypsin, respectively.

the addition of Triton X-100 (Table III). This datum is consistent with the interpretation that plasma membrane isolated via the silica microbead method are oriented such that ATP is accessible to the hydrolytic site on the ATPase, which is on the cytosolic face of the plasma membrane.

If these plasma membrane fractions of different origin expose different leaflets of the plasma membrane, then one would expect them to exhibit a different sensitivity to proteolysis. To establish this, the plasma membrane fractions were incubated with varying concentrations of trypsin and the membrane-associated peptide pattern was evaluated on SDS-polyacrylamide gels. The silica microbead fraction was extremely sensitive to proteolysis, while a 10-fold higher concentration of trypsin was necessary to achieve any proteolysis in the two-phase plasma membrane (Fig. 5). Furthermore, the peptide pattern resulting from proteolysis was different between these two plasma membrane preparations.

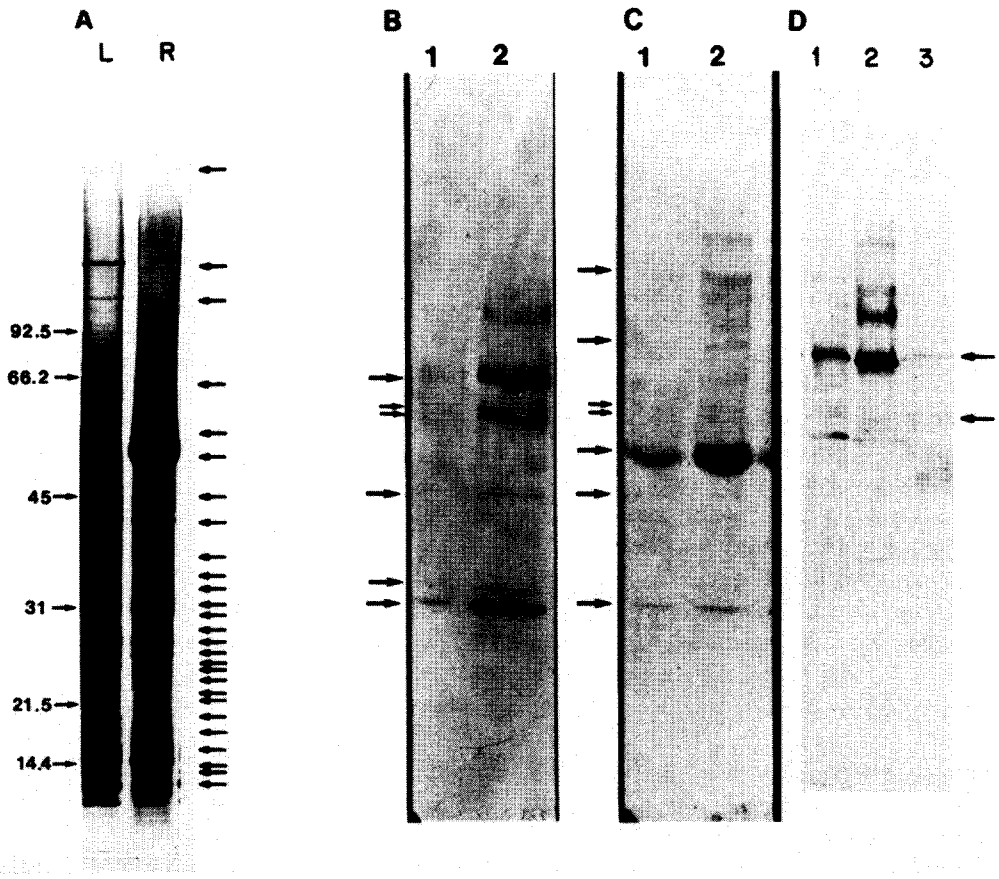

Fig. 6. (A) Silver stained SDS-polyacrylamide electrophoresis gels of (L) phase-partitioned plasma membrane and (R) silica-microbead plasma membrane (arrows indicate homologous peptides). (B) Immunoblot of (1) silica-microbead-associated plasma membrane and (2) phase-partitioned plasma membrane with antiserum raised against the phase-partitioned plasma membrane preparation. Antiserum was used at a 1:200 dilution. Arrows point to fainter staining bands. (C) Immunoblot of (1) silica-microbead plasma membrane and (2) phase-partitioned plasma membrane with antiserum raised against the silica-microbead plasma membrane preparation. Antiserum was used at a 1:50 dilution. Arrows point to fainter staining bands. (D) Concanavalin A affinoblot of (1) microsome, (2) phase-partitioned plasma membrane and (3) silica-microbead plasma membrane. Arrows indicate faint staining bands in lane 3.

Protein and glycoprotein comparison of plasma membrane derived from two-phase vs. silica microbeads

The electrophoretic pattern of plasma membrane proteins was compared to determine extent of homology or how the peptide pattern varied as a result of two such different methods for isolating the plasma membrane. A silver-stained gel is shown in Fig. 6A. While many differences are apparent between the two-phase and silica-microbead isolated plasma membrane, many of the bands are homologous. Several of the homologous peptides were seen to stain positively in the phase partitioned plasma membrane preparation, but stained negatively in the silica microbead preparation. For instance, the three highest molecular weight peptides (indicated by the upper three arrows on Fig. 6A) demonstrated this property. It is difficult to reproduce photographically such negative staining peptides.

To further establish protein homology between these two different plasma membrane preparations, antiserum was raised against each fraction. Each antiserum was then used to immunoblot both preparations. Both antisera were cross-reactive to SDS-solubilized proteins from the other preparation (Fig. 6B), further substantiating the common origin of the peptides associated with both of these plasma membrane preparations.

A glycoprotein stain was also performed on proteins solubilized from each plasma membrane preparation. It can be seen that many of the two-phase-derived plasma membrane bands are glycoproteins (Fig. 6D). Only a very few glycoproteins could be identified from the silica microband-derived plasma membrane. These are indicated by arrows in Fig. 6D, lane 3.

Topographical labeling of plasma membrane proteins

Two impermeant, photoactivable labels were used to label plasma membrane proteins. These probes labeled proteins from both leaflets of the plasma membrane. ANDS, which probes the hydrophilic region of the membrane, labeled two peptides (A and B; Table VI) from the cytosolic leaflet and none from the extracellular leaflet (Fig. 7, lane 2; and data not shown). ANMS, which labels in more hydrophobic regions of the membrane, labeled six peptides from the cytosolic leaflet (C–H; Table VI) and five from the extracellular leaflet (I–M; Table VI; Fig. 7).

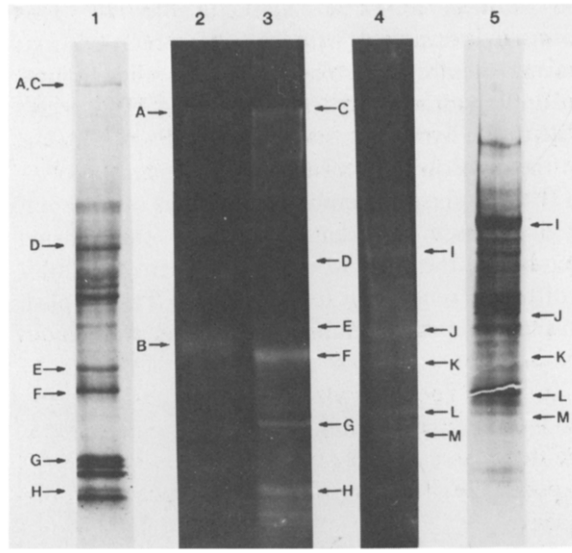

Fig. 7. ANDS and ANMS labeling of plasma membrane proteins. Lanes 2 and 3 represent silica-microbead-derived plasma membrane proteins labeled with ANDS (lane 2) and ANMS (lane 3). Lane 1 shows Coomassie-blue-stained silica-microbead-derived proteins; letters indicate proteins that were labeled with ANDS and ANMS. These were identified by calculating R_F values of the fluorescent bands. Lane 4 shows labeling patterns of two-phase derived plasma membrane labeled with ANMS. Lane 5 identifies Coomassie-blue-stained phase-partitioned plasma membrane proteins that were labeled with ANMS. Lanes 1, 2 & 3, 4, and 5 are all taken from different gels, so direct comparison of M_r on this figure is not possible. Molecular mass standards were run on all of these gels and the molecular weight estimates were calculated from the standards.

The relative mobility (R_F) and molecular mass of the labeled peptides were calculated from the gels and the values are reported in Table VI. Only one cytosol-facing protein, with $M_r = 167000$, was labeled by both ANDS and ANMS. It is possible that peptides D and I, and E and J were labeled from both sides of the membrane, indicating that these may be transmembrane proteins. The M_r estimates were then used to identify the labeled peptides in a Coomassie blue stained gel. Fig. 7 demonstrates which of the peptides were labeled from the cytosolic leaflet and which from the extracellular leaflet of the plasma membrane. It is

also evident from Fig. 7 that many of the major Coomassie-staining peptides were not labeled by either ANDS or ANMS. These may represent integral proteins buried deep in the bilayer where the probes could not penetrate, or proteins having no affinity for the negatively charged sulfonate labels.

Discussion

Plasma membrane-rich fractions were isolated from tomato suspension cultures by two different methods. These isolation techniques, aqueous two-phase partitioning and silica microbeads, yielded plasma membrane with the exterior leaflet exposed and the cytosolic leaflet exposed, respectively.

Since there are no published data on tomato plasma membrane isolation and both phase-partitioning and silica-microbead isolations are relatively recent developments for plasma membrane isolation, we first characterized the plasma membrane fractions obtained by these techniques. Marker enzyme analysis indicated that a single-phase partition step resulted in relatively pure plasma membrane. H^+-ATPase activity, a marker for the plant plasma membrane, was shown to increase significantly over the microsomal activity. This H^+-ATPase activity was stimulated by K^+ over 100%, strongly inhibited by vanadate (approx. 80–90%), and had optimal activity at pH 6.5. Other endomembrane components were predominantly partitioned into the lower phase. When upper phase activity was compared to a microsomal fraction, less than 2% of endoplasmic reticulum and mitochondria marker enzyme activities partitioned into the upper phase. Golgi apparatus was the major contaminant of this plasma membrane fraction with 20–30% of the microsomal activity found in the upper phase. Analysis of the marker enzyme activity for Golgi apparatus for both phase-partitioned vesicles and microsomal vesicles on linear sucrose gradients, however, indicated that the amount of Golgi contamination found in the upper phase was relatively small compared to the amount that would have been found in a typical plasma membrane 'cut' from a gradient. Collectively, these data indicate that this plasma membrane fraction is quite pure when compared to plasma membrane fractions obtained from sucrose gradients. This result is in agreement with Widell [17], Yoshida [18], and other work [15,39,40].

The behavior of phase-partitioned plasma membrane vesicles was characterized on linear sucrose gradients. The distribution of total protein was analyzed, since most of the total protein was plasma-membrane-associated. Although there was a strong peak at 1.1764 g/cm^3, the protein distributed in a peak ranging from 1.1366 to 1.2186 g/cm^3. Yoshida et al. [18], using phase-partitioned plasma membrane vesicles from Orchard grass found a similar distribution ranging from 1.1036 to 1.2025 g/cm^3. These results suggest that the density of plasma membrane isolated from a single species; or in our case, undifferentiated suspension cells of a single species may have a wider density range than previously thought.

Plasma membrane obtained by the silica microbead isolation also was shown to be relatively pure, although endomembrane content was higher than the values obtained from phase partitioning. ATPase activity was enhanced 2–3-fold over microsomal activity. Potassium stimulated this activity, but only by approx. 30%. This suggested that the ATPase activity being measured was not exclusively from the plasma membrane. A pH curve demonstrated a strong optimum at pH 6.5, with some activity at pH 7.5, where tonoplast activity is expected. ATP was the favored substrate; however, ADP, ITP, UTP, UDP and *p*-nitrophenol phosphate were also utilized. When *p*-nitrophenol phosphate was used as a substrate, molybdate was a strong inhibitor. This may indicate the presence of a nonspecific phosphatase in the plasma membrane fraction, which probably explains the lack of K^+ stimulation and substrate specificity.

Marker enzyme activities for other endomembrane components were reduced in the silica microbead plasma membrane fraction. Endoplasmic reticulum and mitochondria were reduced by 85–90% and 88–98%, respectively. Golgi apparatus was reduced by 55–75%. Surprisingly, approx. 15–25% of the original cell protein co-purified with the plasma membrane. This has also been observed in the *Dictyostelium* and red beet systems [12,14] and suggests some degree of non-endomembrane contamination.

Silica microbeads inhibited apparent ATPase activity. This was shown to be due to the silica microbeads binding some essential component in the reaction mixture, probably both ATP and Mg^{2+}. Since the silica microbeads are inhibitory to ATPase activity, this fraction is somewhat richer in plasma membrane than can be estimated from marker enzyme activity alone.

Biochemical characterization of these two plasma membrane fractions was performed by SDS-polyacrylamide gel electrophoretic analysis. Not surprisingly, the peptide pattern from these two fractions showed many homologous peptides when analyzed by silver staining. Coomassie blue staining exhibited less homology, which is probably due to the lower sensitivity of Coomassie blue. Antibodies raised against each plasma membrane preparation also were shown to cross-react with the other. Several glycoproteins were detected in the phase-partitioned plasma membrane, while only faint staining of a few peptides was apparent in the silica microbead plasma membrane. No apparent explanation exists for this latter observation.

Of extreme importance to the establishment of protein topography in a membrane is isolation of the membrane with a defined orientation. Aqueous two-phase partitioning reportedly yields plasma membrane vesicles with the external leaflet exposed [10,29,20,40], while silica microbeads yield sheets of plasma membrane with the cytosolic leaflet exposed (12–14). The external surface of silica microbead-isolated plasma membrane is reportedly impermeant to probes [12–14]. We have established that these conclusions concerning orientation also hold for tomato plasma membrane by two criteria: (1) the effect of Triton X-100 on ATP utilization, and (2) sensitivity to proteolysis. Triton X-100 stimulated H^+-ATPase in the phase-partitioned plasma membrane by 6-fold but had no effect on the silica-microbead plasma membrane fraction. The H^+-ATPase has generally been assumed to have its ATP-binding site on the cytosolic leaflet of the plasma membrane [11,41]. The fact that the H^+-ATPase activity of the phase-partitioned plasma membrane is latent until Triton X-100 is added is strong evidence that these vesicles are tight and oriented with the cytosolic leaflet inside the vesicle. This orientation of phase-partitioned plasma membrane vesicles has also been reported by Larsson [19]. The silica microbead plasma membrane ATPase activity was not stimulated by Triton X-100. Since ATP is completely accessible to the ATP-binding site of the H^+-ATPase, this plasma membrane fraction is oriented with the cytosolic leaflet exposed.

To further establish the orientation of these two plasma membrane fractions, trypsin digest experiments were performed. With different leaflets of the plasma membrane exposed, and hence different proteins or protein domains, one would expect different proteins to be cleaved by the trypsin. This proved to be the case. Furthermore, it was also observed that the silica-microbead plasma membrane was far more sensitive to proteolysis than the right-side-out phase-partitioned plasma membrane. One possible explanation for this might relate to glycoprotein asymmetry across the plasma membrane. It has been established in many systems that glycoproteins are predominantly localized in the external leaflet of the plasma membrane [42,43]. One function of the glycosyl group may be to render the protein more resistant to proteolysis [44,45]. If this is the case, then one might expect the proteins in the exterior leaflet of the plasma membrane to be more resistant to proteolysis.

Having established the orientation of these two plasma membrane fractions, they were used for topographical studies of plasma membrane proteins. The hydrophilic membrane probe, ANDS, labeled two proteins from the cytosolic surface of the plasma membrane. ANMS, which probes more hydrophobic regions of the membrane, labeled six proteins from the cytosolic side. One of these peptides, with a molecular mass of about 167 kDa, was labeled by both ANDS and ANMS.

Phase-partitioned plasma membrane, with the extracellular leaflet exposed, had no proteins labeled by ANDS. The more hydrophobic probe, ANMS, labeled five peptides from the extracellular leaflet. Two of the labeled peptides (D, I and E, J; Table VI) with molecular masses of about 64 and 41 kDa, may have been labeled from both sides of the plasma membrane and hence may represent transmembrane proteins. Although it is not clear why ANDS failed to label any proteins

from the extracellular leaflet of the plasma membrane, one possibility may be that removal of the cell wall or phase partitioning alters the physical parameters of the membrane proteins such that they are not accessible to the ANDS.

In summary, we have isolated plasma membrane with defined orientations from protoplasts isolated from tomato suspension cultures. To our knowledge, this is the first use of phase partitioning to isolate plasma membrane from protoplasts as the starting material. With these defined preparations of plasma membrane, we were able to label specific proteins associated with each surface of the plasma membrane. Probes like the ones used here can be powerful tools to investigate protein topography in the membrane and may prove to be of value when establishing structure and function relationships of specific proteins.

Acknowledgements

We wish to thank Dr. M. Docktor for helpful discussions on the ANDS and ANMS labels. The generous gift from Beatrice Foods is gratefully acknowledged.

References

1 Spanswick, R.M. (1981) Annu. Rev. Plant Physiol. 32, 267–289
2 Willison, J.H.M. and Brown, R.M. (1977) Protoplasma 92, 21–41
3 Anderson, R.L. and Ray, P.M. (1978) Plant Physiol. 61, 723–730
4 Marme, D. (1977) Annu. Rev. Plant Physiol. 28, 173–198
5 Geiger, B. (1983) Biochim. Biophys. Acta 737, 305–341
6 Jacobson, B.S. (1983) Tissue Cell 15, 829–852
7 Randall, S.K. and Ruesink, A.W. (1983) Plant Physiol. 73, 385–391
8 Hodges, T.K. and Leonard, R.T. (1974) Methods Enzymol. 32, 392–406
9 Hodges, T.K., Leonard, R.T., Bracker, C.E. and Keenan, T.W. (1972) Proc. Natl. Acad. Sci. U.S.A. 68, 3307–3311
10 Hodges, T.K. and Mills, D. (1986) Methods Enzymol. 118, 41–54
11 Quail, P.H. (1979) Annu. Rev. Plant Physiol. 30, 452–484
12 Chaney, L.K. and Jacobson, B.S. (1983) J. Biol. Chem. 258, 10062–10072
13 Schmidt, R., Ackermann, R., Kratky, Z., Wasserman, B. and Jacobson, B. (1983) Biochim. Biophys. Acta 732, 421–427
14 Wassermann, B.P., Jacobson, B.S., Schmidt, R., Kratky, Z. and Poole, R.J. (1984) Biochim. Biophys. Acta 775, 57–63
15 Kjellbom, P. and Larsson, C. (1984) Physiol. Plant 62, 501–509
16 Lundborg, T., Widell, S. and Larsson, C. (1981) Physiol. Plant 52, 89–95
17 Widell, S., Lundborg, T. and Larsson, C. (1982) Plant Physiol. 70, 1429–1435
18 Yoshida, S., Kawata, T., Uemura, M. and Niki, T. (1986) Plant Physiol. 80, 152–160
19 Larsson, C., Kjellbom, P., Widell, S. and Lundborg, T. (1984) FEBS Lett. 171, 271–276
20 Kormer, L.E., Kjellbom, P., Larsson, C., and Moller, I.M. (1985) Plant Physiol. 79, 72–79
21 Mueller, T.J. and Morrison, M. (1974) J. Biol. Chem. 249, 7568–7573
22 Philips, D.R. and Morrison, M. (1971) Biochemistry 10, 1766–1771
23 Cabantchik, I.Z., Balskin, M., Breuer, W. and Rothstein, A. (1975) J. Biol. Chem. 250, 5130–5136
24 Maddy, A.H. (1964) Biochim. Biophys. Acta 88, 390–399
25 Berg, H.C. (1969) Biochim. Biophys. Acta 183, 64–78
26 Juliano, R.L. and Rothstein, A. (1971) Biochim. Biophys. Acta 249, 227–235
27 Gahmborg, C.G. and Andersson, L.C. (1977) J. Biol. Chem. 252, 5888–5894
28 Gahmborg, C.G. and Hakomori, S.F. (1973) J. Biol. Chem. 248, 4311–4317
29 Galbraith, D.W. and Northcote, D.H. (1977) J. Cell Sci. 24, 245–310
30 Perlin, D.S. and Spanswick, R.M. (1980) Plant Physiol. 65, 1053–1057
31 Docktor, M.E. (1979) J. Biol. Chem. 254, 2161–2164
32 Linsmaier, E.M. and Skoog, F. (1965) Physiol. Plant 18, 100–117
33 Gogstad, G.O. and Krutnes, M.B. (1982) Anal. Biochem. 126, 355–359
34 Laemmli, U.K. (1971) Nature 227, 680–685
35 Oakley, B.A., Kirsch, D.R. and Norris, N.R. (1980) Anal. Biochem. 105, 361–363
36 Faye, L. and Chrispeels, M.J. (1985) Anal. Biochem. 149, 218–224
37 Towbin, H. Staehlin, T. and Gordon, J. (1979) Proc. Natl. Acad. Sci. U.S.A. 76, 4350
38 Sze, H. (1984) Physiol. Plant 62, 683–691
39 Uemura, M. and Yoshida, S. (1984) Plant Physiol. 75, 818–826
40 Moller, I.M. and Lundborg, T. (1985) Planta 164, 354–361
41 Cantley, LC, Resh, M.D. and Guidotti, G. (1978) Nature 272, 552–554
42 Atkinson, P.H. and Summers, D.F. (1971) J. Biol. Chem. 246, 5162–5175
43 Gahmborg, C.G. (1981) in Membrane Structure (Finean, J.B. and Michel, R.H., eds.), pp. 127–160, Elsevier, Amsterdam
44 Olden, K., Bernard, B.A., Humphries, M.J., Yeo, T.K., Yeo, K.T., White, S.L., Newton, S.A., Bauer, H.C. and Parent, J.P. (1985) Trends Biochem. Sci. 10, 78–82
45 Winkler, J.R. and Segal, H.L. (1984) J. Biol. Chem. 259, 1958–1962