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The Effects of Salt Stress on Polypeptides in Membrane Fractions from Barley Roots[1]

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ABSTRACT

Cell fractions enriched in endoplasmic reticulum, tonoplast, plasma membrane, and cell walls were isolated from roots of barley (*Hordeum vulgare* L. cv CM 72) and the effect of NaCl on polypeptide levels was examined by two-dimensional (2D) polyacrylamide gel electrophoresis. The distribution of membranes on continuous sucrose gradients was not significantly affected by growing seedlings in the presence of NaCl; step gradients were used to isolate comparable membrane fractions from roots of control and salt-grown plants. The membrane and cell wall fractions each had distinctive polypeptide patterns on 2D gels. Silver-stained gels showed that salt stress caused increases or decreases in a number of polypeptides, but no unique polypeptides were induced by salt. The most striking change was an increase in protease resistant polypeptides with isoelectric points of 6.3 and 6.5 and molecular mass of 26 and 27 kilodaltons in the endoplasmic reticulum and tonoplast fractions. Fluorographs of 2D gels of the tonoplast, plasma membrane, and cell wall fractions isolated from roots of intact plants labeled with [^{35}S]methionine *in vivo* also showed that salt induced changes in the synthesis of a number of polypeptides. There was no obvious candidate for an integral membrane polypeptide that might correspond to a salt-induced sodium-proton antiporter in the tonoplast membrane.

Saline soils inhibit the growth of crop plants because of the effects of water stress, ion toxicity, ion imbalance, or a combination of these factors. The extent of this reduction in growth is dependent on the species of plant, the salinity level, and the ionic composition of the soil. Barley is the most salt-tolerant grain of major agricultural importance (19). The ability of barley to tolerate relatively high concentrations of NaCl is correlated with the maintenance of a high ratio of K^+/Na^+ in the cytoplasm, a high ratio of Na^+/K^+ in the vacuoles, exclusion of Na^+ at the PM,[2] accumulation of Na^+ in mature leaves, and redistribution of K^+ from mature to young leaves (14, 23). The selective transport of K^+ and Na^+ undoubtedly involves the PM and the tonoplast. Previously, we observed (13) that NaCl altered polypeptide synthesis in barley roots and preliminary cell fractionation studies showed that some of these changes occurred in polypeptides located in the microsomal membrane fraction. We recently developed methods to isolate fractions enriched in PM, tonoplast, and ER from barley roots (5–7). We are utilizing these enriched membrane fractions to look for salt-induced changes in membrane polypeptides and ion transport that can be correlated with the ability of specific barley cultivars to tolerate salt.

In this study we examined the effect of salt treatment on polypeptide composition of the different membrane fractions. Because the PM and tonoplast may have important roles in the ability of barley to tolerate salt (14, 23), we were interested in identifying specific polypeptide changes in these membranes. The most striking effect of salt on barley roots that we have observed so far is the induction of a Na^+/H^+ exchange in the tonoplast membrane of barley roots when plants were grown in 100 mM NaCl (9). Therefore, we expected to observe an increase in or induction of one or more polypeptides in the tonoplast membrane of salt grown plants that might correspond to the Na^+/H^+ antiporter.

The identities and functions of the polypeptides whose synthesis is altered by salt stress are not known. A first step in determining the function of unknown proteins is to ascertain their intracellular location (4). We were particularly interested in two polypeptide pairs with pIs of approximately 6.3 and 6.5 and M_r of 26 and 27 kD that increased significantly with salt stress (13). The 26- and 27-kD polypeptides were associated with both the soluble and the microsomal membrane fractions. This distribution would result if they were peripheral membrane proteins that were associated with a specific membrane *in vivo*, soluble proteins that became associated with the membranes during homogenization of the tissue, or soluble proteins within the ER, Golgi apparatus, or transition vesicles. The experiments in this paper were designed to characterize the polypeptide patterns of membrane fractions enriched in ER, tonoplast, and PM, to identify salt-induced polypeptide changes in the membrane fractions, to clarify the relationship of the 26- and 27-kD polypeptides to the membrane fractions, and to search for candidates for salt-induced transport proteins.

MATERIALS AND METHODS

Plant Material. Seeds of barley (*Hordeum vulgare* L. cv CM 72) were sown above an aerated solution containing full strength nutrients (8) with or without 100 mM NaCl as described previously (13).

Linear Sucrose Gradients. Microsomal membranes were prepared, separated on linear sucrose gradients, and fractions were collected as described by DuPont and Hurkman (6). ATP-dependent proton transport and NADH Cyt *c* reductase activities were assayed as described previously (6). Transport was assayed at 24°C with 2 μM acridine orange, 1 mM ATP, 1 mM $MgCl_2$, and 0.25 M sucrose in 5 mM Pipes-KOH (pH 7.0) with 50 mM KCl or 50 mM KNO_3 as indicated in the figures.

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[2] Abbreviations: PM, plasma membrane; PM_1, membrane fraction collected from the 30/40% interface of a discontinuous sucrose gradient; PM_2, membrane fraction collected from the 34/40% interface of a discontinuous sucrose gradient; TG, tonoplast-Golgi membrane; PMSF, phenylmethylsulfonyl fluoride; 2D, two-dimensional; DOC, deoxycholate; 1D, one-dimensional; pI, isoelectric point.

Discontinuous Sucrose Gradients. The ER, TG, and PM_1 fractions were isolated using sucrose step gradients and washed with KCl as previously described (5). The ER fraction was collected from the sample/22% sucrose interface, the TG fraction from the 22/30% sucrose interface, and the PM_1 fraction was collected from the 30/40% sucrose interface. The proteins in the washed, pelleted membrane fractions were prepared for 2D PAGE as described below.

***In Vivo* Labeling.** Intact plants were used for the labeling studies to avoid wound-induced changes in protein synthesis (11, 26, 28) that could occur if excised root segments were used. Plants were placed in small vials (three plants per vial) with the roots immersed in 4mL of the same solution that the plants were grown in; 50 μg/mL chloramphenicol and 70 μCi of [^{35}S]methionine (approximately 1100 Ci/mmol or 41 TBq/mmol; New England Nuclear[3]) were then added. Forty-two plants were labeled for each of the control and salt treatments. Labeling was performed for a period of 3 h at room temperature (approximately 24°C).

Isolation of Labeled Membranes. Membranes were prepared from the labeled roots by a modification of the procedure of DuPont *et al.* (7). The roots were excised, rinsed three times with ice cold water, and homogenized in 25 mL of grinding mix containing 150 μM PMSF to inhibit proteases. The homogenate was filtered through four layers of cheesecloth and the material retained was stored at −70°C for isolation of cell walls (below). The 10,000 to 100,000*g* membrane pellet was suspended in 1 mL of suspension buffer (0.25 M sucrose and 1 mM DTT in 5 mM Pipes-KOH [pH 7.2]) and layered onto a step gradient consisting of 1.0 mL each of 22, 30, 34, and 40% (w/w) sucrose in a Quick Seal tube (Beckman). The gradients were centrifuged for 30 min at 115,000*g* in a Beckman VTi80 vertical rotor. The 22/30% (TG) and 34/40% (PM_2) interfaces were collected with a syringe and washed with a 150 mM KCl solution as previously described (7). The proteins in the KCl-washed pellets were prepared for 2D PAGE as described below. The sample/22% interface was not collected because there was not enough protein to analyze on 2D gels. Polypeptide patterns on Coomassie-stained 1D gels of unlabeled membrane fractions prepared by this method were identical to those obtained previously (7).

Isolation of Labeled Cell Walls. Cell walls were prepared by a modification of the method of Nagahashi *et al.* (18). The material retained from the membrane isolation procedure was ground to a fine powder in liquid nitrogen using a mortar and pestle. Ten mL of grinding mix containing 2 mM PMSF were added to the powder and the powder was homogenized by continuous grinding as the mortar warmed to room temperature. When the homogenate reached approximately 4°C, it was transferred to a 15 ml Corex tube (Corning). All further operations were performed at 2 to 4°C. The homogenate was centrifuged at 250*g* for 5 min in a Sorval HB-4 rotor. The pellet was suspended in 5 mL of grinding mix, placed on ice, and sonicated for 5 min with a 50% pulsed cycle at an intensity of 3 (Sonifer Cell Disruptor 350; Branson Sonic Power Co.). The suspension was centrifuged, and the pellet was resuspended in grinding mix and sonicated once more. The suspension was centrifuged at 250*g* for 5 min. The pellet was suspended in 3 mL of grinding mix, layered onto 20 mL of 60% (w/w) sucrose, and centrifuged at 15,000*g* for 60 min. The pellet was washed two times with 20 mL of water. The final pellet contained purified cell walls as determined by light microscopy of samples stained with methylene blue-basic fuchsin (2). The cell wall proteins were prepared for 2D PAGE as described below.

[3] Mention of a specific product name by the U.S. Department of Agriculture does not constitute an endorsement and does not imply a recommendation over other suitable products.

Detergent Washes. Peripheral proteins were removed from TG membranes by a DOC wash procedure previously used for purification of the tonoplast ATPase of corn (16) or a Triton X-100 wash procedure previously used for purification of the plasma membrane ATPase of tomato (1). The TG fraction was divided into three aliquots, which were diluted with the KCl wash solution to 28 mL each, and centrifuged at 100,000*g* for 35 min in a Beckman 42.1 rotor. One pellet was suspended immediately in the electrophoresis extraction buffer (below); this was the control, or total membrane, sample. The other two pellets were suspended in 0.4 mL each of suspension buffer. One was diluted with an equal volume of a buffered solution of DOC to a final concentration of 0.15% DOC, 0.15 M KCl, and 2 mM DTT in 25 mM Tris-HCl (pH 8.0). The other was diluted with an equal volume of a buffered solution of Triton X-100 to a final concentration of 0.1% Triton X-100, 0.5 M KBr, 2 mM DTT and 25 mM Tris-HCl (pH 8.0). The final protein concentration in each of the detergent washes was approximately 1 mg/mL. The membrane samples were incubated on ice for 15 min, then divided into smaller aliquots and centrifuged in an Airfuge (Beckman) at 178,000*g* and 4°C for 30 min. The proteins in the supernatants and pellets were prepared for 2D PAGE as described below.

Protease Treatments. The TG fraction was divided into three aliquots, washed with KCl, and three pellets were obtained as above. Each pellet was suspended in 0.3 mL of suspension buffer. One sample (control) was made to 500 μL with suspension buffer and placed on ice for 30 min. The second sample was made to 150 μg/mL each of chymotrypsin and trypsin and 1% DOC in a volume of 450 μL. Following incubation for 30 min at room temperature, protease activity was blocked by addition of PMSF to 160 μg/mL and aprotinin to 180 μg/mL in a final volume of 500 μL. The third sample was made to 150 μg/mL protease K and 1% DOC in a volume of 450 μL. Following incubation for 30 min at 30°C, protease activity was blocked by addition of PMSF to 250 μg/mL in a final volume of 500 μL. The samples were prepared for 2D PAGE as described below.

2D PAGE. The membrane proteins were prepared for isoelectric focusing by the phenol partitioning method of Schuster and Davies (25) as described by Hurkman and Tanaka (12, 13). Proteins from pellets of unlabeled membrane fractions were prepared exactly as described by Hurkman and Tanaka (12). Pellets of radioactively labeled membranes, detergent washed membranes, and cell walls were suspended in 0.5 mL of extraction buffer (0.7 M sucrose, 0.5 M Tris, 30 mM HCl, 50 mM EDTA, 0.1 M KCl, 2% [v/v] 2-mercaptoethanol; PMSF was added to a final concentration of 2 mM just before use). An equal volume of 2X extraction buffer was added to the protease treated membranes and the supernatants of detergent washed membranes; they were then made to a final volume of 1.5 mL with extraction buffer. The proteins in all samples were partitioned into phenol, precipitated with 0.1 M ammonium acetate in methanol, and solubilized in 9 M urea, 4% (v/v) NP-40, 2% (v/v) 2-mercaptoethanol, and 2% (v/v) ampholytes as described previously (12).

2D PAGE was performed according to O'Farrell (21) with the modifications of Hurkman and Tanaka (12, 13). The first dimension isoelectric focusing gels contained pH 3.5 to 10 and pH 5 to 7 (1:4, v/v) ampholytes. Samples containing approximately 70 μg of protein were loaded for silver-stained gels. For the detergent washes, the proteins extracted from the supernatants and pellets were solubilized in the same volume and samples of equal volumes were loaded onto the focusing gels. Samples containing approximately 200,000 cpm were loaded for fluorography. The second dimension SDS gels contained 10% acrylamide. The 2D gels were fixed and silver stained by the method of Morrissey (17) and destained by the method of Oakley *et al.* (20) or processed for fluorography by the method described by

Garrels (10). Fluorographs were made by exposure of Kodak X-Omat AR film to the dried gels for 3 d at −70°C. Because the polypeptide patterns were highly reproducible, fluorographs could be superimposed on silver-stained gels to positively identify labeled polypeptides.

RESULTS

Characterization of Membrane Fractions. Membrane fractions enriched in ER, tonoplast, and PM were isolated from roots of control and salt-treated plants by sucrose gradient centrifugation. Preliminary experiments were performed to determine if the distribution of membranes on continuous sucrose gradients was altered significantly by growing seedlings in the presence of NaCl. Linear sucrose gradients were used to separate membranes isolated from roots of plants grown in full nutrients or full nutrients with 100 mM NaCl. NADH Cyt *c* reductase was assayed as a marker for the ER, nitrate-sensitive proton transport as a marker for the tonoplast membranes, and nitrate-insensitive proton transport as a marker for the PM (5–7). The distributions of ER, tonoplast, and PM activities were similar on gradients from control (Fig. 1) or salt-treated (Fig. 2) roots, with a peak of ER NADH Cyt *c* reductase from 1.08 to 1.10 g/cm^3, a peak of tonoplast-type H^+-ATPase from 1.10 to 1.13 g/cm^3, and a peak of PM H^+-ATPase from 1.14 to 1.18 g/cm^3. There were some differences in the absorbance traces, but this varied between experiments and may not be significant. The distributions of the marker enzymes on the linear sucrose gradients indicated that step gradients of the same sucrose composition could be used to isolate comparable membrane fractions from roots of plants grown in full nutrients or full nutrients plus 100 mM NaCl.

Previously, we demonstrated (5) that the membrane fraction collected at the sample/22% sucrose interface (ER) was enriched in the ER marker, NADH Cyt *c* reductase. The membranes collected at the 22/30% sucrose interface were enriched in nitrate-inhibited ATPase and also contained polypeptides of 60 and 70 kD that reacted with antibodies to the 60– and 70–kD

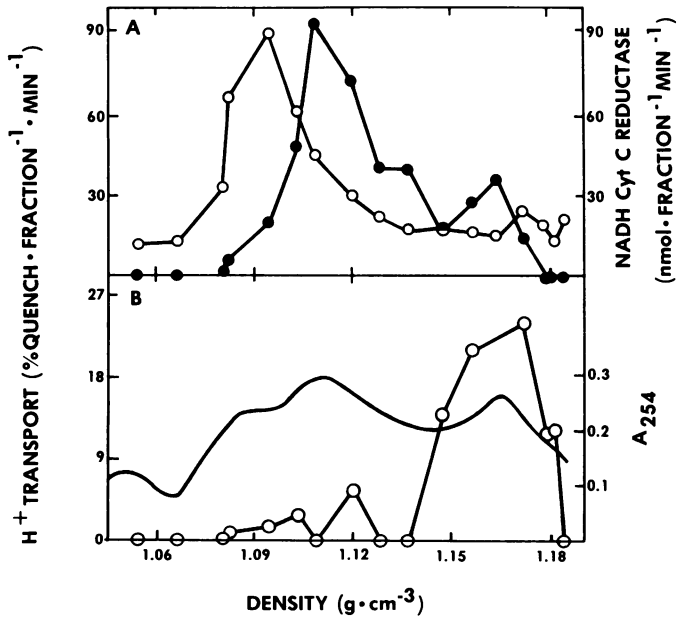

FIG. 1. Distribution of enzyme activities in a continuous sucrose gradient of a 10,000*g* to 80,000*g* pellet prepared from barley roots of control plants. A, NADH Cyt *c* reductase (○); ATP-dependent proton transport assayed n the presence of 50 mM KCl (●). B, UV trace (——); ATP-dependent proton transport assayed in the presence of 50 mM KNO_3 (○).

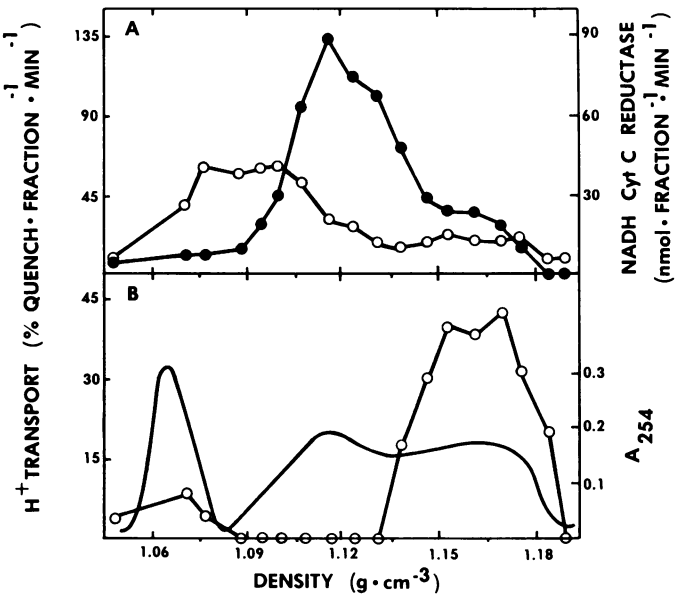

FIG. 2. Distribution of enzyme activities in a continuous sucrose gradient of a 10,000*g* to 80,000*g* pellet prepared from barley roots of plants grown in the presence of 100 mM NaCl. The symbols in A and B are the same as in Figure 1.

subunits of the tonoplast ATPase from corn roots and beet roots (7). Because glucan synthase I, a marker for Golgi membranes, was also present (5), this fraction was referred to as the TG fraction. The membranes that were collected at the 30/40% sucrose interface (PM_1) were enriched in vanadate-inhibited ATPase (5). Contamination by the nitrate-inhibited ATPase was reduced by collecting the membranes on a 34/40% interface and discarding the membranes on the 30/34% interface. The membranes on the 34/40% interface (PM_2) contained a 100-kD polypeptide that reacted with antibodies to the purified PM ATPases from corn roots and from *Neurospora* (7). The PM_2 fraction also contained a set of polypeptides that ranged in M_r from 100 to 200 kD that reacted with mAb to an arabinogalactan protein from the PM of tobacco cells (7).

Polypeptide Patterns of the Membrane Fractions. 2D PAGE was used to compare the polypeptide patterns of the ER, TG, and PM_1 fractions. The purpose of these comparisons was to identify polypeptides (designated by numbers in Fig. 3) and groups of polypeptides (designated by letters in Fig. 3) that were specific to or enriched in each fraction and to determine polypeptide changes induced by salt (Fig. 3). Table I makes it possible to compare easily the effects of different treatments on individual polypeptides and groups of polypeptides and serves as a useful summary of the data. As predicted by the distributions of marker enzymes on the sucrose gradients, 2D PAGE showed that the polypeptide patterns for the ER, TG, and PM_1 fractions were different on silver-stained gels (Fig. 3, A, C, and E). Although the polypeptide patterns of the fractions were complex, two generalizations were readily apparent. First, the PM_1 fraction contained the greatest number of polypeptides; gels of the PM_1 fraction contained approximately 570 polypeptides compared to approximately 420 in the TG fraction and 260 in the ER fraction. Second, there were more polypeptides with basic pIs in gels of the TG and PM_1 fractions than in gels of the ER fraction.

Polypeptides and groups of polypeptides overlapped between the fractions, just as the peaks of enzyme activity overlapped on the linear sucrose gradients (Figs. 1 and 2). Because of this overlap, we made a detailed comparison of the 2D gels and assigned polypeptides to the fraction in which they were enriched. Polypeptides enriched in the ER fraction included 1, 2, 4, 6 to

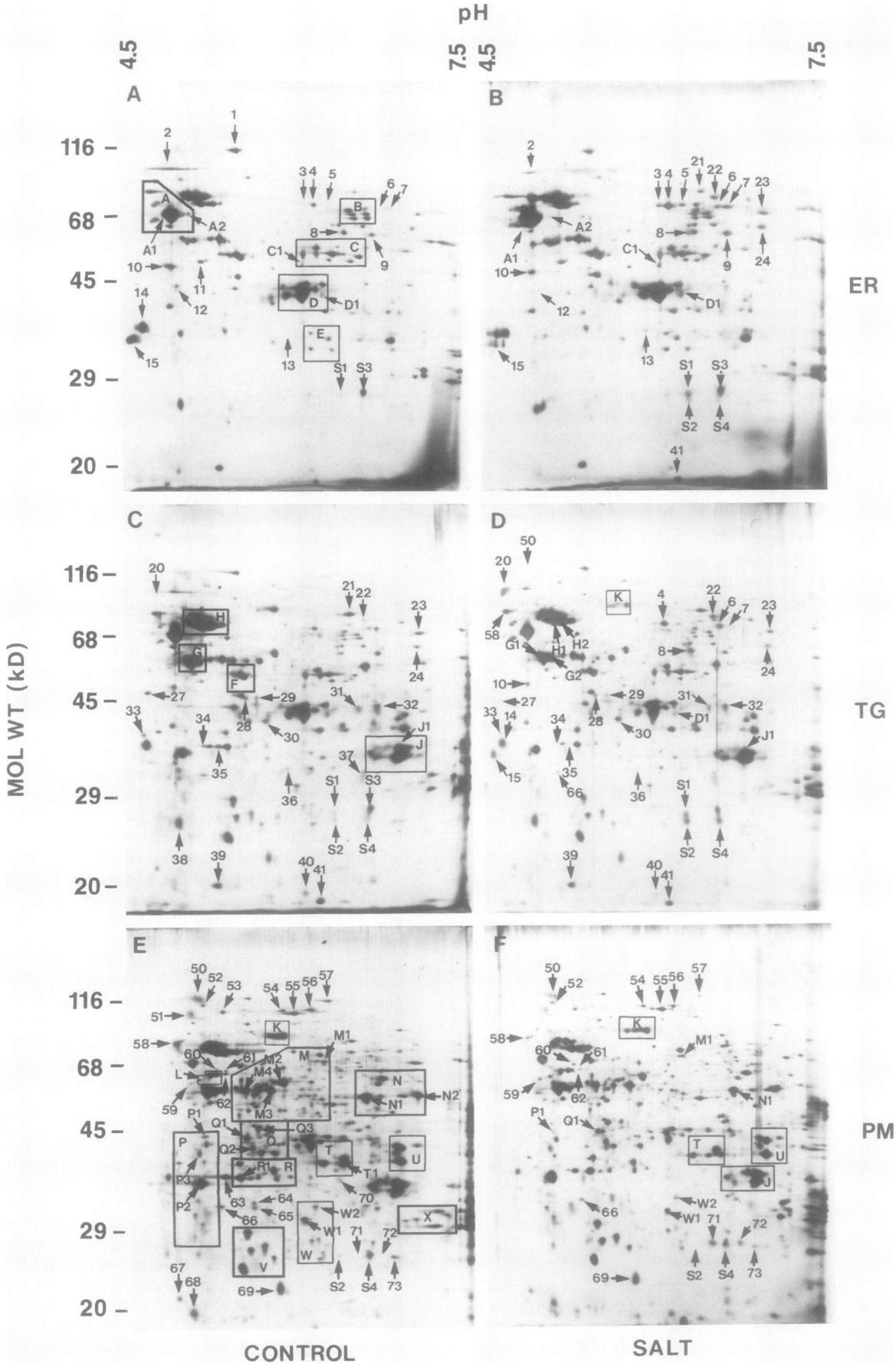

FIG. 3. Characterization of ER, TG, and PM enriched fractions by 2D gel electrophoresis and the effect of NaCl on polypeptide patterns. A, C, and E, silver stained gels of ER, TG, and PM fractions, respectively, isolated from roots of control plants. The numbers and letters in each panel designate polypeptides and groups of polypeptides that are enriched in each fraction. B, D, and F, silver stained gels of ER, TG, and PM fractions, respectively, isolated from roots of plants grown with 100 mM NaCl added to the nutrient solution. The numbers and letters designate polypeptides and groups of polypeptides that increase or decrease with salt treatment (see Table I).

Table I. *Effect of Salt, Detergents, and Proteases on Polypeptides of Membrane Fractions Enriched in ER, Tonoplast (TG), and PM*

The fractions were isolated from roots of barley plants grown in the presence or absence of 100 mM NaCl. Polypeptides (designated by numbers) and groups of polypeptides (designated by letters) are as identified in Figure 3. Polypeptides 54 and 55 correspond to the PM ATPase, G1 and G2 to the 60-kD subunit of the tonoplast ATPase, and H1 and H2 to the 70-kD subunit of the tonoplast ATPase. Data for the table are taken from the 2D gels in Figures 3 to 6.

Protein No.	Effect of NaCl					Detergent Solubilization[c]		Protease Resistant[d]	
	Silver-stained gel[a]			Fluorograph[b]		TX-100	DOC	C + T	PK
	ER	TG	PM	TG	PM				
1	Same	Same							
2	DEC	Same							
3	INC								
4	INC	inc				Pellet	Pellet		
5	INC								
6	INC	INC		Same	Same				
7	INC	dec							
8	INC	inc		inc					
9	INC	Same							
10	DEC	dec							
11	Same								
12	DEC								
13	INC								
14	Same	DEC		DEC	dec	Both	supt		
15	DEC	inc				Both	supt		
20		INC							
21	inc	Same				Pellet	Pellet		
22	inc	INC		DEC	inc				
23	INC	dec				Pellet	Pellet		
24	INC	dec				Pellet			
27		DEC							
28		DEC							
29		DEC							
30		INC							
31		DEC							
32		DEC							
33		DEC							
34		DEC							
35		DEC							
36		DEC							
37		Same				Pellet	Pellet		
38	Same	Same		dec		Pellet	Both		
39	Same	DEC							
40	Same	DEC							
41	inc	DEC							
42				INC					
43				INC					
50		dec	dec	INC	inc	Pellet	supt		
51		Same	Same	inc	INC	Pellet	supt		
52			dec						
53		Same	Same		INC				
54			dec						
55			Same						
56			dec						
57			DEC						
58		dec	DEC		DEC				
59			INC						
60			INC						
61			DEC						
62		Same	DEC						
63		Same	Same						
64			Same						
65			Same		Same				
66		dec	DEC		DEC	Pellet	Pellet		
67		Same	Same			Pellet	Pellet		
68			Same			Pellet			
69		Same	dec			Pellet			
70			Same		DEC				
71			INC						
72			INC						
73			DEC						
74					DEC				
75					Same				
A	Same					supt	supt	Yes	Yes
A1	INC	Same	Same	Same					
A2	DEC								
B	Same	Same	Same			Both	supt		
C		Same	Same	Same	Same				
C1	INC	Same							
D			Same	dec	dec	supt	supt		
D1	INC	dec							
E	Same	Same							
F	Same	Same		Same					
G	Same	Same	Same			Both	Both	Yes	No
G1		Same		inc	inc	Both	Both		
G2		Same		inc	inc	Both	Both		
H	Same	Same	Same	Same	Same	Both	Both	Yes	No
H1		Same				Both	Both		
H2		Same				Both	Both		
J			inc		Same	Pellet	Pellet		
J1		dec		Same					
K		dec	DEC			Pellet	Pellet		
L			Same						
M		Same							
M1			dec	Same	INC				
M2			Same		dec	Pellet	Both		
M3			Same						
M4			Same	Same	Same				
N			Same						
N1			DEC			Pellet	Both		
N2			Same		DEC				
P		Same							
P1			DEC	DEC	Same				
P2		Same	Same	DEC	Same				
P3			Same	DEC					
Q		Same				supt			
Q1		Same	DEC	Same	INC	Both	Pellet		
Q2		Same	Same	Same	INC				
Q3		Same	Same	Same	Same				
R		Same	Same		Same				
R1		Same	Same	inc	Same				
S1	INC	INC		INC	INC	supt	supt	Yes	Yes
S2	inc	INC	dec	INC	INC	supt	supt	Yes	Yes
S3	inc	Same	Same	Same	inc	Pellet	Pellet	No	No
S4	inc	INC	dec	Same	Same	supt	supt	Yes	Yes
T		Same	DEC						
T1					Same				
U		Same	dec						
V		Same	Same			Both	supt		
W1			DEC			Pellet	Pellet		
W2			DEC			Pellet	Pellet		
X		Same	Same			Pellet	Pellet		

[a] DEC, large decrease; dec, small decrease; INC, large increase; inc, small increase; Same, no change; no entry, not present in the cell fraction. [b] Abbreviations are as in a. An entry is made for all polypeptides that were labeled with [^{35}S]methionine. Not all polypeptides that stained with silver were labeled. Some polypeptides that were labeled (42, 43, 74, 75) were not detected with silver stain. [c] The distribution of polypeptides is indicated as follows: supt, supernatant; both, pellet and supernatant. The majority of the TG polypeptides were present in the supernatant following detergent treatments; no entry indicates that the polypeptide was located in the supernatant. Only the supernatant polypeptides mentioned in the text are indicated in the table. TX-100, Triton X-100. [d] No entry, the polypeptide was susceptible to both protease treatments; yes, protease resistant; no, susceptible to the protease treatment; C + T, chymotrypsin plus trypsin; PK, protease K.

15, S3, and groups A to E (Fig. 3A). Polypeptides enriched in the TG fraction included 20 to 24, 27 to 41, S1, S2, and groups F to H and J (Fig. 3C). Polypeptides enriched in the PM_1 fraction included 50 to 73, S4, and groups K to N, P to R, and T to X (Fig. 3E). Some polypeptides (A–D, G, and H) were present in all three fractions, but most polypeptides or groups of polypeptides were found principally in a single fraction with some overlap into an adjacent fraction. Thus groups E and F overlapped the ER and TG fractions, and groups J, K, P, T, U, and V overlapped the TG and PM_1 fractions. Groups K, P, and U increased and groups A to D decreased in amount from the ER fraction to the TG fraction to the PM_1 fraction. Polypeptides and groups of polypeptides assigned to membrane fractions (Fig. 3, A, C, and E) will be called ER, TG, or PM polypeptides for ease of discussion, although it is recognized that their origins have not been verified.

Only a few of the barley membrane polypeptides have been identified: the 60– and 70-kD subunits of the tonoplast ATPase (G1, G2 and H1, H2, respectively, Fig. 3D) and the 100-kD catalytic subunit of the PM ATPase (54 and 55, Fig. 3E) (7). Possible explanations for the detection of two polypeptides for each ATPase subunit were discussed previously (7). Although polypeptides with similar M_r and pI to the tonoplast ATPase subunits were also present in the ER and PM fractions, only slight immunological cross-reaction with these polypeptides was detected (7). No polypeptides with a combination of M_r and pI similar to that of the PM ATPase were present in the ER and TG fractions.

Salt-Induced Changes in Constitutive Polypeptides. A comparison of the polypeptide patterns of ER, TG, and PM_1 fractions prepared from roots of control (Fig. 3, A, C, and E) and salt grown (Fig. 3, B, D, and F) plants was made on silver-stained 2D gels to determine the effect of NaCl on the levels of constitutive polypeptides. Salt did not induce the synthesis of unique polypeptides or cause polypeptides to disappear, but did cause increases or decreases in the amounts of some of the polypeptides in each of the membrane fractions (Table I). In the ER fraction (Fig. 3B), a group of high M_r, basic polypeptides increased (3–9) as well as polypeptides 13, A1, C1, D1, S1, S2, S3, and S4 while several of the more acidic polypeptides (2, 10, 12, 15, and A2) decreased. Other polypeptides that increased in the ER fraction included polypeptides 21 to 24, which were TG polypeptides. In the TG fraction (Fig. 3D), polypeptides 20, 22, 30, S1, S2, and S4 increased and polypeptides 23, 24, 27 to 29, 31 to 36, 39 to 41, and J1 decreased. Changes in the amounts of the 60- (G1 and G2) and 70-kD (H1 and H2) subunits of the tonoplast ATPase were not observed in response to salt treatment. In the PM_1 fraction (Fig. 3F), polypeptides 59, 60, 71, and 72 increased and polypeptides 50, 52, 56 to 58, 61, 62, 66, 69, 73, M1, N1, P1, Q1, S2, S4, W1, and W2 and groups K, T, and U decreased. A slight decrease in polypeptide 54 was observed as a result of the salt treatment; this was previously identified as a subunit of the PM ATPase (7). Some of the polypeptides that were present in two fractions (ER and TG or TG and PM) increased (Table I: 4, 6, 8, 22) or decreased (Table I: 10, 50, 58, 66, K) in both. Several other polypeptides increased in one fraction yet decreased in another (Table I: 7, 15, 23, 24, 41, D1); these differences may be indicative of shifts in vesicle subpopulations between fractions.

Of all the polypeptide changes induced by salt, the most obvious were associated with polypeptides S1, S2, and S4. Polypeptide S1 increased greatly in the ER fraction. Polypeptides S1, S2, and S4 increased greatly in the TG fraction. Polypeptide S4 was prominent in the PM_1 fraction, but it did not change in the PM_1 fraction in response to salt. Polypeptide S3 was prominent in both the ER and TG fractions, but it increased slightly only in the ER fraction.

Detergent Washes. The effect of detergent and protease treatments on polypeptide distributions was tested in the TG fraction from salt-grown plants because the S1 to S4 polypeptides were located predominantly in this fraction. When the TG fraction was treated with 0.1% Triton X-100, a number of polypeptides remained in the detergent-washed pellet (Fig. 4A, Table I), but the majority of the polypeptides were released to the supernatant (Fig. 4B, Table I). Polypeptides in the pellet that were specific to the TG fraction were 21, 23, 24, 37, 38, S3, and group J. The other polypeptides in the pellet were ER and PM polypeptides and are indicated in Table I. A group of polypeptides located on the basic side of group X was also present in the pellet (BP on Fig. 4, A and C). In addition to the polypeptides clearly associated with the pellet or the supernatant, some polypeptides and groups of polypeptides were present in both the pellet and the supernatant (compare Fig. 4, A and B; Table I). Polypeptides in the pellet whose synthesis decreased with salt treatment included polypeptides 23 and 24 of the TG. Results were similar when the TG fraction was washed with 0.15% DOC in place of Triton X-100, but fewer polypeptides were associated with the washed pellet (compare Fig. 4, A and C; Table I). These included polypeptides 21, 23, 37, S3, and group J of the TG.

Both the Triton X-100 and DOC treatments released S1, S2, and S4 from the TG membrane vesicles. Polypeptides that corresponded in M_r and pI to the 60– (G1 and G2) and 70-kD (H1 and H2) subunits of the tonoplast ATPase appeared to be distributed equally in both fractions. Following the detergent washes, the only polypeptide whose synthesis increased with salt treatment was polypeptide 4 of the ER.

Protease Treatments. Although S1, S2, and S4 were released from the membrane vesicles by detergent washes, this was not sufficient information to determine if these polypeptides were contained within sealed vesicles or adhering to the vesicle surface. The TG fraction from salt-grown plants was treated with proteases to determine the accessibility of polypeptides to the external medium. Polypeptides S1 to S4 were not hydrolyzed (data not shown), which suggested that the polypeptides were not accessible to the proteases. To test the hypothesis that S1, S2, and S4 were contained within the interior of the membrane vesicles, the TG fraction was treated with proteases in the presence of detergent. When the TG fraction was treated with 1% DOC and chymotrypsin plus trypsin (Fig. 5A), polypeptides were hydrolyzed to such an extent that few polypeptides characteristic of the membrane fractions were identifiable (Table I). However, S1, S2, and S4 were still present. Other polypeptides that could be identified included group A of the ER and groups G and H of the TG.

When the TG fraction was treated with 1% DOC and protease K, fewer polypeptides were detected on the 2D gels (Fig. 5B), but S1, S2, and S4 and group A remained. Comparison of the gels for both protease treatments also indicated the presence of a number of common unidentifiable polypeptides (arrows, Fig. 5, A and B). Different preparations of the TG fraction were used for the experiments shown in Figure 5, A and B. Polypeptides S1 to S4 increased more in the membranes used for 5A than in those used for 5B. In both preparations, S1, S2, and S4 were present in similar amounts in gels of control and protease treated membranes. The results with the protease treatments showed that polypeptides S1, S2, and S4 were resistant to proteases. Therefore it was not possible to distinguish whether they were proteins associated with the exterior or the interior of the membrane vesicles by this method.

Polypeptide Patterns of [^{35}S]Methionine-Labeled Fractions. Previously, we found (13) that the effects of salt on polypeptide synthesis were most easily detected when proteins were labeled *in vivo*. To determine the effects of salt on the synthesis of newly made membrane proteins, membranes were prepared from roots of control and salt-grown plants that had been labeled *in vivo*

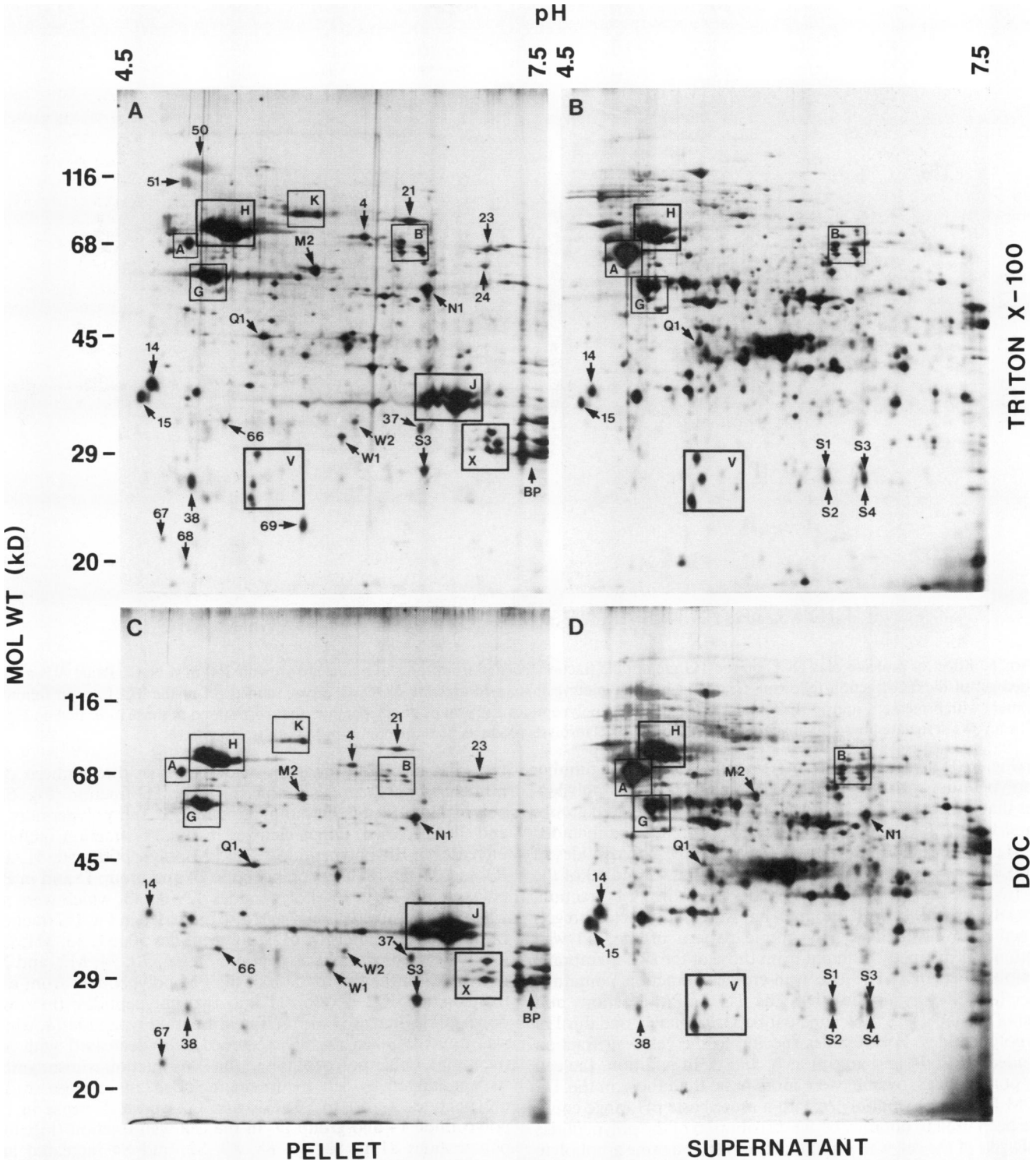

FIG. 4. Effect of detergent treatments on the TG fraction isolated from roots of plants grown with 100 mM NaCl. A and B, Silver stained gels of the pellet and supernatant, respectively, following treatment of the TG fraction with 0.1% Triton X-100. C and D, Silver stained gels of the pellet and supernatant, respectively, following treatment of the TG fraction with 0.15% DOC. The numbers and letters designate polypeptides and groups of polypeptides that correspond to those indicated on Figure 3. BP, basic polypeptides.

with [^{35}S]methionine and the polypeptide patterns were analyzed by fluorography of 2D gels. Plants were incubated for 3 h with the [^{35}S]methionine because this was sufficient time to label polypeptides S1 to S4. A cell wall-enriched fraction was included because the S1 to S4 polypeptides were also present in a 3000*g* fraction (WJ Hurkman, CK Tanaka, unpublished observation). The ER fraction was omitted because it was present in amounts too low to analyze on 2D gels. Comparison of the fluorographs (Fig. 6, A and C) with the silver-stained gels (Fig. 3, C and E) of the TG and PM fractions showed that 38% of the polypeptides in the TG fraction and 42% of the polypeptides in the PM fraction were labeled during the 3 h incubation with the [^{35}S] methionine. The polypeptide patterns of the three fractions were different (Fig. 6, A, C, and E). As in the silver-stained gels, the

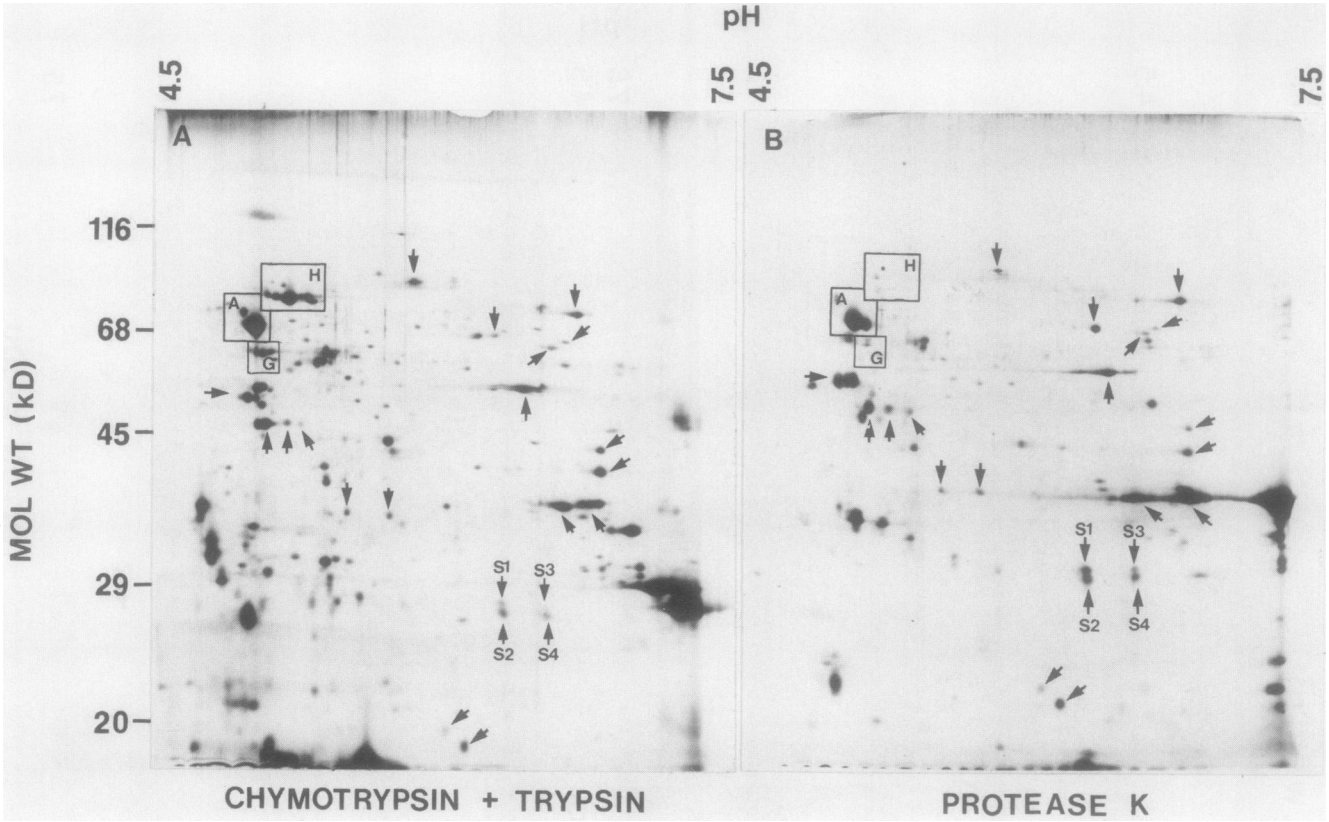

FIG. 5. Effect of protease plus DOC on the TG membrane fraction isolated from roots of plants grown with 100 mM NaCl. Panel A is a silver stained gel of the TG fraction following treatment with chymotrypsin/trypsin and 1% DOC. B, Silver stained gel of the TG fraction following treatment with protease K and 1% DOC. The letters designate polypeptides and groups of polypeptides that correspond to those indicated on Figure 3. The arrows designate unidentifiable polypeptides, probably hydrolysis products, that are common to both treatments.

TG (Fig. 6A) and the PM_2 (Fig. 6C) fractions contained a number of polypeptides with the same M_r and pI (Table I). TG polypeptides that were labeled included polypeptides S1 to S4 and groups G and H (Fig. 6A). PM polypeptides that were labeled included 50, 51, 58, 65, 66, 70, N, and Q1 to Q3, three polypeptides of group R, and Group M (Fig. 6C). Polypeptides 42 and 43 of the TG fraction, and polypeptides 74 and 75 of the PM_2 fraction, were labeled with [^{35}S]methionine, but were not detected on gels stained with silver. The polypeptide pattern of the cell wall fraction was distinctly different from those of the two membrane fractions (Fig. 6E). The cell wall-enriched fraction contained fewer labeled polypeptides than the TG or PM fractions and most of the label was associated with approximately one third of the polypeptides. Polypeptides specific to the cell wall fraction included 80 to 94 and groups a, b, and d. In addition, the cell wall polypeptides, overall, were more basic than those in the TG or PM fractions. Ampholytes with a more basic pH range could have been used to obtain a better distribution of the polypeptides in 2D gels of the cell wall fraction. However, the same ampholyte range was used for all 2D gels in order to compare the labeled polypeptides in the cell wall fraction with those in the tonoplast and PM fractions. When this comparison was made, only two polypeptides, S2 and S4, were found to be present in all three fractions.

Salt-Induced Changes in [^{35}S]Methionine-Labeled Polypeptides. A comparison of the patterns of polypeptides synthesized in salt-grown roots (Fig. 6, B, D, and F) with those of control roots (Fig. 6, A, C, and E) indicated that treatment of plants with 100 mM NaCl did not cause striking qualitative differences in the TG, PM_2, and cell wall fractions. Salt did not induce the synthesis of unique polypeptides or cause polypeptides to disappear, but did cause increases or decreases in the synthesis of a number of polypeptides (Table I). In the TG fraction (Fig. 6B), polypeptides S1, S2, G1, and G2 increased and polypeptides 22 and 38 decreased. Other changes in the TG fraction included increases in ER polypeptide 8 and PM polypeptides 50, 51, and R1 and decreases in ER polypeptide 14 and group D and in PM polypeptides P1 to P3. Polypeptides 42 and 43, which were not visualized on silver-stained gels, also increased in the TG fraction. In the PM_2 fraction (Fig. 6D), polypeptides 50, 51, 53, M1, Q1, and Q2 increased and polypeptides 58, 66, 70, 74, M2, and N2 decreased. In the fluorograph of the PM polypeptides from salt-treated roots, Q2 is resolved into two polypeptides; the lower polypeptide increased with salt treatment. Polypeptide 74, which was not visualized on silver stained gels, decreased with salt treatment. Other polypeptides in the PM_2 fraction whose synthesis was altered by salt treatment included an increase in TG polypeptides 22, S1 to S3, G1, and G2 and a decrease in ER polypeptide 14 and group D. In the cell wall fraction (Fig. 6F), polypeptides 81 to 83, 86, 88, 89, S2, and S4 increased and polypeptides 80, 84, 85, 92 to 94, a1 to a3, d1, and all polypeptides in group b decreased.

DISCUSSION

When the effect of salt stress on polypeptide synthesis in barley roots was examined, changes that could easily be detected on 1D polyacrylamide gels were not found (WJ Hurkman, CK Tanaka, unpublished observations). These results are in contrast to the striking changes caused by heat shock (15) and anaerobiosis (24), where synthesis of most proteins ceases and the synthesis of a new set of proteins is induced. Although salt stress did not cause

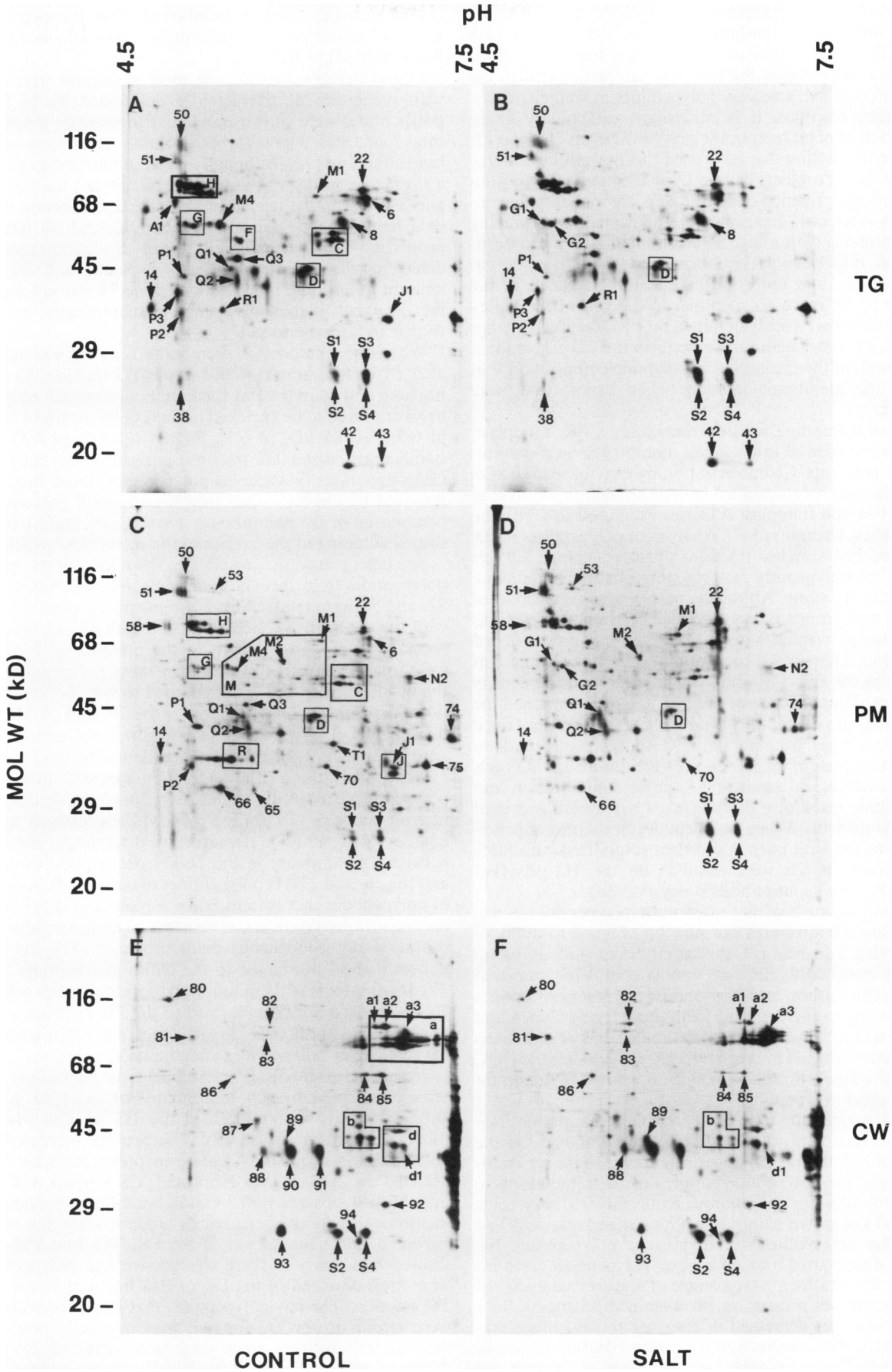

FIG. 6. Effect of NaCl on net protein synthesis in cell fractions following labeling of barley roots with [^{35}S]methionine. A, C, and E, Fluorographs of TG, PM, and cell wall fractions, respectively, isolated from roots of control plants. The numbers and letters designate polypeptides and groups of polypeptides indicated on Figure 3. B, D, and F, Fluorographs of TG, PM, and cell wall fractions, respectively, isolated from roots of plants grown with 100 mM NaCl. The numbers and letters designate polypeptides and groups of polypeptides that increase or decrease with salt treatment (see Table I).

the synthesis of unique polypeptides in barley roots, the subtle changes in polypeptide synthesis could be detected on high resolution 2D gels of soluble and microsomal fractions (13). In this paper, the microsomal fraction was subfractionated to examine the effect of salt stress on polypeptides in well characterized membrane fractions. It is particularly difficult to obtain good resolution of plant membrane polypeptides on 2D gels (12) and there are few studies that have used this powerful technique (3, 7, 22, 29, 30). Problems caused by inadequate sample solubilization and the presence of nonprotein components that interfered with isoelectric focusing were alleviated by extracting membrane proteins with a high ionic strength buffer and partitioning them into phenol (12). Improved solubilization, enhanced resolution, and more sensitive staining resulted in the visualization of far more polypeptides in 2D gels of the barley PM (12) than were previously obtained for PM fractions of other plants (3, 22, 29, 30). The improvements in the 2D gels made it possible to analyze the changes in polypeptide composition that occurred in the membrane fractions when barley roots were grown in salt.

We isolated membrane fractions enriched in ER, tonoplast, and PM by separation of microsomal membranes on discontinuous sucrose gradients. Characterization by enzyme assays, SDS PAGE, and immunoblots assayed with antibodies raised to subunits of the PM and tonoplast ATPases were used to verify the identities of these fractions (5–7). Analysis of polypeptides in the ER-, TG-, and PM-enriched fractions on silver-stained 2D gels showed that the polypeptide patterns were qualitatively unique for each of the fractions. Although there was some overlap of polypeptides and groups of polypeptides between the fractions (Table I), many polypeptides were clearly specific to or enriched in each fraction. There was a correlation between the number of polypeptides in the ER, TG, and PM_1 fractions and the density of the fraction. Thus, 2D gels of the PM_1 fraction contained the greatest number of polypeptides, and the ER fraction contained the fewest. Previously we observed fewer polypeptide bands in the PM_2 fraction than in the ER or TG fractions on 1D SDS gels (7), but this was due to inadequate sample solubilization and poor polypeptide resolution that occurred when SDS was used to extract PM proteins. When polypeptides of the PM_2 fraction were first extracted with phenol and then solubilized with SDS, more polypeptide bands were resolved on the 1D gels (WJ Hurkman, CK Tanaka, unpublished observation).

The function of most of the membrane polypeptides is unknown, but specific attributes can now be assigned to many of the polypeptides. For example, the major polypeptide in group A was highly enriched in ER, was almost completely removed by the detergent washes, and was one of the few polypeptides that was resistant to the protease treatments. The polypeptides in J were present in large quantities in the PM and TG fractions and were greatly enriched in the membrane pellets after the DOC Triton X-100 washes. Similar generalizations can be made for many of the other polypeptides.

Identical step gradients were used to isolate membrane fractions from roots of control and salt-treated plants because the distribution of membranes on linear sucrose gradients was similar. On 2D gels, the polypeptide patterns of each fraction were qualitatively identical for membrane fractions isolated from roots of control and salt grown plants. Salt stress caused relatively few changes in the net synthesis of membrane polypeptides. No polypeptides disappeared and no unique polypeptides were induced by the salt treatment. An average of approximately 5% of the total polypeptides present on silver-stained gels or on fluorographs increased or decreased in response to salt. More dramatic changes in PM composition were found during cold acclimation of rye seedlings; a similar low percentage of polypeptides (6%) increased or decreased with acclimation, but, unlike our results with salt stress, a number of other polypeptides (14%) appeared or disappeared (percentages were calculated from Fig. 8 and Table II in Ref. 29).

Of the changes detected, the most significant were associated with the group of polypeptides designated S1 to S4. These polypeptides were shown previously to increase significantly in total root extracts and in both soluble and microsomal membrane fractions (13). Although this set of four polypeptides forms a distinctive group in soluble and membrane fractions, S1, S2, and S4 may not be related in structure and function to S3. The data in this paper indicate that S1, S2, and S4 have similar properties: they were removed from the membranes by mild detergent washes, were protease resistant, and increased in amount in response to salt. Polypeptide S3 was not removed by the detergent washes, was sensitive to proteases, and did not change in response to salt.

When the microsomal membrane fraction was subfractionated, S1 and S2 were enriched in the TG fraction, S3 in the ER fraction, and S4 in the PM fraction. Following salt treatment, S1 to S4 appeared to be enriched in the TG fraction and S1 (27-kD, pI 6.3), S2 (26–kD, pI 6.3), and S4 (26-kD, pI 6.5) increased significantly in the TG fraction, indicating that they could be Golgi apparatus or vacuolar polypeptides. Since they were not removed by the KCl wash that is a standard procedure in the preparation of the membranes, it is unlikely that S1 to S4 were simply adhering to the surface of the membrane vesicles.

The detergent washes resulted in the release of the majority of polypeptides from the TG membrane vesicles. Polypeptides S1, S2, and S4 were released from the membrane, but S3 remained with the detergent washed pellet. Because S1, S2, and S4 were resistant to proteolysis, even in the presence of detergent, we were unable to distinguish whether these polypeptides were on the outside of the vesicles or trapped within the vesicles. Questions about the cellular location of the 26- and 27-kD polypeptides may be answered by isolating the polypeptides, raising antibodies against them, and using the antibodies for immunocytochemical studies (27). One might speculate that the barley polypeptides are similar to the 26-kD polypeptide that accumulates in vacuoles of cultured tobacco cells adapted to grow in high levels of salt (27). However, we detected no cross-reaction between the antibody to the 26-kD polypeptide from tobacco and the 26- and 27-kD polypeptides of barley (13). Another class of polypeptides that is induced by a biological stress, PR (pathogenesis-related) proteins, is resistent to commercially available proteases and endogenous plant proteases (31), but it is not known if they are related to the barley polypeptides S1 to S4. The relationships of these four polypeptides to each other, to the 26-kD tobacco polypeptide, and to the PR proteins will not be resolved until antibodies, cDNA clones, and DNA sequences for the four barley polypeptides are obtained.

We were also searching for candidates for membrane transport proteins affected by salt treatment. For example, a Na^+/H^+ antiport activity was induced in the TG fraction when barley roots were grown in salt (9). The activity increased at least sevenfold and a onefold increase in polypeptide levels can be detected on 2D gels (WJ Hurkman, CS Fornari, CK Tanaka, unpublished observation). A number of polypeptides in the membrane fractions increased in amount when the roots were grown in salt. Comparison of the ER, TG, and PM fractions showed that many of these changes were in polypeptides that were most enriched in the ER or PM fractions rather than the TG fraction. The few polypeptides that increased with salt and were specific to the TG fraction were not likely candidates for the Na^+/H^+ antiport since they were removed by the mild detergent washes. The most prominent changes were in polypeptides S1, S2, and S4. These were unlikely candidates for the $Na^+/$

H^+ antiport since they were of relatively low M_r and were also present in the cell wall and soluble fractions.

The gels also provided some information on the subunits of the tonoplast and PM ATPases. Polypeptides 54 and 55 cross-reacted with antibodies to the PM ATPase of *Neurospora* (7). On silver-stained gels, polypeptides 54 and 55 were restricted to the PM fraction, but they were not detected in fluorographs. Apparently, the 3 h labeling period was not long enough to label the PM ATPase polypeptides. Polypeptide 54 appeared to decrease in response to salt on silver-stained gels. Polypeptides H1 and H2 cross-reacted with antibodies to the 70-kD subunit and polypeptides G1 and G2 cross-reacted with antibodies to the 60-kD subunit of the tonoplast ATPase of red beet (7). Polypeptide groups G and H were present in all three fractions, although antibodies to the subunits of the tonoplast ATPase did not react with any polypeptides in the PM_2 fraction and cross-reacted slightly with polypeptides in the ER fraction (7). Further work would be needed to clarify the relationship of the polypeptides in groups G and H to the three membrane fractions. The polypeptides in groups G and H were only partially removed from the membrane by the detergent treatments, were relatively insensitive to trypsin plus chymotrypsin, and were almost completely digested by protease K. The effects of salt on groups G and H were not easily detected in the silver-stained gels, but they did not appear to change in response to salt. Labeling of G1 and G2 with [^{35}S]methionine appeared to increase in response to salt. Although the data suggest that some of the polypeptide subunits of the tonoplast and PM ATPases were affected by salt, the changes were subtle and it is premature to speculate on their significance.

Analysis of barley membrane fractions on high resolution 2D gels made it possible to detect the changes in polypeptide composition that occurred in response to salt. Minor changes in putative subunits of the tonoplast and PM ATPases were observed, as well as changes in amounts of other polypeptides specific to ER, TG, or PM. Induction of completely new polypeptides was not observed, nor was there a striking increase in any polypeptide in the TG fraction that would be a likely candidate for the Na^+/H^+ antiporter. The most obvious change was an increase in several polypeptides of 26– and 27–kD that were differentially associated with ER, TG, PM, and cell wall fractions, were insensitive to proteases, and were easily removed with detergents.

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