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Selective Inhibition of Active Uptake of Sucrose into Plasma Membrane Vesicles by Polyclonal Sera Directed against a 42 Kilodalton Plasma Membrane Polypeptide[1]

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ABSTRACT

Several polyclonal sera were raised in rabbits and in mice against putative sucrose carrier proteins, *i.e.* a 42 kilodalton (O Gallet, R Lemoine, C Larsson, S Delrot [1989] Biochim Biophys Acta 978: 56–64) and a 62 kD (KG Ripp, PV Viitanen, WD Hitz, VR Fransceschi [1988] Plant Physiol 88: 1435-1445) polypeptide of the plasma membrane. The effects of these sera on the active uptake of sucrose and of valine into purified plasma membrane vesicles from sugar beet (*Beta vulgaris* L.) leaves and roots were studied. At a dilution of 1/50, the anti-42 kilodalton sera consistently inhibited sucrose uptake in plasma membranes from leaves or from roots. They had no effect on valine uptake. Under the same experimental conditions, the anti-62 kilodalton sera had no effect on active uptake of sucrose. The data further support the view that a 42 kilodalton polypeptide is a component of the transport system mediating sucrose uptake across the plasma membrane of plant cells.

According to the mass-flow mechanism of translocation, long distance transport of sugars in the plant depends on the build-up and on the maintenance of a pressure gradient between the phloem of the source and the phloem of the sink. In this model, accepted by most authors, the path is thought to play a relatively passive role, whereas phloem loading and unloading govern the intensity and the direction of phloem transport by controlling sucrose fluxes. Although recent work indicates the importance of the symplastic pathway in some steps of assimilate transport (18, 25–28), transport of sugars across the plasmalemma is involved in the control of major processes such as assimilate leakage from the mesophyll, loading (or retrieval) of sucrose in the phloem, and sugar unloading in mature sinks. The carrier protein(s) mediating sucrose transport through the plasma membrane therefore play(s) a major physiological role for the distribution of carbohydrates, and indirectly for all the phloem-mobile solutes moved by mass-flow. Unfortunately, these proteins have not yet been identified unequivocally. A sucrose photolyzable derivative, 6′-deoxy-6′-(4-azido-2-hydroxy) benzamidosucrose was used to label a sucrose binding protein in microsomal preparations from soybean cotyledons (24). This polypeptide appeared in the microsomal fraction of soybean cotyledon cells concomitantly with the onset of active sucrose influx (24). Polyclonal antibodies raised against the 62 kD polypeptide from soybean cross-reacted with a 62 kD polypeptide in purified plasma membrane vesicles from spinach leaves and immunolocalization studies in fully expanded leaves of this species showed that this polypeptide is specifically located in the plasma membrane of the sieve tube. The sieve tube plasma membranes were not immunolabeled in young leaves where the phloem contained no mature sieve tube member (29). The same localization was found in mature leaves of sugar beet with the same antibodies, but no Western blot was provided to show what proteins of sugar beet cross-reacted with the serum directed against the anti-62 kD polypeptide from soybean (29). Although these data correlate well with the localization and with the synthesis pattern expected for the sucrose carrier involved in phloem loading, the evidence is still indirect, and no functional proof is available to conclude with certainty that this protein is actually involved in sucrose transport. Moreover, the sequence of the polypeptide does not show any homology with any known bacterial or animal sugar carrier (11).

On the other hand, differential labeling of broad bean microsomes (22) and purified plasma membrane vesicles from broad bean (21) and sugar beet (10) leaves by N-ethylmaleimide in the presence of sucrose allowed the identification of an integral 42 kD polypeptide which was specifically protected by sucrose. Polyclonal antibodies raised against the 42 kd polypeptide partially purified from sugar beet plasma membranes selectively inhibited the uptake of sucrose into broad bean mesophyll protoplasts, while they had no effect on the uptake of hexoses or of amino acids (16). Although the latter data gave some functional evidence for the involvement of this polypeptide in sucrose transport, the fact that the antibodies used recognized a rather large band around 42 kD, plus some other minor bands (16), as well as the discrepancy with the conclusions given above concerning the 62 kD polypeptide led us to test further the identity of the 42 kD and of the 62 kD polypeptides by an immunological approach. A recent technique allowing the study of active uptake of sucrose in plasma membrane vesicles artificially energized (2, 3, 15) was adapted and used to test the effects of several

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polyclonal antibodies raised against the 42 kD or the 62 kD polypeptides.

MATERIALS AND METHODS

Preparation and Characterization of the Plasma Membrane Vesicles

Growth of plants, preparation of plasma membrane vesicles and assays for marker enzymes were conducted as described by Gallet *et al.* (10). The average diameter of the vesicles was 291 nm, and their internal volume was 2.2 μL mg protein^{-1}. Eighty percent of the vesicles were oriented right-side out, as determined both from measurements of ATPase latency and from the binding of Concanavalin A (17).

Protein was determined according to Bearden (1) using 0.01% (w/v) Triton X-100 to solubilize membrane proteins, with bovine serum albumin as a standard.

Preparation of the 42 and 62 kD Polypeptides

The 42 and 62 kD polypeptides were obtained by preparative electrophoresis of plasma membrane vesicles from sugar beet leaves. The plasma membrane proteins were separated in preparative slab gels (0.16 × 14 × 16 cm) according to Laemmli (13). The separation gel consisted of a linear gradient of acrylamide (10 to 22%) and was covered by a stacking gel at pH 6.8. Before electrophoresis, all samples were incubated for 3 min at 90°C in a 25 mM Tris/HCl buffer (pH 6.8) containing 11% (v/v) glycerol and 2.16% (w/v) SDS. The samples were then cleared by centrifugation (15 min at 13 000 g) before being deposited on the gel. Two to three mg (1 mL) of plasma membrane proteins were deposited in a large preparative well (10 cm wide), and the electrophoresis was run at a constant intensity of 10 mA per gel for 1 h, and 15 mA for 13 h. After electrophoresis, proteins were detected by negative staining with 0.25 M $CuCl_2$ (14). Molecular masses were determined with the Dalton VII marker kit from Sigma (lactalbumin, 14.2 kD; trypsin inhibitor, 20.1 kD; trypsinogen, 24 kD; carbonic anhydrase, 29 kD; glyceraldehyde 3-phosphate dehydrogenase, 36 kD, egg albumin, 45 kD, bovin serum albumin, 66 kD). A 2 to 3 mm band corresponding either to the 42 kD or to the 62 kD polypeptides was cut out from the gel, sliced and electroeluted for 8 h at 200 V in a Biotrap device (Schleicher and Schull, Paris). The electroelution buffer contained 50 mM Tris/boric acid and 0.2% SDS (pH 8.0). The yield of electroelution, as determined in parallel experiments where the proteins were labeled with ^{3}H-N-ethylmaleimide, was 86%. The 42 kD (or the 62 kD) eluates recovered from 10 to 16 gels were pooled, and dialyzed and concentrated in a Microprodicon (Biomolecular Dynamics, Beaverton, Or.) or in an Amicon MultiMacrofiltration system equipped with Diaflo ultrafilters (Amicon, Paris) against phosphate buffered saline, pH 7.2. Due to large losses during the dialysis and concentration step, 20 to 30 gels had to be processed for the preparation of one antigenic dosis for the rabbits, and 50 to 70 gels were necessary to immunize each mouse.

Preparation of Polyclonal Antisera

The 42 and 62 kD antigens prepared as described above were used to immunize the animals according to Figure 1. For convenience, these sera will be referred to as the anti-42 kD and anti-62 kD sera respectively (but see Discussion on the specifity of these sera). Western blots against the plasma membrane proteins were prepared according to Lemoine *et al.* (16). In some experiments, periodate sensitivity of the epitopes recognized by the antibodies was determined by incubating the blots 1 h in 10 mM periodate (20) before incubation with the rabbit polyclonal anti-42 kD serum.

Rabbit antibodies directed against the 62 kD polypeptide of plasma membranes from soybean cotyledons were prepared (24) and kindly supplied by Dr. William Hitz (Dupont de Nemours).

Uptake Experiments

Uptake experiments were run at 25°C as described by Lemoine and Delrot (15) and Gaillard *et al.* (9) except that dithiothreitol was omitted in the incubation media. For uptake experiments in energized conditions, the concentrated vesicle solution was first preincubated for 30 min in K-buffer (pH 7.5) with 5 μM valinomycin. Vesicles were then diluted in Na-buffer (50 mM Na-phosphate, pH 5.5) containing 5 μM valinomycin and 1 mM [6,6′(n)-^{3}H]sucrose (26 kBq). The combination of K-buffer (pH 7.5) inside the vesicles and of Na-buffer (pH 5.5) in the incubation medium generates a ΔpH (transmembrane pH, outside acidic) and a K^+ diffusion gradient (due to the presence of valinomycin) towards the outside of the vesicles, resulting in a $\Delta\Psi$ (transmembrane electrical gradient, inside negative). To study the effects of the sera, a 100 μL aliquote (1 mg protein) of membranes suspended in K-buffer, pH 7.5, were incubated with 10 mL of a serum solution (final dilution 1/50, 1/100 or 1/200) at room temperature. Thirty min later, valinomycin (5 μM) was added

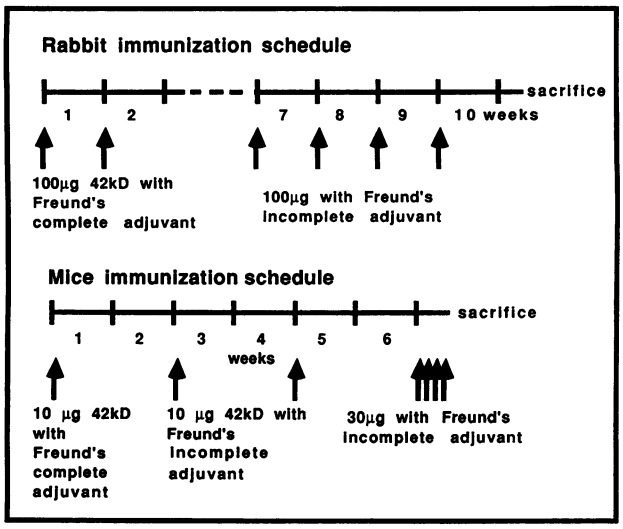

Figure 1. Immunization schedules used to prepare the polyclonal sera.

and the samples were transferred on ice until beginning of the uptake assay (within 30 min). The assay was run as described above. Duration of sucrose uptake was 2 min, which corresponds to the maximum of absorption (15). Sucrose uptake was measured as the difference between uptake at 2 min and time zero (*i.e.*, the samples were immediately diluted with an ice-cold blocking medium containing 1 mM $HgCl_2$ and filtrated). The same procedure was used to study the uptake of valine, except that the incubation medium contained 1 mM L-[3,4(n)-^{3}H] valine (18.5 kBq). All data presented deal with total uptake of sucrose or of valine, including a passive component, which is independent on the presence of ionic gradients and accounts for about 10 to 20% of total uptake (9, 15).

RESULTS

Rabbit Polyclonal Serum

The anti-42 kD serum previously prepared and used in studies on sucrose uptake by broad bean protoplasts (16) was tested. Whatever the serum dilution used, from 1/200 to 1/50 a preimmune serum did not significantly inhibit active uptake of sucrose in sugar beet plasma membrane vesicles, compared to untreated samples (Fig. 2A), while the anti-42 kD serum induced a concentration-dependent inhibition of sucrose uptake (Fig. 2B), with significant effects observed for 1/100 and 1/50 dilutions. At a 1/50 dilution, the inhibition measured accounted for 50% of total uptake. At the same dilution, the polyclonal anti-42 kD serum from rabbit did not inhibit the uptake of valine (Fig. 3).

The effect of the rabbit anti-42 kD serum was also tested with plasma membranes prepared from sugar beet roots (Fig. 4). In this experiment, the membranes were pretreated with mixtures containing various proportions of preimmune and anti-42 kD sera. The anti-42 kD serum clearly inhibited active uptake of sucrose by plasma membranes from sugar beet root for dilutions ranging between 1/100 and 1/25, and the maximal inhibition reached 80% compared to untreated membranes.

A Western blot prepared against the plasma membrane proteins from sugar beet leaf shows that the anti-42 kD serum from rabbit recognized a single but rather broad region around

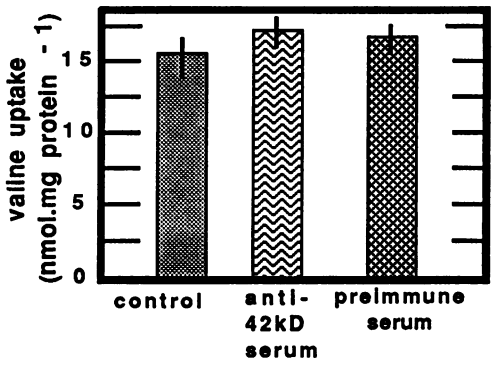

Figure 3. Effects of rabbit preimmune and anti-42 kD sera on the active uptake of valine into plasma membrane vesicles energized by ΔpH and ΔΨ. The sera were used at a 1/50 dilution. Mean of 2 independent experiments (8 measurements ± SE)

42 kD (Fig. 5A). After treatment of the gels with periodate, the signal obtained upon incubation with the anti-42 kD serum was fainter and somewhat narrower, suggesting that part of the antibodies were directed against glycan groups of membrane glycoproteins (Fig. 5B). At a 1/5000 dilution (giving very clear pictures in Western blot with a crude homogenate from soybean cotyledon, [24]), the antibodies directed against the 62 kD polypeptide from soybean plasma membranes showed no cross-reactivity with the plasma membrane proteins from sugar beet (Fig. 5C) and at a 1/200 dilution, these antibodies gave a very faint reaction at 62 kD (Fig. 5D).

Mice Polyclonal Sera

Figure 6A summarizes the effects of 5 different anti-42 kD sera prepared independently. At a 1/100 dilution, sucrose uptake measured with leaf plasma membranes pretreated by the anti-42 kD sera was not significantly different from that measured either with the control or with plasma membranes pretreated with a preimmune serum at the same concentration as the anti-42 kD sera. At a dilution of 1/50, the mean inhibition exerted by the anti-42 kD sera on sucrose uptake was 63%, compared to the control, and only 41% compared

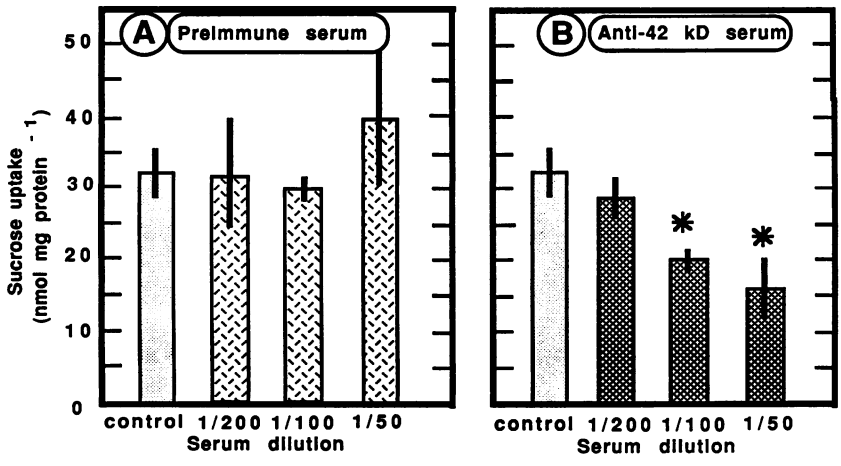

Figure 2. Effects of rabbit preimmune and anti-42 kD sera on sucrose uptake by plasma membranes from sugar beet leaf energized by ΔpH and ΔΨ. Preincubation in the sera lasted 30 min and duration of uptake was 2 min. The asterisks indicate that the results are significantly different from the untreated control and also from the preimmune control at the same dilution, at the 0.01 level. Mean of 3 independent experiments (12 measurements ± SE)

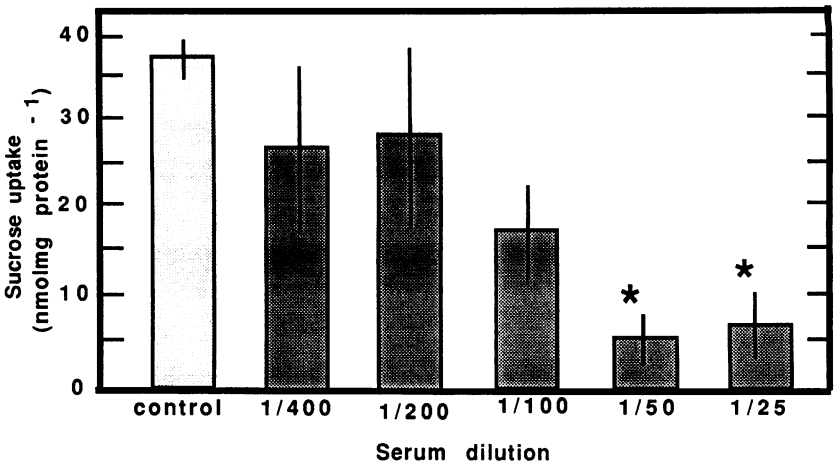

Figure 4. Effects of rabbit preimmune and anti-42 kD sera on sucrose uptake by plasma membranes from sugar beet root energized by ΔpH and ΔΨ. Preincubation in the sera lasted 20 min and duration of uptake was 2 min. In this experiment, the anti-42 kD serum was diluted with increasing amounts of preimmune serum, but the total volume of preimmune + anti-42 kD sera was kept constant (4 μl). The asterisks indicate that the results are significantly different from the control (0.05). Mean of 4 measurements ± SE.

to the membranes pretreated by preimmune serum at the same concentration and under the same experimental conditions. Indeed, even if this was not statistically significant, the preimmune sera from mice exerted some inhibition of sucrose uptake (Fig. 6B). Nevertheless, the uptake of sucrose measured in the presence of the anti-42 kD sera at a 1/50 dilution was significantly different from that measured in the control samples and from that measured in the samples treated with the preimmune sera. Compared to untreated membranes, the inhibition of sucrose uptake induced by individual anti-42 kD sera ranged between 22 and 88%. Given the very poor cross-reaction between sugar beet plasma membranes and the anti-62 kD serum raised against the soybean protein, several sera directed against the 62 kD region of plasma membranes from sugar beet leaves were raised, and their effect on sucrose uptake was tested. The sera directed against the 62 kD polypeptides from mature sugar beet leaves did not affect sucrose uptake (Fig.6C).

At a 1/50 dilution, the anti-42 kD sera which inhibited sucrose uptake did not significantly affect valine uptake by leaf plasma membranes (Fig. 7).

Western blots prepared against the plasma membrane proteins show that the mice sera prepared present a good specificity for the polypeptides of the 42 kD and 62 kD regions respectively (Fig. 8). The serum used to prepare the blot shown in Figure 6 exerted a 72% inhibition of sucrose uptake by leaf plasma membranes.

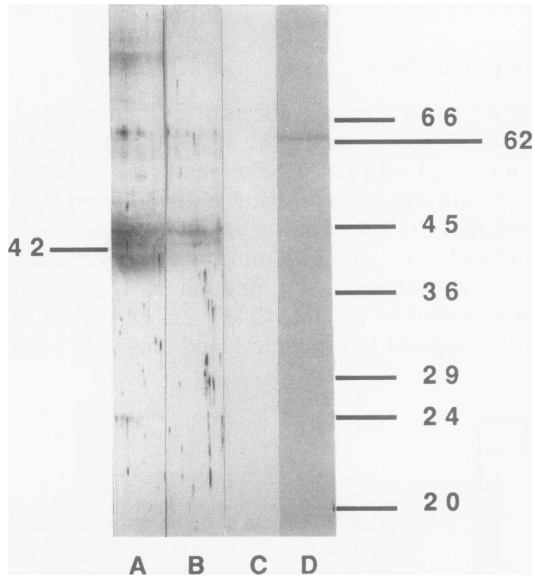

Figure 5. Western analysis of proteins from sugar beet plasma membrane with anti-42 kD and anti-62 kD sera raised in rabbits. One hundred μg protein were deposited in each lane. Lanes A and B, blots made with the anti-42 kD serum. In B, the proteins were treated by periodate after the transfer step. In lanes C and D, the blot was made with antibodies raised against the 62 kD polypeptide from soybean cotyledon, at a dilution of 1/5000 and 1/200 respectively. Incubation with the primary and the secondary antibody was for 2 h and for 90 min respectively. Position of the molecular mass markers is shown on the right.

DISCUSSION

We have recently shown that a polyclonal anti-42 kD serum was able to inhibit selectively sucrose uptake by broad bean protoplasts (16). However, because of the discrepancy between our conclusions and those presented by Ripp *et al.* (24), we found it necessary to test further the identity of the 42 kD and of the 62 kD polypeptides by an immunological approach. Several sera were raised either in rabbit or in mice, and the uptake was assayed with sugar beet plasma membrane vesicles instead of broad bean protoplasts. In the previous work, we showed that the supernatant remaining after precipitation of the antibodies did not inhibit sucrose uptake. Yet, the purified antibodies did not inhibit the uptake either (16). This was ascribed to the presence in the serum of components affecting synergistically the binding of the antibodies to the membranes, confirming earlier results from a similar work on lac permease of *E. coli* (30). For this reason, and due to the limited amounts of the sera available, no attempt was made to purify the antibodies in the present study.

The anti-42 kD serum from rabbit used previously to inhibit sucrose uptake in protoplasts (16) also inhibits sucrose uptake in leaf or root plasma membranes artificially energized (Figs. 2 and 4). This technique may therefore now be used to study

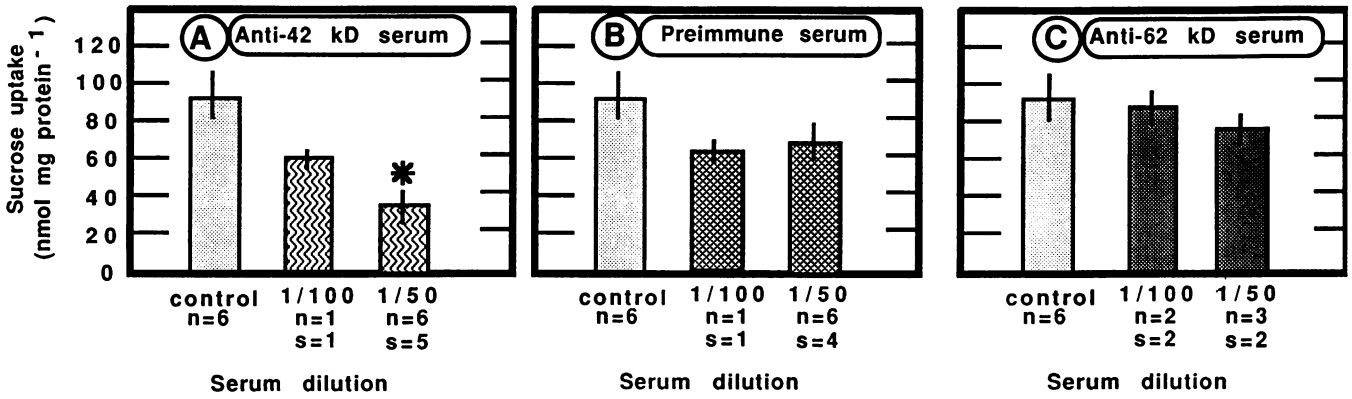

Figure 6. Effects of mice preimmune, anti-42 kD, and anti-62 kD sera on the active uptake of sucrose into plasma membrane vesicles from sugar beet leaf energized by ΔpH and Δψ. *n* = number of experiments (4 measurements per experiment). s = number of different sera tested. The asterisks indicate that the results are significantly (0.01 level) different from the control and from the corresponding preimmune serum.

the effect of sera directed to carrier proteins. The amounts of sera needed are much lower than those used in the protoplast assay used previously (16). Interestingly, the preimmune serum which induced some inhibition of sucrose uptake in the protoplast assay did not exert such an effect in the plasma membrane assay. The anti-42 kD serum from rabbit did not inhibit the uptake of valine, which is also energized by the proton motive force in this system (9). The selective inhibition of sucrose uptake can therefore be ascribed to the effects of the serum on the transport system, and not on the energization of the vesicles.

This selective inhibition was found again with various sera raised in mice. Indeed, the mice anti-42 kD sera also significantly inhibited sucrose uptake in leaf plasma membrane vesicles (Fig. 6), while they did not inhibit the uptake of valine (Fig. 7). Although the extent of inhibition observed depended on the mouse serum used (between 22 and 88%), if one takes into account the variability of the immunological response, these data lend further support to the view that a 42 kD polypeptide is involved in sucrose uptake.

The percentages of inhibition given above are minor estimates of the actual inhibition of active uptake, since the passive component of uptake which represents 10 to 20% of total uptake in the control was substracted neither from the control values nor from the "serum-treated" values. The incomplete inhibition of sucrose uptake generally observed makes it impossible to conclude that no carrier other than a 42 kD polypeptide is involved. In this regard, it is important to note that, as in other materials, kinetic studies reveal two independent phases for sucrose uptake into sugar beet leaf discs (19).

However, incomplete inhibition may also simply be due to incomplete blocking of the 42 kD polypeptide by the serum used. Studying the effect of monoclonal antibodies on lactose uptake into proteoliposomes containing the purified lactose carrier, Carrasco *et al.* (4) noted that the inhibition observed was never greater than 80%, even for a carrier/antibody ratio (w/w) as low as 4. Testing the effects of monoclonal antibodies on the uptake of hexoses by plant plasma membrane vesicles purified on a sucrose-gradient, Rausch *et al.* (23) used a protein/antibody ratio of 10 to study its effect on glucose uptake. Similarly, monoclonal antibodies against the Na^+-D-glucose transporter, used at a 1/1 ratio (w:w) on brush border membrane vesicles, induced a 70% inhibition of glucose uptake (12). Although the antibody concentration of all sera was not systematically investigated in the present work, the mouse anti-42 kD serum giving a 72% inhibition of sucrose uptake and used in Figure 6, contained 1.9 mg IgG mL^{-1}, and 1.4 mg IgM mL^{-1}. According to our experimental conditions, at a 1/50 dilution, the plasma membrane protein/antibody ratio was about 125, and assuming that the 42 kD polypeptide accounts for 0.5% of total plasma membrane polypeptide (10), the 42 kD/antibody ratio was about 1. This value is relatively low compared to the values given above (12, 23) but further increase in the serum concentration would have not necessarily led to a stronger inhibition (12).

Being established that the anti-42 kD sera selectively inhibit sucrose uptake, the next question concerns the target of these

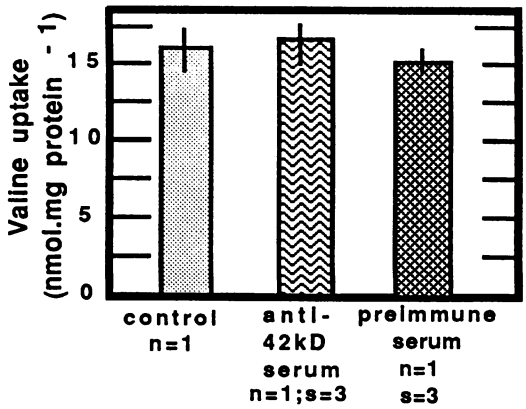

Figure 7. Effects of mice preimmune, anti-42 kD, and anti-62 kD sera on the active uptake of valine into plasma membrane vesicles energized by ΔpH and Δψ. *n* = number of experiments (4 measurements per experiment). s = number of different sera tested. The dilution of the sera was 1/50.

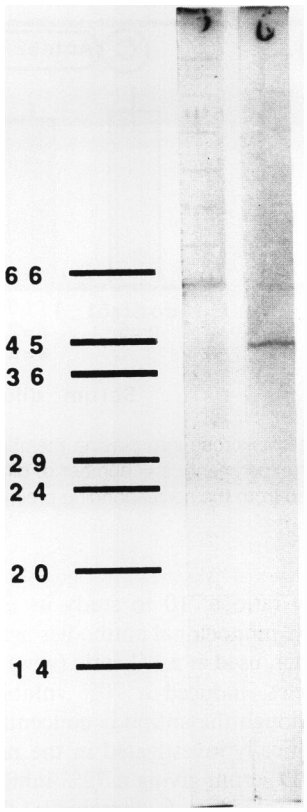

Figure 8. Immunoblots prepared with an anti-42 kD mouse serum (right) and of an anti-62 kD mouse serum (left) after separation of total plasma membrane proteins from sugar beet leaf. The sera were used at a 1/1000 dilution. Incubation with the primary and the secondary antibody was for 2 h and for 90 min respectively. Position of the molecular mass markers is shown on the left.

sera, as assessed by the Western blots. The concentrations of sera efficient in the uptake experiments (1/50 or 1/100) appear high compared to the concentrations used for the blot (1/200 for the rabbit sera, or 1/1000 for the mice sera), and it might be argued that during the uptake experiments, the sera recognize other polypeptides in addition to those appearing on the blots. In fact, if one takes into account the relative concentrations of the antigen and of the serum during both kinds of experiments, it is likely that the reverse is true, *i.e.*, the sera bind to a smaller number of polypeptides in the uptake experiments than in the blot experiments. Indeed, the typical conditions used for pretreating the membranes by the sera in the uptake experiments are 1 mg membrane in only 0.1 mL of serum at a 1/50 dilution. In the blot experiments, only 100 μg protein were electrophoresed, transferred (not with 100% efficiency) and incubated in 4 mL of serum at a 1/200 or 1/1000 dilution. The ratio antigen/serum is therefore much higher in uptake experiments than during the blots. Another point concerns the possibility that the serum might act in a non specific way by binding to glycans present in glycoproteins of the plasma membrane. The calculations made above show that in any case these glycans, if any, must be present in the regions recognized in the blot experiments. Deglycosylation experiments showed that part of the antibodies contained in the anti-42 kD serum from rabbit recognized some glycans present in the 42 kD region. Therefore, at least for this serum, we cannot exclude that part of the inhibition is due to the binding of the antibodies to glycans. Yet, in the case of the human Na^+-glucose cotransporter, which is a glycoprotein, it has been clearly shown that the glycan part is absolutely necessary for the transport function (8). The possibility that some of the antibodies contained in the rabbit anti-42 kD serum may be directed towards glycans of the 42 kD region glycoproteins therefore does not contradict the conclusion that a 42 kD polypeptide (possibly a glycoprotein) is involved in the active transport of sucrose.

Lack of inhibition of sucrose uptake by the anti-62 kD sera tested does not necessarily imply that this polypeptide is not involved in sucrose uptake. It may be that this polypeptide is poorly antigenic, or that the vesicles taking up sucrose in our system come mainly from mesophyll cells. In soybean, the 62 kD polypeptide seems to be more concentrated in the plasma membrane of the sieve tubes (29). The inhibition of sucrose uptake exerted by the anti-62 kD sera in the plasma membrane vesicles originating from the sieve tubes could be masked by the uptake due to the vesicles originating from the parenchyma cells.

In conclusion, we have adapted the energization technique to the study of antibodies raised against proteins putatively involved in sugar transport. Anti-42 kD sera raised from mice or rabbit consistently and selectively inhibited active uptake of sucrose into plasma membrane vesicles from sugar beet leaf or root, while anti-62 kD sera had no effect. These data provide strong and detailed support to the previous results obtained with a single serum on broad bean protoplasts (16). These data allow us to conclude, on a functional basis, that a 42 kD polypeptide is involved in sucrose uptake in the leaf and in the root cells of sugar beet. However, the high concentrations of sera needed for inhibition, and the partial inhibition generally observed do not allow us to exclude that other polypeptides may be involved in sucrose uptake. In any event, definite identification of the sucrose carrier(s) must await reconstitution experiments.

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