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myo-INOSITOL BINDING AND TRANSPORT IN BRUSH BORDER MEMBRANES OF RAT KIDNEY

TADAOMI TAKENAWA, EIKO WADA and TORU TSUMITA

Department of Cell Chemistry, Institute of Medical Science, The University of Tokyo, Takanawa 108, Tokyo and Institute of Basic Medicine, University of Tsukuba, Niiharu, Ibaragi (Japan)

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Summary

Using hypotonically treated brush border membranes, binding and transport of *myo*-inositol were examined.

By hypotonic treatment, both total and non-specific uptake decreased significantly, but specific uptake was not affected.

myo-Inositol release from membranes preloaded by incubation for 2 min was very rapid and about 98% of preloaded *myo*-inositol was released in 5 min of incubation. However, *myo*-inositol release from membranes preloaded by incubation for 20 min was fairly slow and 50% of *myo*-inositol remained in the membranes even after 10 min of incubation.

Uptake of *myo*-inositol decreased by the increase of osmolarity in the medium. However, effect of osmolarity on the uptake was less significant when *myo*-inositol concentration was lower.

Under conditions in which mainly binding occurred, *myo*-inositol binding to the membranes was measured. Two binding systems were demonstrated and high affinity site could bind 22 pmol/mg protein at most and the apparent K_m value was 8.3 μM.

Both binding and transport processes were dependent on Na^+ and enhanced by Na^+-gradient.

Introduction

Previous physiological studies on the transport of *myo*-inositol in the kidneys of intact animals [1] and in renal cortical slices [2—6] showed that the transport was an energy- and Na^+-dependent, stereospecific uphill transport processes which could be inhibited by analogues of *myo*-inositol, scyllitol and inosose-2.

Recently techniques for isolation of renal brush border membranes have

been developed [7—11]. The membrane preparations have been used for biochemical analyses of sugar uptake [12—17], because the use of such membrane preparations excluded the involvement of sugar metabolism and made it possible to impose non-physiological conditions for cells on the membrane.

Studies with the isolated membrane vesicles clarified some molecular aspects of sugar transport such as binding of sugars to the membranes, stimulation of the transport by Na^+-gradient and counter transport. However, there seemed to be some arguments among the investigators on the interpretation of uptake of sugars by membrane vesicles, which represented the sum of the binding to the membrane and the transport into intravesicular spaces. Some [14,18,19] reported that the most part of the sugar taken up by the membrane was merely bound to the membrane, because the uptake was hardly influenced by osmolarity in the medium. Others [20—22] described on the contrary that the transport into intravesicular space overwhelmed the simple binding because they found that sugar uptake drastically decreased with the increase of osmolarity in the medium and also that sugar uptake was enhanced by counter transport reaction.

In a previous paper [23], we reported stereospecific uptake of *myo*-inositol using isolated brush border membranes. In this case, *myo*-inositol taken up by isotonically prepared membranes was predominantly transported into intravesicular space.

These discrepancies may be caused by differences of preparation method of the brush border membranes and of the experimental conditions. It was necessary for the consistent understanding of the phenomena to analyse the causes of these discrepancies.

In the present report, effect of the experimental conditions on the binding and the transport of *myo*-inositol was studied using hypotonically treated membrane preparations. The presence of Na^+-gradient-dependent mediated transport of *myo*-inositol, which was well characterized in glucose transport, is documented.

Materials and Methods

All chemicals used in the experiment were of the highest grade available from commercial sources. [^{14}C]Mannitol (59.2 Ci/mol) was purchased from New England Nuclear Corporation, Boston, and *myo*-[^{3}H]inositol (15 Ci/mol) was a gift from Dr. Komai (National Institute of Health, Tokyo).

Rat renal brush border membranes were isolated in isotonic sucrose solution by a procedure described by Takenawa [23]. Purity of the preparation was monitored routinely by specific enzyme markers, namely alkaliphosphatase for brush border membrane, (Na^+ + K^+)-ATPase for basal-lateral membrane, succinic-dehydrogenase for mitochondria, glucose-6-phosphate for endoplasmic reticulum.

Protein was determined by the method of Lowry et al. [24] using bovine serum albumin as the standard.

Isolated brush border membranes were suspended in 0.025 M sucrose containing 0.01 M Tris · HCl buffer (pH 7.4) at 0°C for 1 h and centrifuged for 20 min at 20 000 × g at 4°C. The pellets were suspended in Tris buffer (0.01 M,

pH 7.4) containing 100 mM NaCl and 32 mM $MgCl_2$ unless otherwise described.

Uptake of *myo*-inositol was measured by Millipore filtration technique. The brush border membranes were incubated at 30°C in 0.5 ml of Tris buffer (0.01 M, pH 7.4) containing 100 mM NaCl, 32 mM $MgCl_2$ and various concentrations of *myo*-[^{3}H]inositol. The amount of membranes varied between 0.3 mg and 0.6 mg. After incubation for various periods of time, the membranes were immediately diluted with 3 ml of ice-cold buffer and filtered through a Millipore filter of 45 mm diameter. The residue was subjected to two successive washes with 3 ml of ice-cold buffer and the washed filter was dried and counted for radioactivity in a liquid scintillation counter.

The amount of radioactivity which could not be displaced by addition of non-labeled *myo*-inositol was regarded as non-specific uptake and specific uptake was calculated by subtracting the non-specific uptake from the total uptake. Blank experiments were carried out adding *myo*-[^{3}H]inositol to the membrane suspension after 3 ml of the cold buffer was added. The mixture was filtered, washed and the residual radioactivity was measured as described above. This blank value was subtracted from the total and non-specific uptake.

Efflux of *myo*-inositol taken up by the membranes was measured as follows. After loading the mebranes with *myo*-[^{3}H]inositol by incubating them in 110 μM *myo*-[^{3}H]inositol for 2 min or for 20 min at 30°C, the incubation mixture was immediately filtered. To the filter on the funnel, a medium containing 10 mM or 100 mM non-labeled *myo*-inositol was placed. The filter was incubated in situ at 30°C for various incubation periods and then washed with 3 ml of the buffer. The radioactivity retained on the filter before and after the incubation was measured. The amount of the radioactivity non-specifically bound to filter was subtracted individually in each case.

Results

Effect of hypotonic treatment of brush border membranes on myo-inositol uptake

Effect of osmolarity of media used during preparation of the membranes on the *myo*-inositol uptake was examined by treating the isotonically isolated preparation with a hypotonic medium (0.025 M sucrose) at 0°C for 1 h. After the treatment, the membranes were suspended in the buffer containing 100 mM NaCl and 32 mM $MgCl_2$. Uptake of *myo*-inositol by the treated membranes was measured at two substrate concentrations, 110 and 1 μM of *myo*-[^{3}H]inositol. When the untreated membranes were incubated with 110 μM substrate, both the specific and the non-specific uptake increased with time (Fig. 1A). By the hypotonic treatment, the total and non-specific uptake decreased significantly, but the specific uptake was not affected (Fig. 1B). After 20 min of incubation, the contribution of non-specific uptake in the total uptake was approx. 60 and 22% when the untreated and treated membranes were used, respectively. The value was only 3% when uptake by the treated membranes was measured with 1 μM substrate. These results clearly showed that the ratio of the specific and the non-specific uptake was significantly influenced by the method of preparation of the membranes and also by the concentration of the substrate in the medium.

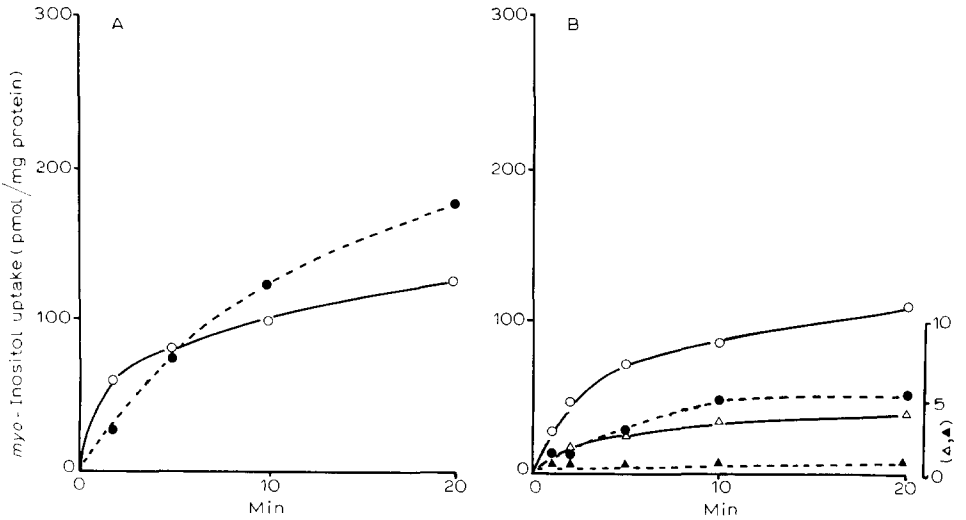

Fig. 1. Effect of the hypotonic treatment of brush border membranes on *myo*-inositol uptake. (A) Isotonically prepared membranes were incubated with 110 μM *myo*-[^{3}H]inositol. Specific uptake, (———) and non-specific uptake, (- - - - -) were determined as described in Materials and Methods. (B) Hypotonically treated membranes were incubated with 110 μM *myo*-[^{3}H]inositol, (○,●) or 1 μM *myo*-[^{3}H]-inositol (△,▲). Specific uptake, (———) and non-specific uptake, (- - - - -) were determined.

myo-[^{3}H]*Inositol efflux from the preloaded brush border membranes*

Brush border membranes were incubated with *myo*-[^{3}H]inositol for 2 or 20 min at 30°C. After the incubation, membranes were transferred into a medium containing unlabeled *myo*-inositol and the release of *myo*-[^{3}H]inositol from the preloaded membranes was examined. *myo*-[^{3}H]Inositol efflux from membranes preloaded by incubating for 2 min was very rapid (Fig. 2). Approx. 98% of the preloaded *myo*-[^{3}H]inositol was released in 5 min of incubation in the presence

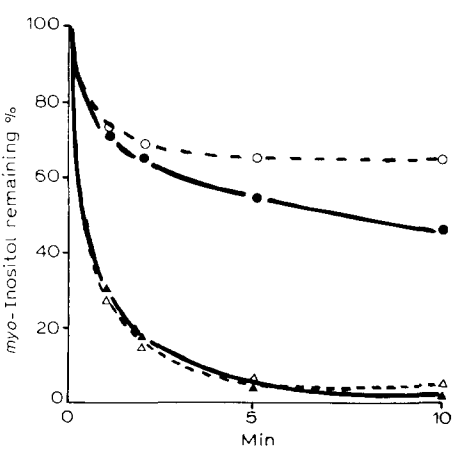

Fig. 2. Release of *myo*-[^{3}H]inositol from preloaded brush border membranes. Hypotonically treated membranes were preincubated with 110 μM *myo*-[^{3}H]inositol for 2 min, (△,▲) or 20 min , (○,●); and efflux from preloaded membranes was determined in the presence of 10 mM, (- - - - -) or 100 mM, (———) unlabeled *myo*-inositol.

of 10 mM or 100 mM unlabeled *myo*-inositol. However, efflux from the membranes preloaded by incubation for 20 min, was fairly slow. By the addition of 10 mM unlabeled *myo*-inositol, only 30% of *myo*-[^{3}H]inositol was released in 10 min of incubation (Fig. 2). Even with 100 mM unlabeled *myo*-inositol, approx. 50% of *myo*-[^{3}H]inositol remained in the membranes after 10 min of incubation.

Effect of medium osmolarity on myo-inositol uptake

It is generally considered that transport into intravesicular space is more sensitive to increased osmotic pressure of the medium than the binding to the membrane. To determine the contribution of binding in the total uptake of *myo*-inositol, effect of osmotic pressure on the uptake was examined. As shown in Fig. 3, uptake of *myo*-inositol in 20 min of incubation decreased with the increase of the concentration of sucrose in the medium. The results indicated an inverse relationship of uptake and osmolarity. However, the sensitivity of the uptake on the osmolarity was less significant when the substrate concentration was lower. When the osmolarity was extrapolated to infinity, under which condition the intravesicular space could be assumed to be zero, *myo*-inositol uptake did not become negligible at both low and high concentrations of the substrate. With respect to mannitol, which is known not to have a specific carrier, the extrapolated value was zero. These results suggested that binding to membranes rather than transport into intravesicular space took place predominantly, even after incubation for a long period when *myo*-inositol concentration was low. Therefore, under experimental conditions usually employed, the uptake included not only transport into the intravesicular space but also binding to membranes.

Effect of myo-inositol concentration on the binding

As described above, binding to membranes occurred mainly when *myo*-

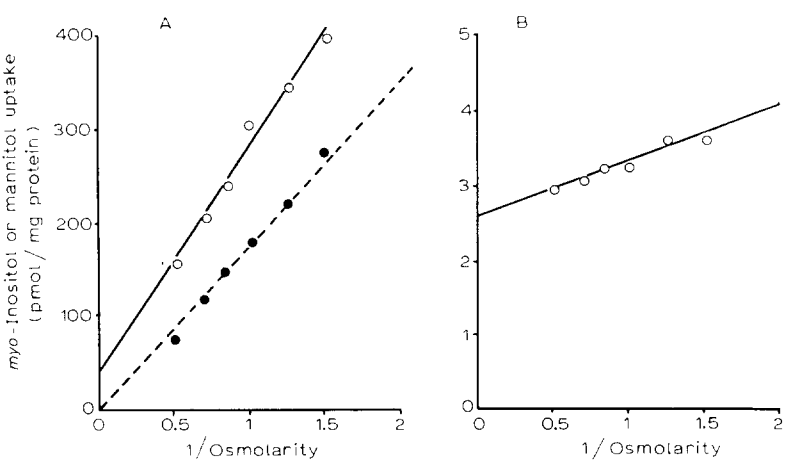

Fig. 3. Effect of medium osmolarity on *myo*-inositol and mannitol uptake. The uptake was determined in the presence of 100 mM NaCl and various concentrations of sucrose. The values given represent the uptake after 20 min of incubation. (A) 168 μM *myo*-[^{3}H]inositol and [^{14}C]mannitol were used. (B) 1.68 μM *myo*-[^{3}H]inositol was used.

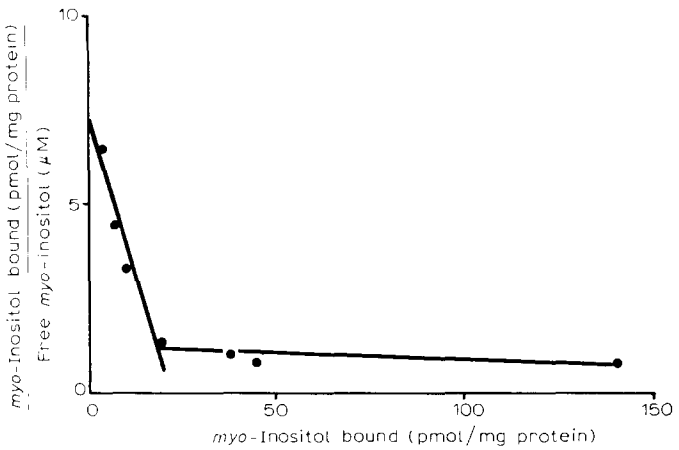

Fig. 4. A Scatchard plot showing the relationship between the concentration of *myo*-inositol and its binding. Binding assay was carried out with various concentrations of *myo*-[^{3}H]inositol for 60 s.

inositol concentrations were low. For this reason, *myo*-inositol binding to the membranes was measured by 1 min of incubation. Binding of *myo*-inositol expressed as pmol bound per mg of membrane protein in 1 min was dependent on the concentrations of *myo*-inositol in the medium. Scatchard plot of the results revealed that there were two distinct binding systems (Fig. 4). One system, which had a high affinity to *myo*-inositol, could bind 22 pmol/mg protein. Another system, which had a low affinity to *myo*-inositol, indicated the presence of non-specific binding or transport into intravesicular space when *myo*-inositol concentration was high. As shown in Fig. 5A, the high affinity site

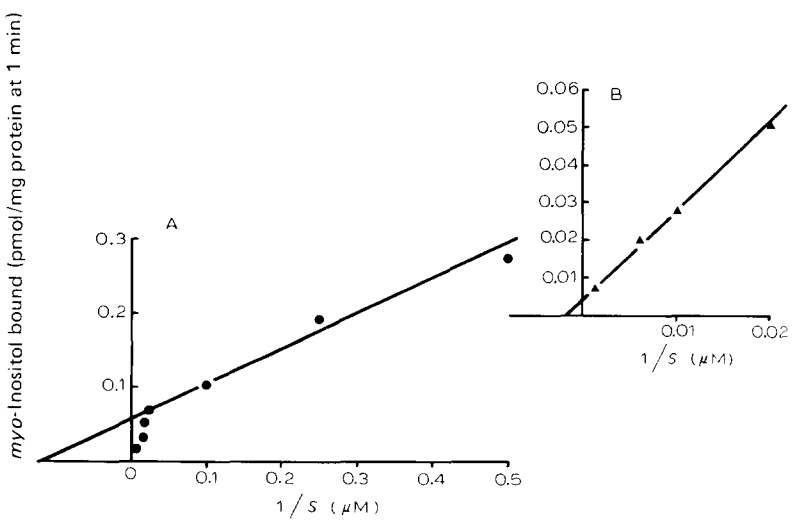

Fig. 5. Double reciprocal plot of bound *myo*-inositol. Binding assay was the same as described in Fig. 4. A and B represent the high and low affinity region, respectively.

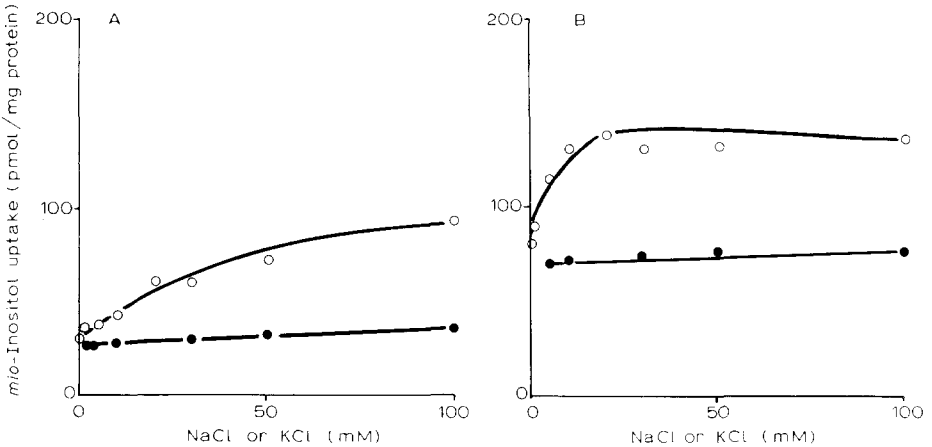

Fig. 6. Effect of Na^+ on the binding and transport of *myo*-inositol to brush border membranes. (A) Brush border membranes were incubated with 110 μM *myo*-[^{3}H]inositol for 2 min in the presence of various concentrations of NaCl, (○) of KCl, (●). (B) Brush border membranes were incubated with 110 μM *myo*-[^{3}H]inositol for 20 min in the presence of NaCl, (○) of KCl, (●). In both cases, osmolarity in the reaction medium was regulated to be constant by addition of mannitol.

reached saturationed at about 20 μM, with a concentration at half saturation of 8.3 μM. Fig. 5B shows the low affinity site which was saturated at 1—1.3 mM with a K_m value of 500 μM.

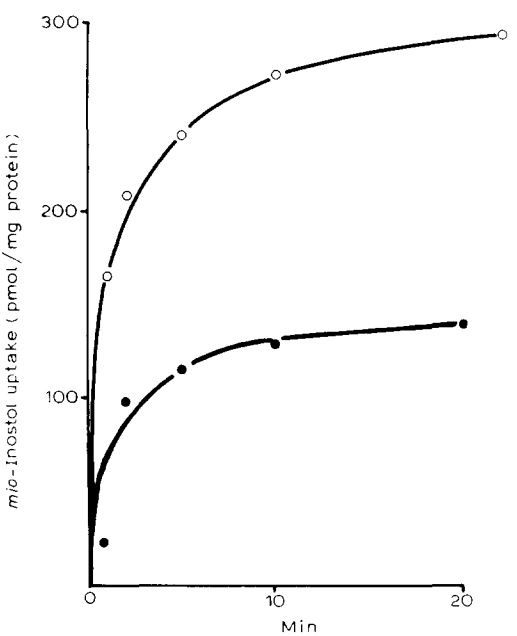

Fig. 7. Effect of Na^+-gradient on *myo*-inositol uptake. The uptake of 110 μM *myo*-[^{3}H]inositol by brush border membranes was examined in the presence of a Na^+-gradient, (○) or in the presence of Na^+ with no gradient, (●) obtained by preincubation in NaCl.

Effect of Na^+ on the binding step and the following transport step

Effect of various concentrations of NaCl on the two phases of uptake was examined. By addition of NaCl, both steps were activated (Fig. 6). However, the transport process was more strongly dependent on Na^+ than the binding process. Activation of the transport attained a plateau at 20 mM NaCl. On the other hand, the binding of *myo*-inositol increased with concentration of NaCl.

Effect of Na^+-gradient on myo-inositol transport

The sodium ion dependency of *myo*-inositol uptake may be due either to Na^+-gradient which exists between the medium and the intravesicular space or to an interaction of Na^+ with the membrane which facillitates *myo*-inositol transport. To examine the first possibility, the following experiment was carried out. Isolated brush border membranes were preincubated in Na^+-free medium or in a medium containing 100 mM NaCl for 20 min at 30° C. After the incubation, the treated membranes were transferred to a medium containing 100 mM NaCl. By these procedures, membranes which had a Na^+-gradient and those which did not have a gradient were prepared. Using these membranes, *myo*-inositol transport, during 10 min of incubation at 30° C, was determined. The presence of Na^+-gradient induced a marked stimulation of *myo*-inositol transport (Fig. 7).

Discussion

Brush border membranes isolated in isotonic media could take up *myo*-inositol more rapidly than those treated in hypotonic media. In that case, both specific and non-specific uptake were fairly rapid and non-specific uptake contributed to a significant portion of the total uptake. These membranes may be rather leaky to small molecules such as *myo*-inositol. However, by treating membranes in hypotonic media, non-specific uptake was suppressed significantly. In addition, with incubation for 2 h, the amounts taken up non-specifically did not reach the level contained in the medium. This finding showed that the membrane vesicles treated hypotonically were relatively tight. Busse et al. [26] reported that brush border membranes obtained by hypotonic lysis formed vesicles and that small molecules such as glycerol and erythritol could penetrate into them but polyethylen glycol 4000 could not. The degree of tightness of the membrane vesicles seems to be dependent on the isolation method and the medium in which the reaction was carried out. Vesicles prepared by Kinne et al. [21] were relatively tight and after 20 min of incubation, the L-glucose level inside the vesicles, which seemed to be derived from leaking, was approximately 40% of that of D-glucose. On the other hand, Evers et al. [27] reported that the amounts taken up non-specifically, which were due to leaking, reached the level in the medium after 20 min of incubation. This result indicated that their preparation was fairly leaky to small molecules.

Detailed data on the relationship between binding and transport have not been reported. In general, at a low substrate concentration, binding to membrane receptor contributed a significant portion of the total uptake. Various trials were made in studies of sugar uptake to minimize contribution of binding. Busse et al. [28] used sugar at high concentrations such as 1 mM to mea-

sure only the amount taken up into intravesicular space. Kinne et al. [21] stopped the uptake reaction by adding a solution containing phlorizin which completely displaced glucose from its binding site and thus determined only the amount transported into intravesicular space.

There have been many investigations on the effect of osmolarity on binding and transport of sugars with brush border membranes [20—22]. According to the results, uptake into intravesicular space could be reduced by increasing osmolarity, whereas, binding to the membranes was influenced little by osmolarity. Our results, using hypotonically treated membranes, that uptake could not be reduced to zero by increasing osmotic pressure, showed that the uptake included binding and transport at the same time. Especially at a very low concentration of *myo*-inositol (1 μM), a minor decrease of the uptake was caused by increasing the osmolarity. This finding was consistent with the idea that at a low substrate concentration binding to membranes contributed a larger portion of the total uptake. In addition, the efflux experiment shown in Fig. 2, also suggested that during a short term incubation, simple binding rather than transport into intravesicular space mainly occurred.

A number of publications concerning the uptake of D-glucose by the brush border described Na^+-dependent transfer of the substance [20—22,25]. Binding of phlorizin to brush border membranes was also stimulated by the addition of NaCl [29]. Recently, Aronson et al. [22] and Kinne et al. [21] have reported that the presence of Na^+-gradient between the incubation medium and the intravesicular space induced a marked stimulation of D-glucose transport. Likewise, both binding and transport processes of *myo*-inositol with brush border membranes were dependent on Na^+, and the latter was enhanced by Na^+-gradient.

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