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myo-INOSITOL TRANSPORT IN PLASMA MEMBRANE OF RAT KIDNEY

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SUMMARY

The *myo*-inositol transport system in kidney plasma membrane preparation was investigated. *myo*-Inositol uptake was more rapid than that due to non-specific uptake. Specific *myo*-inositol uptake was temperature dependent and pH sensitive; the optimum was at pH 7.4. Specific *myo*-inositol uptake was inhibited by scyllitol and inosose-2 but not (+)-inositol, D-glucose, D-galactose or mannitol. Inhibition of *myo*-inositol uptake by scyllitol was of the competitive type. It showed that the transport system is stereospecific and that *myo*-inositol shares the transport system with scyllitol. Moreover, the specific *myo*-inositol uptake was inhibited by phlorizin. Counter transport of *myo*-inositol was demonstrated. The results indicate that *myo*-inositol uptake by the membrane preparation represents the entry into the intravesicular spaces rather than binding to the membrane.

It was concluded that the plasma membrane of rat kidney has a cyclitol carrier system specific to *myo*-inositol and scyllitol.

INTRODUCTION

It is well known that the *myo*-inositol content of mammalian tissue is extremely high in kidney, brain, thyroid, seminal fluid and seminal vesicle, although it is maintained at a low level in blood [1]. Howard and Anderson [2] reported the uptake of *myo*-inositol by kidney slices against a concentration gradient. Hauser [3–5] demonstrated that *myo*-inositol was actively transported in rat kidney slices and that the uptake was Na^+ and energy dependent and inhibited by phlorizin. According to Johnstone and Sung [6], the active transport of *myo*-inositol was also found in Ehrlich ascites tumor cells and the uptake of *myo*-inositol disappeared by removing Na^+ or K^+ from the medium. The uptake was inhibited by 2,4-dinitrophenol but not by D-glucose, D-galactose, galactitol, 3-*O*-methyl-D-glucose, xylitol and L-xylose. Caspary and Crane [7] found that *myo*-inositol was actively transported in hamster intestine and the uptake was Na^+ dependent and phlorizin sensitive. In this case, in contrast to Ehrlich ascites tumor cells, D-glucose inhibited the active transport of *myo*-inositol.

Recently, using isolated plasma membrane, the properties of the glucose transport system have been studied [8–11]. D-Glucose transport into fat cell membranes was investigated under limited conditions, in which no demonstrable metabolism occurred. Evidence was presented that D-glucose transport is temperature dependent and is inhibited by 3-*O*-methylglucose and phlorizin but not by L-glucose [8]. Hopfer et al. [9] investigated D-glucose transport in rat small intestine brush border. With the isolated membrane, it was demonstrated that D-glucose was transported more rapidly than L-glucose and the initial uptake of D-glucose was increased 3- to 5-fold by the addition of Na^+. Moreover, counter transport of D-glucose was also demonstrated. According to Carter et al., plasma membranes from adipose cells of rat [10, 11] and human blood cells [12] demonstrated facilitated diffusion of glucose in a manner identical to sugar transport in the intact cells.

However, cyclitol transport has not been demonstrated with isolated membranes and the mechanism of *myo*-inositol transport remains obscure. This paper demonstrates a *myo*-inositol transport system in the plasma membrane of rat kidney.

MATERIALS AND METHODS

Materials

myo-Inositol was purchased from Nippon Rikagaku Yakuhin Co., Ltd. D-Glucose, D-galactose, mannitol and phlorizin were obtained from Wako Pure Chemicals Industries, Ltd. Bio-Solve was from Beckman Instruments, Inc. Scyllitol and inosose-2 were supplied by Mitsui Pharmaceutical Co., Ltd. *myo*-[^{3}H]Inositol (35600 dpm/nmole) and [^{3}H]scyllitol (7960 dpm/nmole) were generously given by Dr T. Komai, National Institute of Health, Tokyo. (+)-Inositol was prepared from a hydrolysate of Kasugamycin as described before [13].

Methods

Protein was determined by the method of Lowry et al. [14] after heating the membrane samples in a boiling water bath in 1.0 M NaOH.

The following enzyme assays were employed in this study. Glutamate dehydrogenase (EC 1.4.1.2) was measured according to Kinne and Kinne-Saffran [15]. Glucose-6-phosphatase (EC 3.1.3.9) was assayed as described by Nordline and Arion [16] and the method of Post and Sen [17] was used to determine the (Na^+, K^+)-ATPase (EC 3.6.1.3) activity.

The isolation of *myo*-inositol 1-phosphate was carried out by the method of Clarke and Dawson [18].

Preparation of plasma membrane fraction from kidney

Male rat kidney cortex was separated from the remainder with a razor blade and the tissues were homogenized in 0.25 M sucrose with eight strokes in a Potter homogenizer with a Teflon pestle. The $500 \times g$ (10 min) supernatant was centrifuged at $16\,000 \times g$ (30 min). The upper layer of the pellet (pink layer) was homogenized in 0.25 M sucrose with three strokes. The homogenate was recentrifuged at $16\,000 \times g$. This step was repeated three times. The pellet thus obtained was used as a plasma membrane source. The transport assay was carried out with this membrane preparation unless otherwise described. This membrane preparation had an (Na^+, K^+)-

ATPase specific activity three times higher than that of the starting homogenate and this fraction had the highest (Na^+, K^+)-ATPase activity. However, the glucose-6-phosphatase activity did not change, whereas the specific activity of glutamate dehydrogenase was reduced 2.5-fold. Further purification of plasma membrane and mitochondria was achieved by the method of Fitzpatrick et al. [19].

Uptake experiment

The usual incubation mixture consisted of 3–5 mg of membrane protein and *myo*-[^{3}H]inositol or [^{3}H]scyllitol in 0.5 ml of Krebs–Ringer phosphate buffer (pH 7.4). After incubation of the mixture at 37 °C for the indicated time, it was rapidly centrifuged at 16 000 × *g* for 20 min. The pellet was washed with ice-cold buffer and then solubilized with 0.3 ml of 0.5 M NaOH in a boiling water bath. After cooling, 0.6 ml of Bio-Solve was added. After mixing vigorously, 0.5 ml of the solution was transferred to a vial. Radioactivity was measured with a liquid scintillation counter.

Specific *myo*-inositol and scyllitol uptake was calculated by subtracting from the total radioactivity an amount due to the non-specific uptake, which was not displaced by high concentrations of unlabeled cyclitol (usually 200 times more unlabeled cyclitol than labeled cyclitol).

Paper chromatography

Paper chromatography of carbohydrates was performed on Whatman No 1 paper in an ascending system of pyridine–n-butanol–water (1 : 1 : 1, by vol.). The R_F of *myo*-inositol was 0.36 in this system.

RESULTS

Uptake of myo-inositol and scyllitol in rat kidney plasma membrane

The plasma membrane preparation showed a rapid *myo*-[^{3}H]inositol uptake as shown in Fig. 1. It was also demonstrated that [^{3}H]scyllitol was taken up by the membrane almost in the same manner as *myo*-inositol. By the addition of a large amount of unlabeled *myo*-inositol or scyllitol to the system, the uptake rate decreased significantly in both cases and the value was used as the non-specific uptake. The specific uptake was calculated from the total uptake value by subtracting the non-specific uptake value. The specific uptake of *myo*-inositol and scyllitol increased linearly within the first 20 min during the incubation. It shows that the kinetic analysis can be done only within 20 min. It appears that the specific uptake of scyllitol was slightly more rapid than that of *myo*-inositol during the first period.

Identification of radioactive compounds in the membrane after incubation with myo-[^{3}H]-inositol

Distribution of *myo*-[^{3}H]inositol incorporated into the membrane preparation was examined to elucidate whether the incorporated *myo*-[^{3}H]inositol remained in the free or in the combined form as inositol phosphatide. The radioactivity due to the incorporated *myo*-[^{3}H]inositol was mostly recovered in the water-soluble fraction. In the chloroform–methanol fraction, the radioactivity was almost negligible. To ascertain whether the radioactivity in the water-soluble fraction was due to *myo*-[^{3}H]-

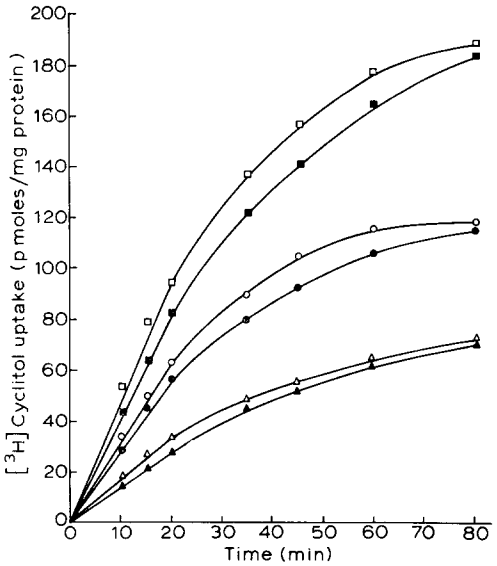


Fig. 1. Time course of *myo*-[^{3}H]inositol and [^{3}H]scyllitol uptake by rat kidney membranes. Kidney membranes were incubated with 110 μM of *myo*-[^{3}H]inositol or [^{3}H]scyllitol in the presence or absence of 11 mM of unlabeled *myo*-inositol or scyllitol in a total volume of 0.5 ml of Krebs–Ringer phosphate buffer at 37 °C for the indicated time. After centrifugation, the pellet was washed with ice-cold buffer (not containing unlabeled cyclitol). ■, *myo* [^{3}H]inositol uptake, in the absence of unlabeled *myo*-inositol; □, [^{3}H]scyllitol uptake in the absence of unlabeled scyllitol; ▲, *myo*-[^{3}H]-inositol uptake in the presence of unlabeled *myo*-inositol; △, [^{3}H]scyllitol uptake in the presence of unlabeled scyllitol; ●, specific uptake of *myo*-[^{3}H]inositol; ○, specific uptake of [^{3}H]scyllitol. Specific uptake was determined as described in Materials and Methods. Assays were performed triplicate.

inositol, the preloaded membrane preparation was extracted with trichloroacetic acid and the extract was subjected to paper chromatography, followed by autoradioscanning. Over 97 % of the radioactivity was recovered in one spot with the same R_F value as authentic *myo*-inositol. In addition, the trichloroacetic acid extract was passed through a column of an anion-exchange resion (Dowex 1-OH). The radioactivity was mostly found in the flow-out fraction. In the eluates, no radioactivity was found. Therefore, it became clear that the radioactivity incorporated into the membranes was mainly retained as *myo*-[^{3}H]inositol without being metabolized.

Subcellular localization of myo-inositol uptake

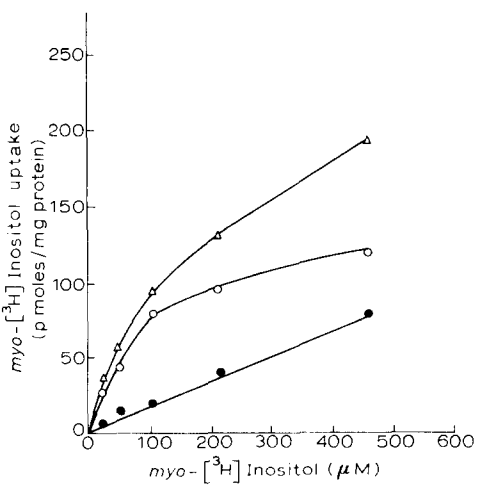
To ascertain the distribution of *myo*-inositol uptake activity among subcellular fractions, rat kidney tissue was fractionated as shown in Table I. It was found that the *myo*-inositol uptake activity was most active in the 12 000 $\times g$ precipitate, which contained mainly plasma membranes and mitochondria. The latter two fractions were further separated from each other as in the Materials and Methods section. Using the purified plasma membrane and mitochondria fractions, the uptake activity was examined. *myo*-Inositol uptake activity was only found in the purified plasma membrane fraction and the specific activity increased 2.6-fold over that of the starting homogenate. It was evident that the *myo*-inositol uptake activity was associated with or specific to the plasma membrane fraction.

TABLE I

DISTRIBUTION OF *myo*-INOSITOL UPTAKE ACTIVITY IN SUBCELLULAR FRACTIONS

Kidneys were homogenized in 0.25 M sucrose with a Potter homogenizer. The homogenate was centrifuged at 600 × *g* for 10 min and the supernatant was centrifuged at 12 000 × *g* for 20 min. The supernatant was centrifuged at 30 000 × *g* for 20 min. The initial homogenate, the 12 000 × *g* ppt. and the 30 000 × *g* ppt. fraction were subjected to the assay. Purified plasma membrane and mitochondria were isolated by the method described in Materials and Methods. Each fraction was incubated with 56 μM of *myo*-[^{3}H]inositol in 0.5 ml of Krebs–Ringer phosphate buffer at 37 °C for 40 min. Besides these treatments, the initial homogenate was centrifuged at 80 000 × *g* directly and this ppt. fraction was used as a homogenate fraction. The specific uptake was determined as described in Materials and Methods. Values are the average of three determinations.

Subcellular fraction	Specific *myo*-inositol uptake (cpm/mg protein)
Homogenate	353
12 000 × *g* ppt.	553
30 000 × *g* ppt.	240
Plasma membrane	913
Mitochondria	45



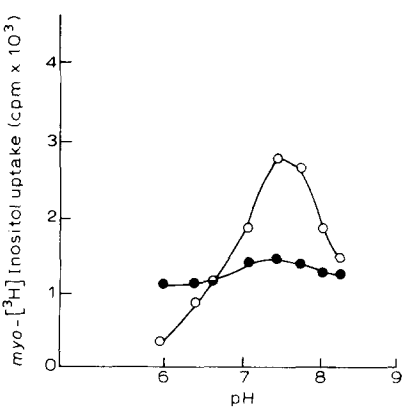


Fig. 2. Effect of concentration of *myo*-[^{3}H]inositol on its uptake. Kidney membranes were incubated with various concentrations of *myo*-[^{3}H]inositol in 0.5 ml of Krebs–Ringer phosphate buffer at 37 °C for 40 min in the presence or absence of 11 mM unlabeled *myo*-inositol. △, *myo*-[^{3}H]inositol uptake in the absence of unlabeled *myo*-inositol; ●, *myo*-[^{3}H]inositol uptake in the presence of unlabeled *myo*-inositol; ○, specific *myo*-[^{3}H]inositol uptake. Specific uptake was determined as described in Materials and Methods. Assays were performed in triplicate.

Fig. 3. Optimum pH for *myo*-[^{3}H]inositol uptake. Kidney membranes were incubated with 56 μM of *myo*-[^{3}H]inositol in 0.5 ml of Krebs–Ringer phosphate buffer at 37 °C for 40 min. Krebs–Ringer phosphate buffer was adjusted to the indicated pH with phosphate buffer. Specific uptake (○) and non-specific uptake (●) were determined as described in Materials and Methods. Assays were performed in triplicate.

Properties of the myo-inositol uptake system

The specific uptake of *myo*-inositol and scyllitol was temperature dependent. At 0 °C, almost no uptake was observed. The uptake of *myo*-inositol increased directly proportional to the membrane concentration within a range of 0.25–10 mg protein. Usually the uptake experiment was carried out using samples of 3–5 mg protein. *myo*-[^{3}H]inositol uptake increased with increasing *myo*-inositol concentration. Non-specific uptake increased linearly with increasing substrate concentration. The specific uptake was, as shown in Fig. 2, saturated at high concentrations. The optimum pH of the uptake activity was examined (Fig. 3). The specific uptake took place within a relatively narrow pH range. Maximum uptake occurred at pH 7.4 and the activity decreased rapidly at pH values other than 7.4. However, non-specific uptake did not change over the pH range examined.

Inhibition of myo-inositol uptake

The effect of various carbohydrate derivatives on the *myo*-inositol uptake was investigated (Table II). Scyllitol and inosose-2 completely inhibited *myo*-[^{3}H]inositol uptake. On the other hand, (+)-inositol did not affect the uptake at all. These results clearly indicate that the OH orientation at the C-2 position of cyclitols is not essential for transport. In addition, the OH orientation at the C-4 and C-5 positions seemed to be important for the uptake. D-Glucose, D-galactose and mannitol did not affect the specific uptake of *myo*-inositol. It was also found that phlorizin inhibited the uptake significantly at a 1.1 mM concentration but only slightly at 0.5 mM.

TABLE II

EFFECT OF VARIOUS CARBOHYDRATES ON *myo*-[^{3}H]INOSITOL UPTAKE

Kidney membranes were incubated at 37 °C for 40 min in a total volume of 0.5 ml of Krebs–Ringer phosphate buffer containing 56 μM of *myo*-[^{3}H]inositol and the indicated material. Other details were described in Materials and Methods. The degree of inhibition was expressed as a percentage relative to that with 11.1 mM unlabeled *myo*-inositol.

Material	Relative inhibition (%)
Inosose-2 (11.1 mM)	98
Scyllitol (11.1 mM)	102
(+)-Inositol (11.1 mM)	0
Mannitol (11.1 mM)	8
D-Glucose (11.1 mM)	8
D-Galactose (11.1 mM)	2
Phlorizin (1.1 mM)	56
Phlorizin (0.5 mM)	12

In order to elucidate the mechanism of the inhibition by scyllitol, the specific uptake of *myo*-inositol at various concentrations was measured in the absence and in the presence of 1.12 mM scyllitol. Lineweaver–Burk plots (Fig. 4) showed a straight-line relationship and the inhibition of specific *myo*-inositol uptake by scyllitol was of the competitive type. The apparent K_m of *myo*-inositol was found to be 2 mM and the apparent K_i with scyllitol was 1.7 mM.

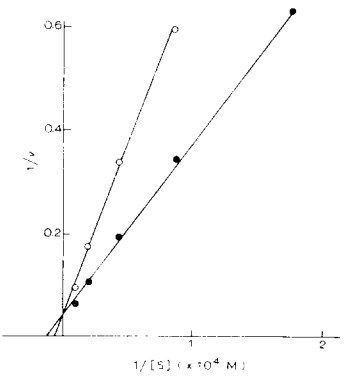


Fig. 4. Lineweaver–Burk plot of the specific uptake of *myo* [^{3}H]inositol and the inhibition by scyllitol. Kidney membranes were incubated with various concentrations of *myo*-[^{3}H]inositol in the presence (○) or absence (●) of 1.12 mM scyllitol at 37 °C for 15 min. v is velocity expressed as 10^{-11} moles/mg protein per 15 min and has been corrected for non-specific uptake.

Counter transport of myo-inositol

In plasma membranes of several kinds of cells, counter transport of sugars [8–12] has been demonstrated. To ascertain whether or not a similar mechanism is involved in the *myo*-inositol uptake process, the possibility of counter transport was examined with *myo*-inositol and the membrane preparation used in this study, as follows. The membrane preparation was incubated with *myo*-inositol, scyllitol or mannitol to accumulate each of these materials into the intravesicular spaces. Then the preloaded membrane preparation was diluted rapidly to produce a reverse concentration gradient between the medium and intravesicular spaces. To these preparations, *myo*-[^{3}H]inositol was added and the incorporation rates were compared. The results are listed in Table III. It is clearly shown that *myo*-[^{3}H]inositol was incorporated by the membrane preparation which had been preloaded with *myo*-inositol. In the case of a membrane preparation pretreated with scyllitol or mannitol,

TABLE III

EFFECT OF PRELOADING WITH UNLABELED POLYOLS ON THE UPTAKE OF *myo*-[^{3}H]INOSITOL

Membranes were incubated in 0.2 ml of Krebs–Ringer phosphate buffer with *myo*-inositol, scyllitol or mannitol (20 mM) for 30 min at 20 °C, then 20 min at 0 °C. They were rapidly diluted with 19 vol. of the buffer containing 1 mM *myo*-[^{3}H]inositol. The incubation medium for membranes preloaded with *myo*-inositol or scyllitol received 1 mM mannitol and the one for the control membrane preparation (preloaded with mannitol) 1 mM unlabeled *myo*-inositol. The mixture was incubated for 15 min at 37 °C. The uptake of *myo* [^{3}H]inositol was determined as described in Materials and Methods. Values are given as the mean ± S.D. with three determinations.

Preloading polyol (20 mM)	*myo*-Inositol uptake (cpm/mg protein)
myo-Inositol	6936 ± 404
Scyllitol	6456 ± 233
Mannitol	5600 ± 70

a significant uptake was also detected. It was also found that the uptake of *myo*-[^{3}H]-inositol was more rapid in the preparation preloaded with *myo*-inositol or scyllitol than that with mannitol. The demonstration of accelerated uptake suggests the occurrence of counter-flow phenomena with respect to the uptake of *myo*-inositol and scyllitol. Taking into consideration that the rates of uptake of *myo*-[^{3}H]inositol in Table III were not corrected for the amount due to non-specific uptake, a real counter-flow rate would be more distinct than that shown in the table.

DISCUSSION

We observed that *myo*-inositol transport by rat kidney slices was competitively inhibited by the addition of scyllitol or inosose-2. But (+)-inositol showed no inhibitory activity. From a comparison of the scyllitol and *myo*-inositol uptake in the slices, some evidence was presented to show that both cyclitols are incorporated by rat kidney by means of a shared transport system [20].

In this communication, the participation of the plasma membrane of rat kidney was demonstrated. It was shown that the uptake of *myo*-inositol by the plasma membrane had similar features to that by slices. With respect to the inhibition activity of cyclitol isomers, scyllitol and inosose-2 inhibited the uptake competitively. No inhibition was detected with (+)-inositol, mannitol, D-glucose and D-galactose. Therefore, the *myo*-inositol transport system may be different from the glucose transport system but specific to cyclitols by means of recognizing the stereospecific OH orientation. In this connection, it was of interest that phlorizin, a potent inhibitor of D-glucose transport, also interfered with the *myo*-inositol uptake.

For the mechanism of D-glucose uptake by membrane preparations, a counter-transport phenomenon has been reported with plasma membranes of rat adipose tissue [10, 11], brush border preparations of rat small intestine [9] and red blood cell ghosts [12]. In these cases, vesicular membrane preparations pretreated with D-glucose incorporated labeled D-glucose from the medium more rapidly than those pretreated with L-glucose, mannitol or other compounds. The acceleration of the substrate uptake was considered to be due to the presence of a specific counter flow. The same phenomenon occurred in *myo*-inositol uptake. In fact, the rat kidney membrane preparation preloaded with *myo*-inositol was able to take up *myo*-[^{3}H]-inositol more efficiently than the mannitol-treated sample. This indicates the occurrence of counter flow as in the case of D-glucose uptake. Therefore, it appeared that the vesicular structure of the kidney membrane was responsible for the specific accumulation of *myo*-[^{3}H]inositol. The fact that the uptake showed a very narrow range of pH optimum seemed to be another feature of the intravesicular accumulation. In contrast, non-specific uptake did not show such a pH optimum but gave a very flat pH–activity curve (Fig. 3). The uptake was very sensitive to the incubation temperature and at 0 °C the uptake was almost negligible. This fact would indicate the participation of membrane vesicles rather than specific binding to the active sites of the membrane. Moreover, it was observed that the specific uptake activity decreased significantly after keeping the preparation in cold conditions overnight. It was considered to be due to the partial destruction, at least, of the vesicular structure during the storage. In addition, a striking decrease in the specific *myo*-inositol uptake was

observed in hyperosmotic medium. These results also suggest intravesicular accumulation.

The plasma membrane preparation used in this experiment contained brush border membranes. It is important to ascertain whether the specific uptake observed here was mainly derived from the properties of plasma membranes or brush border membranes. The plasma membrane must have an important role to maintain high *myo*-inositol levels in tissues other than kidney and intestine. However, in kidney and intestine, the possibility of the participation of the brush border membrane should be examined using a purified preparation.

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