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# Oral ingestion of mannose alters the expression level of deaminoneuraminic acid (KDN) in mouse organs

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**Abstract** Deaminoneuraminic acid (KDN) is a unique member of the sialic acid family. We previously demonstrated that free KDN is synthesized *de novo* from mannose as its precursor sugar in trout testis, and that the amount of intracellular KDN increases in mouse B16 melanoma cells cultured in mannose-rich media [Angata *et al.* (1999) J. Biol. Chem. 274, 22949–56; Angata *et al.* (1999) Biochem. Biophys. Res. Commun. 261, 326–31]. In the present study, we first demonstrated a mannose-induced increase in intracellular KDN in various cultured mouse and human cell lines. These results led us to examine whether KDN expression in mouse organs is altered by exogenously administered mannose. Under normal feeding conditions, intracellular free KDN was present at very low levels (19–48 pmol/mg protein) in liver, spleen, and lung, and was not detected in kidney or brain. Oral ingestion of mannose, both short-term (90 min) and long-term (2 wk), resulted in an increase of intracellular KDN up to 60–81 pmol/mg protein in spleen and lung and 6.9–18 pmol/mg protein in kidney and brain; however, no change was observed in liver. The level of KDN in organs appears not to be determined only by the KDN 9-phosphate synthase activity, but might also be affected by other enzymes that utilize mannose 6-phosphate as a substrate as well as the enzymes that breakdown KDN, like KDN-pyruvate lyase. In blood, the detectable amount of free KDN did not change on oral ingestion of mannose. These findings indicate that mannose in the diet affects KDN metabolism in various organs, and provide clues to the mechanism of altered KDN expression in some tumor cells and aged organs.

**Keywords** Biosynthesis of sialic acid · Mannose · KDN · Deaminoneuraminic acid · Mannose therapy

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## Introduction

Deaminoneuraminic acid (KDN, 2-keto-3-deoxy-D-*glycero*-D-*galacto*-nononic acid) is a member of sialic acids (Sia) in which an acylamino group at the C-5 position of *N*-acylneuraminic acid is replaced by a hydroxyl group. KDN was initially discovered in rainbow trout eggs [1], and is found in glycoproteins and glycolipids of organisms ranging from bacteria to mammals [1–12]. KDN residues have unique properties distinct from *N*-acetylneuraminic acid (Neu5Ac) or *N*-glycolylneuraminic acid (Neu5Gc). For example, KDN ketosidic linkages are resistant to bacterial and viral sialidases [1,13]. The KDN residue is a stop signal for the elongation of polysialyl chains of fish egg polysialoglycoproteins by capping the non-reducing terminus of the polysialic acid chain [14].

In mammals, KDN expression is very low compared with that of Neu5Ac and Neu5Gc [15,16]. Increased KDN expression, however, occurs in certain cancer cells [11], in liver of aged rats [17], and in fetal erythrocytes [11]. These altered KDN expression patterns might be the

result of changes in KDN metabolism. Thus, our aim is to understand the molecular mechanism underlying altered KDN expression in mammals. In 1999, we demonstrated that free KDN monosaccharide is synthesized by three sequential reactions in rainbow trout testis [15], where mannose is a key 6-carbon-sugar precursor for the synthesis of KDN: Reaction 1, mannose + ATP → mannose 6-phosphate (Man-6-P) + ADP; Reaction 2, Man-6-P + phosphoenolpyruvate → KDN 9-phosphate (KDN-9-P) + Pi; Reaction 3, KDN-9-P → KDN + Pi. We further demonstrated that the amount of intracellular KDN in mouse melanoma B16 cells and African green monkey kidney COS-7 cells increased when the cells were cultured in mannose-rich media, while these cells contained only a minute amount of KDN when cultured under control conditions [16]. This finding led to the hypothesis that KDN biosynthesis in mammalian cells is regulated by the amount of extracellular mannose.

To test this hypothesis, we examined whether the mannose increases intracellular free KDN in various mammalian cell lines. We also investigated if this phenomenon occurs in mouse organs following oral ingestion of mannose.

## Materials and methods

### Materials

Mannose was purchased from Nacalai Tesque (Kyoto, Japan). 2-Aminopyridine and 1,2-diamino-4,5-methylenedioxybenzenne (DMB) were purchased from TaKaRa (Kyoto, Japan) and Dojindo (Kumamoto, Japan), respectively. BCA protein assay reagent (PIERCE, Rockford, IL, USA) was used for quantifying the protein amount. Dulbecco's Modified Eagle Medium (DMEM) was obtained from Sigma (St. Louis, MO, USA). Fetal bovine serum (FBS) was purchased from Thermo Trace (Melbourne, Australia). KDN and ManNAc 6-phosphate (ManNAc-6-P) were prepared as described previously [18,19].

### Cell lines and animals

Cultured cell lines used were mouse melanoma B16, mouse fibroblastic preadipocyte 3T3-L1, mouse leukemic monocyte RAW-264, mouse hepatocellular carcinoma Hepa 1–6, mouse neuroblastoma Neuro2A, mouse mammary epithelial COMMA1-D, chronic human myelogenous leukemia cells K562, and human cervical carcinoma HeLa. DMEM (1 g/l glucose) containing 10% FBS, 100 units/ml Penicillin G and 0.1 mg/ml Streptomycin was used for B16, Hepa, RAW, Neuro2A, and HeLa cells. DMEM (4.5 g/l glucose), instead of DMEM (1 g/l glucose), was used for 3T3-L1, Neuro2A, and COMMA 1D cells. For K562 cells, F12-Nutrient mixture (Sigma) was used instead of DMEM. Mice (ddY, female, 14 weeks old) were purchased from Japan CLEA (Tokyo Japan), and were fed on a normal diet (CE-2, Japan CLEA) with water.

### Fluorometric high performance liquid chromatography (HPLC) for analyses of sialic acids and hexoses

The sialic acids were subjected to derivatization with DMB as described [20,21]. DMB derivatives of sialic acids were quantitatively analyzed by the fluorometric HPLC. Briefly, the DMB derivative solution was directly applied to an ODS column (250 × 4.6 mm *i.d.*, Wakopak Handy-ODS, Wako, Japan) and eluted isocratically with methanol/acetonitrile/water (7/9/84, v/v/v) at 1.0 ml/min at a column temperature of 26°C on a JASCO LC-900 HPLC system equipped with a JASCO FP-920 fluorescence detector (excitation, 373 nm; emission, 448 nm). Mannose and glucose were determined by a pyridylamination/HPLC method as described previously [22]. Pyridylaminated sugars were subjected to HPLC on a TaKaRa PALPAK Type A column (4.6 × 150 mm *i.d.*) on the HPLC-fluorescence detector system (excitation, 310 nm; emission, 380 nm). The column was eluted with 0.7 M potassium borate, pH 9.0: acetonitrile (9:1, v/v) at 65°C at 0.4 ml/min for 200 min.

### Quantification of sialic acids in cells cultured in the presence and absence of mannose

Each cell line was grown in a 9-cm plastic dish (TPP, Trasadingen, Switzerland) in DMEM medium supplemented with 10% FBS. Cells were incubated in the medium supplemented with 20 mM mannose for 24 h. After the medium was discarded, the cells were washed three times with cold PBS and harvested by scraping with a plastic policeman in ice-cold PBS. After centrifugation at 850 × *g* for 5 min, the cell pellet was suspended in ice-cold water and homogenized by sonication at 50 watts for 30 sec on Branson Sonifier 250. An aliquot of the homogenate was analyzed for protein amounts by the BCA protein assay reagent. The homogenate was centrifuged at 100,000 × *g* for 60 min and the pellet was used for analysis of bound Sia as described [15]. The supernatant was mixed with ethanol (final 70%), kept on ice for 30 min, and centrifuged at 15,000 × *g* for 15 min. An aliquot of the supernatant was lyophilized and used for analysis of free Sia as described [16].

### Quantification of sialic acids in organs from mice orally administered with mannose

Mannose or glucose was orally ingested to mice (ddY, female, 14 weeks old) under the following conditions. (i) control group: Two mice were free to access to a usual diet and water;

(ii) Man/90 m group: Two mice, after fasting overnight, were intragastrically ingested with 0.2 ml mannose solution containing 18 mg mannose with a sonde tube and left for 90 min before collecting organs; (iii) Glc/90 m group: Two mice, after fasting over night, were intragastrically ingested with 0.2 ml glucose solution containing 18 mg glucose and left for 90 min before collecting organs; (iv) Two mice were free to access to the usual diet and water containing 5% mannose for 2 weeks. After the treatment, brain, lung, liver, kidney, and spleen were excised, immediately frozen, and kept at $-80^{\circ}$C until use. Each organ was minced and homogenized in 10 mM Tris-HCl (pH 7.5) using a Polytron homogenizer (Kinematica, Switzerland). The homogenate was centrifuged at $100{,}000 \times g$ for 60 min. The supernatant was mixed with ethanol (final 70%) and kept on ice for 30 min. After centrifugation at $15{,}000 \times g$ for 15 min, the supernatant was lyophilized and used for analysis of free Sia as described above.

### Determination of concentrations of mannose and KDN in blood

Two mice (ddY, female) were fasted overnight, and ingested with mannose or glucose as described above. Blood was collected from tail vein, and centrifuged at $15{,}000 \times g$ for 15 min. The supernatant was mixed with ethanol (final 70%), kept on ice for 30 min, and centrifuged at $15{,}000 \times g$ for 15 min. The supernatant was lyophilized and used for analysis of mannose and KDN. Mannose and KDN were determined as described above.

### Assay for KDN 9-phosphate synthase activity in various mouse organs

Brain, lung, liver, kidney, and spleen were excised from mice and homogenized in Tris-HCl (pH 7.5) containing 1 mM dithiothreitol, 10% glycerol and protease inhibitors (1 $\mu$g/ml each of aprotinin, leupeptin and pepstatin) using a Polytron homogenizer. The homogenate was centrifuged at $100{,}000 \times g$ at 4°C for 60 min. The supernatant was determined for protein amounts by the BCA protein assay reagent and used as the enzyme fraction. KDN-9-P synthase activity was assayed as described [16]. The reaction mixture (10 $\mu$l) consisted of 4 $\mu$g of enzyme fraction and 10 mM Man-6-P, 10 mM PEP, 20 mM $MgCl_2$ and 1 mM sodium vanadate for KDN-9-P synthase (KPS) activity. For the Neu5Ac-9-P synthase (NPS) activity, 1 mM ManNAc-6-P, instead of Man-6-P, and 1 mM PEP was used. The reaction mixture was mixed with ethanol (final 70%), kept on ice for 30 min, and centrifuged at $15{,}000 \times g$ for 15 min. The supernatant was lyophilized, and was subjected to derivatization with DMB and fluorometric HPLC. Thus, the sample was applied to a resource Q column (6.5 × 30 mm; Amersham Biosciences, Piscataway, NJ, USA), and eluted at 1.0 ml/min with a linear gradient of 0–0.3 M NaCl in 20 mM Tris-HCl (pH 8.0) in 15 min. The elution profile was monitored on fluorescent detector as described above. One unit of the enzyme activity was defined as the amount of enzyme that produces 1 $\mu$mol of KDN-9-P or Neu5Ac-9-P per 30 min under these conditions.

## Results

### The amount of intracellular free KDN increases in various cells cultured in mannose-rich media

Previously, we demonstrated that the amount of intracellular free KDN in B16 and COS-7 cells increased when these cells were cultured in mannose-rich media [16]. To further demonstrate that this phenomenon commonly occurs in various cells, we further analyzed several mouse and human cultured cell lines. Under control culture conditions, the concentration of intracellular free KDN in these cells ranged from 0.011 to 0.076 nmol/mg protein (Figure 1a), and the levels of the bound form of KDN were barely detectable. The amount of intracellular KDN increased in all cell lines that were cultured in mannose-rich media, although the extent of the increase differed between cell lines (Figure 1a).

In mouse RAW-264 and Neuro2A cells cultured in mannose-rich media, the intracellular free KDN markedly increased up to 1.0 and 2.0 nmol/mg protein, respectively. In Neuro2A, the amount of intracellular free KDN was greater than that of free Neu5Ac. These results indicate that mammalian cells have the potential to synthesize free KDN, and suggest that extracellular mannose induces an increase in intracellular free KDN. In all the cell lines tested, the bound form of KDN was expressed at very low levels before and after exogenous addition of mannose, although the amount of the bound form of KDN increased very slightly (data not shown).

In contrast, the levels of intracellular free Neu5Ac were not changed in these cell lines by culture in mannose-rich media (Figure 1b). No free Neu5Gc was detected (data not shown).

### Oral ingestion of mannose raises the blood mannose concentration, but has no effect on the blood KDN concentration in mice

To determine the effects of mannose ingestion on KDN metabolism in mouse organs, we first investigated the concentrations of mannose and KDN in blood before and after oral ingestion of mannose. Mannose (4.5 or 18 mg dissolved in 0.2 ml water) was intragastrically administered to ddY

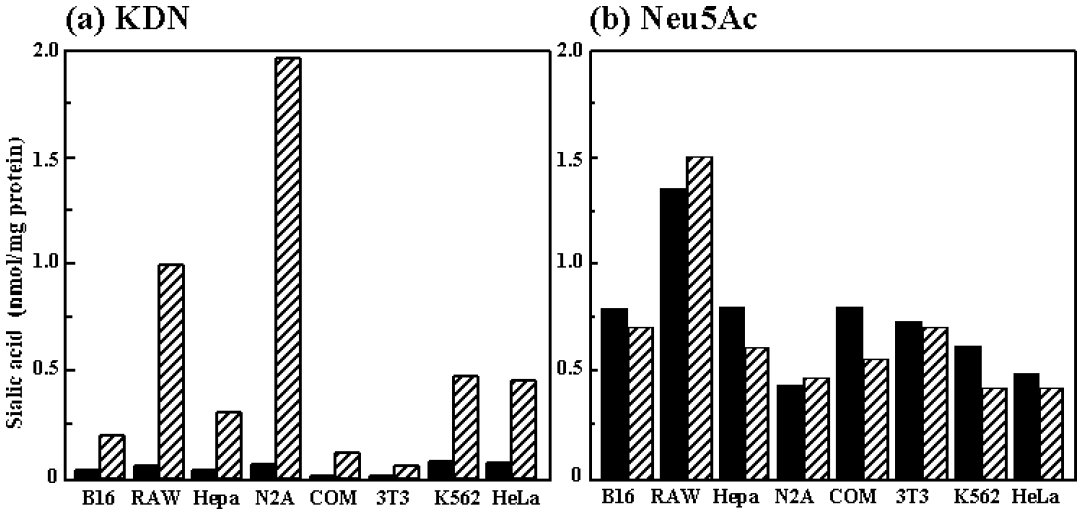


**Fig. 1** Effects of exogenously added Man on the amount of intracellular KDN (a) and Neu5Ac (b). Cells were cultured in the basal media supplemented with (stripe) or without (black) 20 mM Man for 15 h, and the amount of intracellular free KDN (a) and Neu5Ac (b) was determined as described under Materials and methods. *B16*, mouse melanoma cell B16; *RAW*, mouse leukemic monocyte RAW-264; *Hepa*, mouse hepatocellular carcinoma cell Hepa 1–6; *N2A*, mouse neuroblastoma cell Neuro2A; *COM*, mouse mammary epithelial COMMA1-D; *3T3*, mouse fibroblastic preadipocyte 3T3-L1; *K562*, chronic human myelogenous leukemia cells K562; *HeLa*, human cervical carcinoma HeLa.

mice, and the mannose concentration in blood was determined (Figure 2a). Blood mannose concentrations ranged from 100 to 135 $\mu$M before ingestion, and rapidly increased after ingestion. When 4.5 mg/0.2 ml mannose was administered, the blood mannose concentration first increased up to 190 $\mu$M in 15 min following the ingestion, and then decreased rapidly to the original level (square). In contrast, administration of glucose (4.5 mg/0.2 ml) to mice had no effect on the blood mannose concentration (Figure 2a, triangle). When 18 mg mannose was administered, the blood mannose concentration gradually increased up to 310 $\mu$M within 90 min following the ingestion, and then decreased (circle). When mice were also fed a 5% mannose solution for 2 wk, the blood mannose concentration was approximately 120 $\mu$M. In these mice, there were no obvious differences in water intake between mice fed 5% mannose and control mice (data not shown).

The blood KDN concentration was also determined after the oral ingestion of mannose. As shown in Figure 2b, the blood KDN concentration was approximately 2 $\mu$M before ingestion, and remained unchanged after ingestion (Figure 2b, circle).

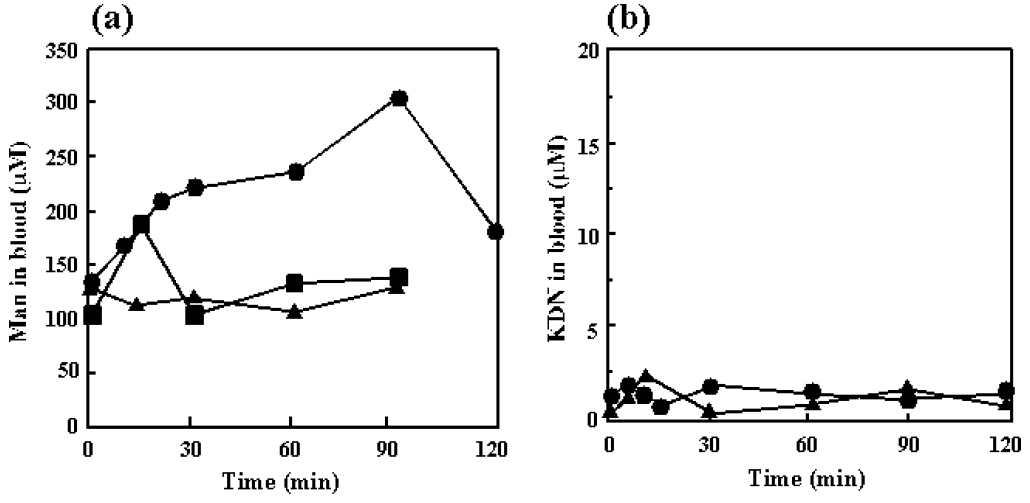


**Fig. 2** Concentrations of mannose and KDN in blood before and after the mannose- and glucose-ingestion. (a) Blood mannose concentration. Blood was collected from the tail vein to analyze the blood mannose concentration at indicated time after intragastric ingestion of 0.2 ml of sugar solution: 4.5 mg mannose (*square*); 18 mg mannose (*circle*); and 4.5 mg glucose (*triangle*). (b) Blood KDN concentration. Blood was also analyzed for the blood KDN concentration at indicated time after intragastric ingestion of 0.2 ml of water (*triangle*) or 18 mg mannose (*circle*).

### Effects of oral ingestion of mannose on the amount of intracellular KDN in mouse organs

Oral ingestion of mannose increased the mannose concentration in blood. Therefore, we investigated whether increased blood mannose concentrations affect KDN metabolism in mouse organs. Mice were fed a normal diet and water as a control. To investigate the long-term effect of oral ingestion of mannose on the level of sialic acids (KDN, Neu5Gc, and Neu5Ac), mice were fed a normal diet and 5% mannose for 2 wk. There was no significant difference in the intake of 5% mannose and water. For investigation of the short-term effect, mice were left for 90 min following a single ingestion of 18 mg mannose or glucose. Liver, lung, spleen, kidney, and brain were excised from the mice to determine the amount of sialic acids. The results are shown in Figure 3. In those organs, the bound form of KDN was detected, although the level was low. It should be noted that body weight and behavior were not changed by mannose ingestion (data not shown). Previous reports also indicate that mannose intake in mice had no obvious effects on growth, health, behavior of mice, organ weights, histology, litter size, or pup growth [23].

(i) **Liver:** Under control conditions, intracellular free KDN was detected at a low level (19 pmol/mg protein), and proportions of the free forms of KDN, Neu5Gc, and Neu5Ac were 4.7%, 81.5%, and 13.8%, respectively (Figure 3a, Control). In liver, there was no short-term or long-term effect of oral ingestion of mannose on the amount of free KDN (Figure 3b).
Neu5Gc expression was 5.9 times higher than that of Neu5Ac in the control condition. Glucose ingestion had no effect on the levels of free KDN and Neu5Ac in liver like under short-time mannose ingestion (Figure 3a). In mice fed mannose for 2 wk, there was a 44% decrease of Neu5Gc and a 25% increase of Neu5Ac, while the level of KDN was constant (Figure 3a, Man/2 wk). Notably, the Neu5Gc/Neu5Ac ratio decreased from 6:1 to 1:1 (mol/mol) during long-term mannose feeding. The effects of long-term mannose ingestion on the expression of CMP-Neu5Ac hydroxylase and de novo synthesis of Neu5Ac in liver remain to be examined.

(ii) **Lung:** In lung, in controls the amount of intracellular free KDN was 48.4 pmol/mg protein, and the proportions of free forms of KDN, Neu5Gc, and Neu5Ac were 10.6%, 51.6%, and 37.8%, respectively (Figure 3c, Control). In lung, there was a 2.5 times higher KDN expression than in liver (48.4 vs 19.2 pmol/mg protein). The amount of free KDN was increased by short-term mannose-ingestion (Figure 3d, Man/90 m), while there was no change in the KDN expression following short-term glucose-ingestion (Figure 3d, Glc/90 m). Long-term ingestion of mannose increased KDN expression to more than 1.3 times higher than that under control conditions (Figure 3d, Man/2 wk).
Neu5Ac and Neu5Gc expression levels were comparable, and appeared to remain constant following both short-term and long-term ingestion of mannose or glucose (Figure 3c).

(iii) **Spleen:** In spleen, in controls the amount of intracellular free KDN was 44.8 pmol/mg protein, comparable with that in lung (Figure 3e, f, Control). The proportion of free forms of KDN, Neu5Gc, and Neu5Ac were 10.1%, 30.9%, and 59.0%, respectively (Figure 3e, Control). With short-term mannose ingestion, but not glucose ingestion, the amount of free KDN increased by 42% (Figure 3f, Man/90 m, Glc/90 m). With long-term mannose ingestion, KDN expression increased by 82% (Figure 3f, Man/2 wk).
The expression level of Neu5Ac to Neu5Gc was about two-fold. There were no obvious differences in the expression of Neu5Gc and Neu5Ac in either the short-term or long-term ingestion of mannose or glucose (Figure 3e).

(iv) **Kidney:** KDN was not detected in kidney in the control condition or in the glucose-ingestion condition (Figure 3g, Control, Glc/m). Following both short and long-term mannose ingestion, however, 10.3 and 17.9 pmol/mg protein of KDN was detected, respectively (Figure 3h).
The molar proportion of Neu5Gc/Neu5Ac amounts was 3:2, and this ratio remained unchanged following short-term mannose or glucose ingestion (Figure 3g). The amount of Neu5Gc increased and that of Neu5Ac decreased following long-term mannose ingestion (Figure 3 g, Man/2 wk).

(v) **Brain:** Intracellular free KDN was not detected in brain in the control condition or in the glucose ingestion condition (Figure 3i). Although the amount was very low (around 10 pmol/mg rotein), KDN was detected in brain following short-term and long-term ingestion of mannose (Figure 3j). These features were similar to those of kidney.
In brain, sialic acid is exclusively Neu5Ac, and no or very low levels of Neu5Gc were detected in all of the conditions tested (Figure 3i). The Neu5Ac expression level increased in the brain following oral mannose or glucose ingestion.

### KDN 9-phosphate synthase activity in various mouse organs

The KDN expression level under both control and mannose-rich culture conditions was different among organs (Figures 3). To examine how KDN 9-phosphate synthase

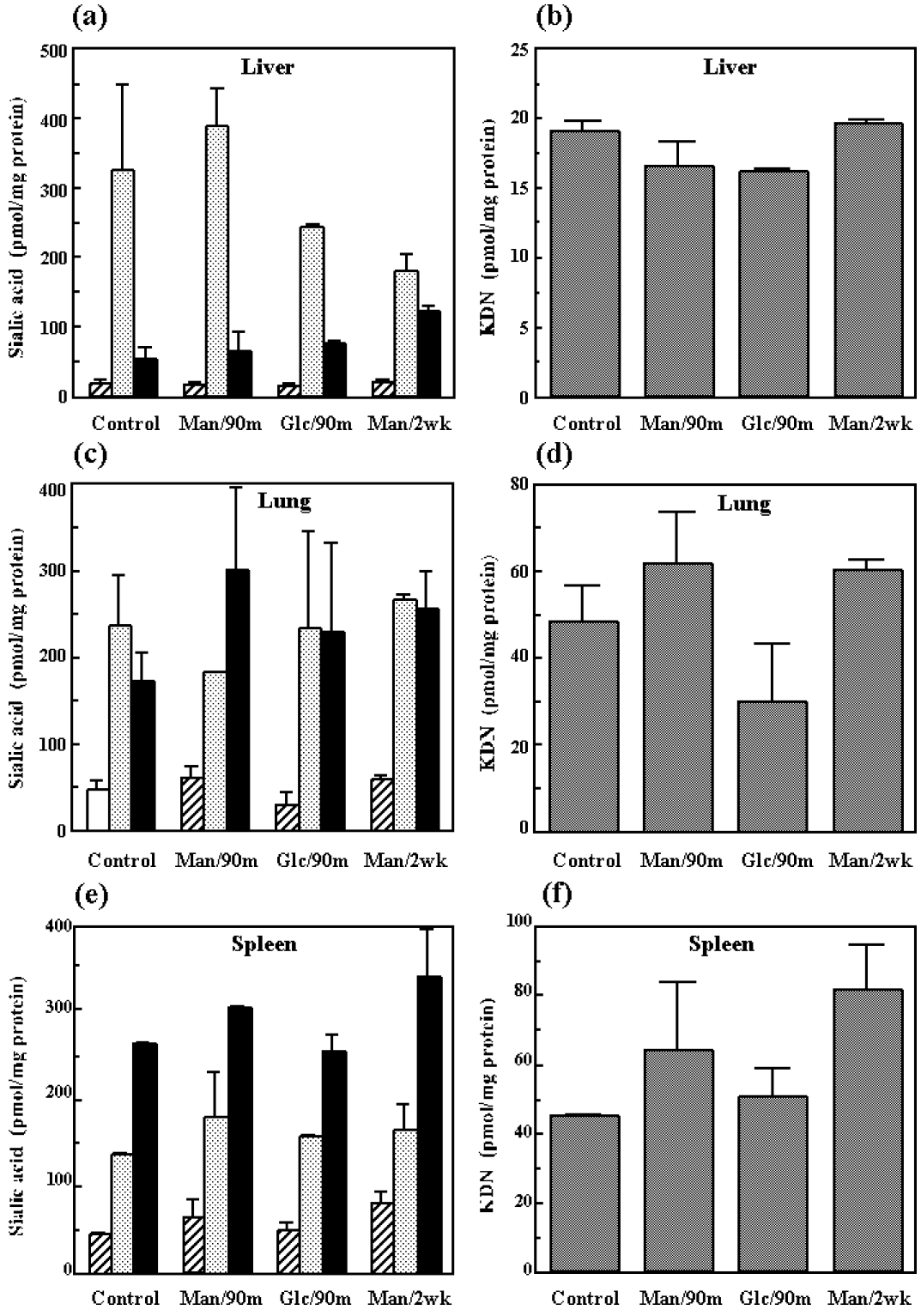


**Fig. 3** Effects of short-term and long-term ingestions of mannose and glucose on the amount of sialic acids in mouse organs. Two mice were kept on a normal diet with water (Control). For the short-term ingestion, 0.2 ml of solution containing mannose (18 mg) was intragastrically administered and the mice were kept for 90 min (Man/90 m). Instead of mannose, glucose (18 mg) was also used in the same experiments (Glc/90 m). For the long-term ingestion, mice were fed on the normal diet with 5% mannose in water for 2 weeks (Man/2 wk). Liver (a, b), lung (c, d), spleen (e, f), kidney (g, h), and brain (i, j) were excised and analyzed for the amount of sialic acids: KDN (stripe), Neu5Gc (stipple), and Neu5Ac (black). Data for KDN are summarized in (b) liver, (d) lung, (f) spleen, (h) kidney, and (j) brain (*Continued*).

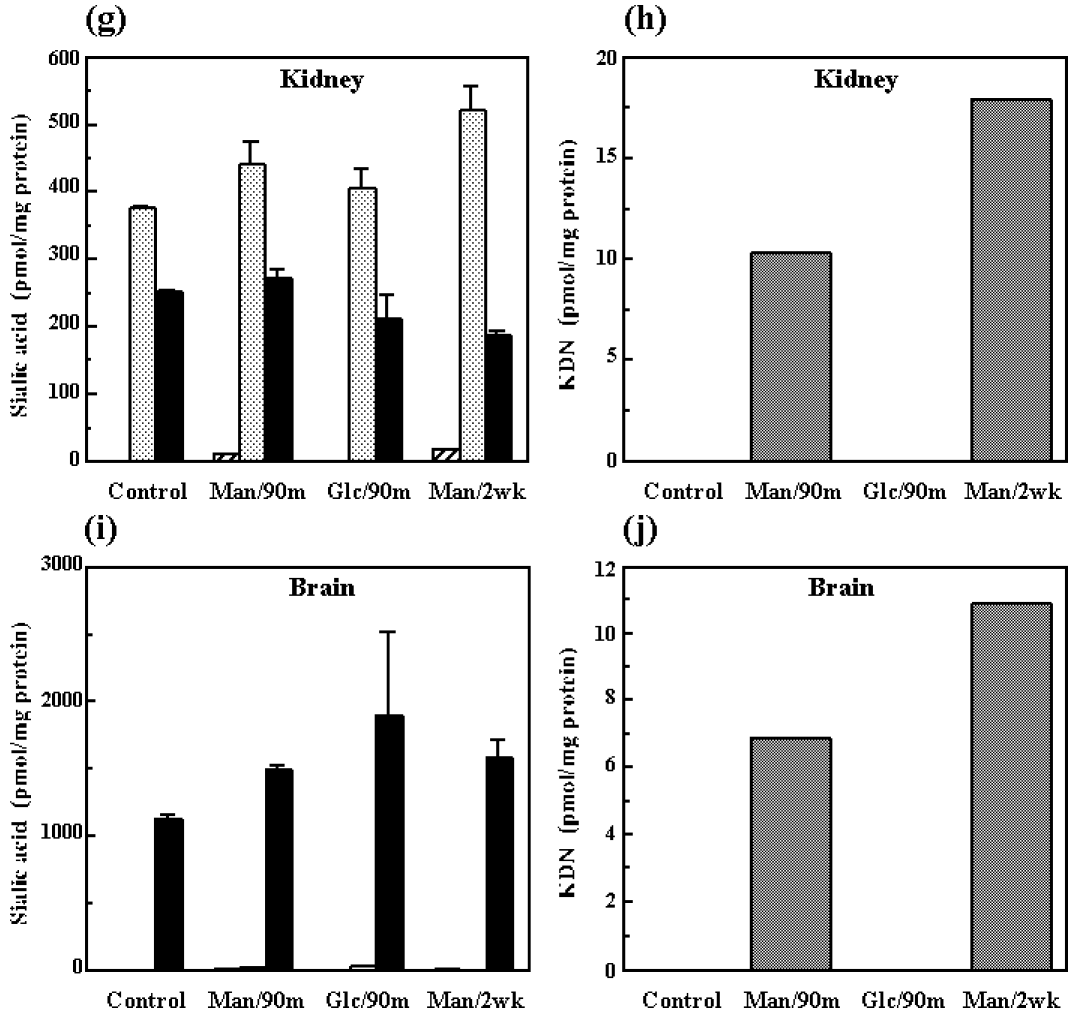


**Fig. 3** *Continued*

(KPS) activity is involved in the expression level of free KDN, KPS activity in various organs was determined. As shown in Figure 4a, KPS activity was detected in all organs examined and the specific enzyme activity ranged from 0.012 to 0.017 mU /mg protein. The Neu5Ac 9-phosphate synthase (NPS) activity was also determined, and specific NPS activities of the organs were 7 to 16 times higher than those for KPS. Notably, the KPS activity level in organs was not always parallel with the mannose-induced increase in free KDN in the same organs. For example, the synthesis of free KDN remained unchanged in liver and increased in spleen, while the KPS activity in liver was much the same as that in spleen. These results suggest that the level of KPS activity does not always determine the synthetic level of free KDN in organs, although it is responsible for the KDN synthesis [15].

In B16 and HeLa cells, the KPS activity did not change before or after mannose-supplementation to the culture media (data not shown), which suggests that KPS activity is not enhanced by mannose-administration in mammalian cells, and possibly in organs.

## Discussion

We previously demonstrated that the amount of intracellular free KDN increased in B16 and COS-7 cell lines cultured in mannose-rich media [16]. In continuation with these experiments, we first demonstrated that the mannose-induced increase of intracellular free KDN ubiquitously occurs in various cell lines (Figure 1a), suggesting that there are common mechanisms for mannose-induced KDN production in mammalian cells. We also confirmed that mannose is an important precursor of KDN (Figure 1a). The oral ingestion experiments, further demonstrated that mannose induces an increase in intracellular free KDN in mouse organs such as lung, spleen, brain, and kidney, but not liver (Figure 3). Following both short-term (90 min) and long-term (2 wk) mannose ingestion, the amount of intracellular free KDN increased in these organs. Free blood KDN levels did not change after mannose ingestion (Figure 2b), thus indicating that KDN is synthesized *de novo* in these organs, but not in blood. The findings that the

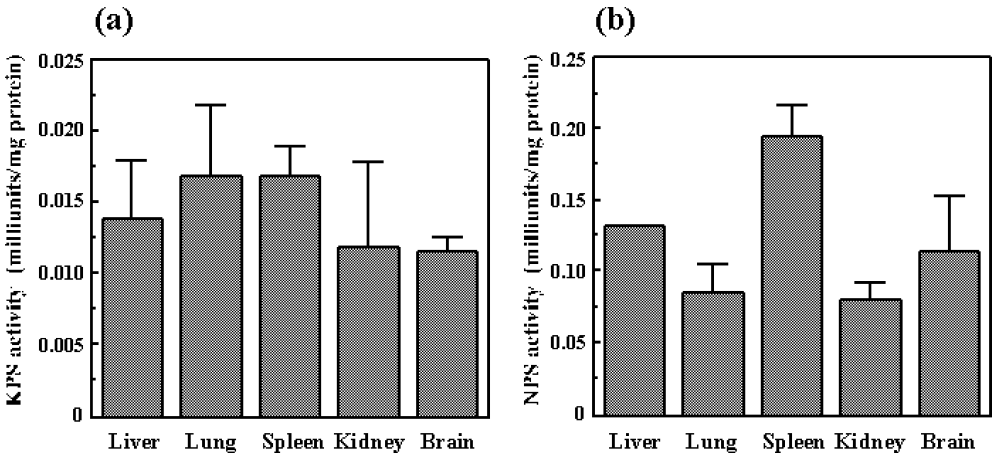


**Fig. 4** KPS and NPS activities in mouse organs. The specific activities for KPS (a) and NPS (b) are shown. All the experiments were performed as described in Materials and methods in duplicate. Errors are indicated by the bars.

mannose-induced increase in KDN synthesis occurred not only in cells cultured in mannose-rich media, but also in organs of mice administered mannose orally, suggest that the expression of free KDN in mammalian cells is regulated by mannose. Even a 90 min-pulse ingestion of mannose results in an increase of free KDN in these organs. These results indicate that the transmigration of mannose into the blood and various organs rapidly proceeds after oral ingestion, consistent with previous observations [23–25].

Notably, the extent of mannose-induced KDN synthesis is different from cell to cell. In addition, free KDN is not elevated in the liver following oral ingestion of mannose. KPS catalyzes the reaction, Man-6-P + phosphoenolpyruvate (PEP) → KDN 9-phosphate (KDN-9-P) + Pi. This reaction is similar to the NPS-catalyzed reaction, where ManNAc-6-P, instead of Man-6-P, is used as a substrate [15,26]: ManNAc-6-P + PEP → Neu5Ac-9-P + Pi. It is conceivable that the KPS activity of various organs determined the increase in the level of free KDN in response to the exogenously added mannose (Figure 4). The KPS activity, however, does not solely determine the level of free KDN, although it is necessary. Rather, it is suggested that the extent of mannose-induced KDN synthesis is regulated by other mannose-related metabolic and transport pathways. These pathways should affect the metabolic flow from mannose to KDN. As shown in Figure 5, the following enzymes and transporters might

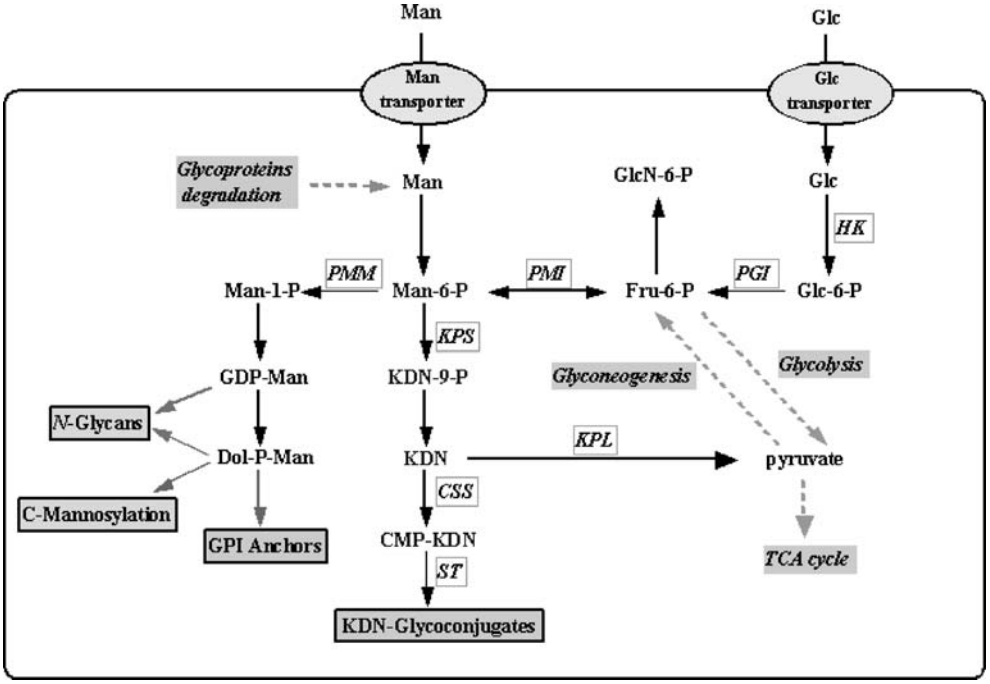


**Fig. 5** A newly proposed metabolic pathway of mannose that includes biosynthetic pathway of KDN. phosphoglucose isomerase (PGI), phosphomannose isomerase (PMI), phosphomannomutase (PMM), hexokinase (HK), CMP-sialic acid synthethase (CSS), sialyltransferase (ST), KDN 9-phosphate synthase (KPS), KDN-pyruvate lyase (KPL).

affect the mannose-induced enhancement of KDN synthesis. Glucose transporters (GLUTs), which efficiently transport glucose into the cell, transport extracellular mannose into the cells to increase the intracellular mannose concentration [27]. A specific mannose transporter is also suggested to be involved in the transport of mannose into the cell [28,29]. Transported mannose is first phosphorylated at the C-6 position by a hexokinase or a putative mannokinase to synthesize mannose 6-phosphate (Man-6-P). A conversion of Man-6-P back to mannose might occur through dephosphorylation by a putative mannose phosphatase. Then, Man-6-P is converted to KDN-9-P by the action of KPS [14,26] and further to KDN by KDN 9-phophatase (Figure 5). These pathways were incorporated into the mannose metabolic network, based on our metabolic study of KDN in rainbow trout [14], and here confirmed in mammals. Two other enzymes might competitively act on Man-6-P as a substrate. One is a phosphomannomutase (PMM), which produces mannose 1-phosphate (Man-1-P). The metabolic flow from this reaction leads to the biosynthesis of glycan chains on glycoproteins, glycosylphosphatidylinositol-anchored proteins, and C-mannosyl glycoproteins through the GDP-Man synthesis. Another is a phosphomannose isomerase (PMI), which catalyzes the conversion of Man-6-P to fructose 6-phosphate. This reaction links mannose metabolism to glucose metabolism, such as glycolysis and *de novo* synthesis of glucosamine. Once synthesized, KDN is converted to CMP-KDN by CMP-sialic acid synthetase (CSS) [30–32] and incorporated into KDN-glycoconjugates by sialyltransferases (ST) [33]. Alternatively, KDN might be degraded into mannose and pyruvate by a putative KDN-pyruvate lyase (KPL), or the known sialate-pyruvate lyase (Neu5Ac aldolase) [34,35].

The liver has the ability to synthesize KDN without exogenous mannose (Figure 3). The mannose-induced enhancement of KDN synthesis, however, does not occur in liver following either short-term or long-term mannose ingestion. On the other hand, the mannose-induced KDN synthesis does occur in other organs, although the level of KPS activity in these organs is similar to that in liver (Figure 4). In this regard, it is interesting to note that Man-6-P is a common substrate of KPS, PMM, and PMI (Figure 5), and there are higher PMM and lower PMI activities in mouse liver than in other mouse organs [23]. Also, in rat, the specific activity of PMM in liver is 1.9 to 3.8 times higher than in other organs [25]. The ratio of PMM to PMI activity in liver is 4 to 13 times higher than that in other organs [25]. Therefore, the higher PMM activity relative to that of PMI or KPS in liver leads to an excess amount of precursor mannose to flow into the PMM pathway rather than into the PMI or KPS metabolic pathway, and subsequently results in the failure to increase KDN levels in liver. It is also possible that the level of free KDN is balanced by the synthesis and degradation of KDN, and KPL activity in liver might be relatively higher than in other organs. These aspects remain to be examined.

Mannose is used for a therapy of a congenital disorder of glycosylation (CDG) type Ib, PMI deficiency [36–38]. In CDG-Ib patients, mannose is not synthesized *de novo* due to a PMI defect and, therefore, mannose required for the glycosylation of proteins can only be supplied by the salvage pathway. A rationale for mannose therapy is to orally supplement CDG-Ib patients with mannose to rescue the mannose deficiency. Previous reports indicate that mannose has no obvious effects on growth, health, behavior of mice, organ weight, histology, litter size, or pup growth [23]. Similar results were obtained in our mannose ingestion experiments in mice. Our results, however, indicated that oral mannose ingestion results in an increase in intracellular free KDN in various organs in mice. Thus, there were no apparent effects of increased intracellular KDN on viability or growth of the cultured cells or mice in the period of this study.

Irrespective of recent success in the mannose therapy for CDG-Ib patients, some side effects attributed to the ingestion of mannose, however, have been reported. For example, mannose increases glycated HbA1c, which can lead to diabetic-like complications. CDG-1b cells (insufficient PMI activity), which absorb high levels of mannose, suffer energy depletion due to the exhaustive consumption of ATP by phosphorylation of mannose, while being deficient in ATP derived from mannose via glycolysis (see Discussion in reference 23). Whether an increase in free KDN in response to the mannose increase is involved in these phenomena remains to be examined.

In contrast to free KDN, the expression of bound KDN in glycoproteins and glycolipids was very low or negligible, even though the level of intracellular free KDN increased beyond the level of intracellular free Neu5Ac. This phenomenon can be explained by the very low activity of CMP-sialic acid synthetase (CSS) toward KDN [32], and the conversion of KDN to CMP-KDN catalyzed by CSS is considered to be, at least in part, a rate-limiting step for the synthesis of bound KDN in glycoproteins and glycolipids downstream from the synthesis of free KDN. Therefore, the increase in intracellular free KDN in response to excess mannose appears to be simply a metabolic consequence. We do not know, however, if this phenomenon is functionally important, and studies to elucidate the function of KDN are underway in our laboratory.

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