

Manganese source affects manganese transport and gene expression of divalent metal transporter 1 in the small intestine of broilers

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(Submitted 13 January 2011 – Final revision received 14 September 2011 – Accepted 15 September 2011 – First published online 15 December 2011)

Abstract

In the present study, two experiments were conducted to investigate the effect of Mn source on Mn transport and the expression of a Mn transporter, divalent metal transporter 1 (DMT1), in the small intestine of broilers. In Expt 1, *in situ* ligated duodenal loops from Mn-deficient chicks (29-d-old) were perfused with solutions containing 0–8.74 mmol Mn/l from either MnSO₄, or one of two organic chelates of Mn and amino acids with moderate (OM) or strong (OS) chelation strength (Q_f) up to 30 min. In Expt 2, Mn-deficient intact broilers (14-d-old) were fed a control diet (12.45 mg Mn/kg) or the control diet supplemented with 100 mg Mn/kg as one of all Mn sources for 14 d. The uptake kinetics of Mn from different Mn sources in the ligated duodenal loops followed a saturable process as determined by regression analysis of concentration-dependent uptake rates. The maximum transport rate (J_{max}) and K_m values, and DMT1 mRNA levels in the ligated duodenal loops were higher ($P < 0.01$) for OM and OS than for MnSO₄. DMT1 mRNA levels were much higher ($P < 0.01$) in the duodenum than in the jejunum and ileum. Both DMT1 mRNA levels in the duodenum and plasma Mn contents from the hepatic portal vein of intact chicks on day 14 post-feeding increased ($P < 0.05$) in the following order: control < MnSO₄ < OM < OS. These results indicated that organic Mn sources with stronger Q_f showed higher Mn transport and absorption, and DMT1 might be involved in the regulation of organic Mn transport in the proximal small intestine of broilers.

Key words: Organic manganese transport: Divalent metal transporter: Gene expression: Small-intestinal loops: Broilers

Mn is an essential cofactor for numerous enzymes or proteins, such as superoxide dismutase, transferases, hydrolases and lyases, and it is involved in respiration, defence against oxidative stress, bone formation and amino acid metabolism^(1–4). Approximately 3–5% of ingested Mn is absorbed across the intestinal wall, and excess Mn is readily excreted via the bile in animals⁽²⁾. Rapidly growing chicks have a high demand for Mn, and some organic Mn sources, including amino acid complexes, have been developed as supplements in livestock feeds. It has been reported that organic Mn sources with optimal chelation strengths (Q_f) are more bio-available than inorganic sources^(5–7). The utilisation of organic Mn was closely correlated with Q_f between Mn and different ligands^(6,8,9). However, limited research has been done on the mechanism of organic Mn transport in the small intestine of animals, especially chickens.

Both *in vitro* and *in vivo* studies with reticulocytes⁽¹⁰⁾ and rodents^(11,12) have been conducted to investigate the molecular mechanisms involved in Mn transport. Divalent metal transporter 1 (DMT1) is an electrogenic transporter located on duodenal enterocytes and transports divalent cations, including Mn²⁺, from the extracellular to intracellular side⁽¹⁰⁾. It has been found that solubilised Mn released from the stomach into the duodenum was transported across the microvilli by DMT1 in rats^(10–12). In chickens, DMT1 has also been found in the digestive tract^(9,13), but it remains unknown whether the expression of DMT1 is regulated by Mn.

The objective of the present study was to investigate the uptake kinetics of Mn from different Mn sources using *in situ* ligated duodenal loops of chicks, as well as the effect of different Mn sources on the intestinal DMT1 mRNA level in ligated duodenal loops and intact broiler models.

Abbreviations: AIC, Akaike information criterion; DMT1, divalent metal transporter 1; $E_{1/2}$, half-wave potential; J_{max} , maximum uptake rate; OM, organic chelates of Mn and amino acids with moderate chelation strength; OS, organic chelates of Mn and amino acids with strong chelation strength; Q_f , chelation strength.

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Materials and methods

Materials

4-Morpholineethanesulfonic acid and *N,N*-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid were of biochemical grade (Beijing Jingke Chemical Reagent Company). Phenol red was chemically pure (Sigma-Aldrich). Similar to our previous study⁽¹⁴⁾, the following three Mn sources were used in the present study: one inorganic manganese sulfate ($\text{MnSO}_4 \cdot \text{H}_2\text{O}$, reagent grade; Beijing Biochemical Reagent Company) and two organic chelates of Mn and amino acids with moderate Q_f (OM, Mn-AA, feed grade, $Q_f = 16.85$ between 10 and 100; Sanbao Additive Company) or with strong Q_f (OS, Bioplex Mn, feed grade, $Q_f = 147.00$ between 100 and 1000; Alltech), respectively. Mn-AA was a chelate of ionised Mn (9.06%) and 29.06% amino acids (containing 3.43% aspartic acid, 0.91% serine, 3.05% glutamic acid, 0.61% threonine, 1.34% glycine, 1.09% arginine, 2.36% alanine, 2.26% tyrosine, 1.24% proline, 1.77% valine, 1.75% phenylalanine, 0.75% isoleucine, 4.11% leucine, 1.82% histidine, 2.25% lysine and 0.32% methionine). Bioplex Mn was a chelate of ionised Mn (10.18%) and 45.26% amino acids (containing 6.11% aspartic acid, 1.91% serine, 7.22% glutamic acid, 1.37% threonine, 1.59% glycine, 2.36% arginine, 1.79% alanine, 4.60% tyrosine, 2.30% proline, 1.90% valine, 1.06% phenylalanine, 2.15% isoleucine, 4.24% leucine, 1.37% histidine, 3.11% lysine, 1.35% methionine and 0.84% cystine). Q_f is a quantitative measurement of chelation or complex strength between metals and ligands according to the shift in half-wave potential ($E_{1/2}$) in polarography, as described by Holwerda *et al.*⁽¹⁵⁾ and Li *et al.*⁽⁶⁾. Briefly, the saturated solution for each organic Mn source was prepared in 50 ml of deionised water and the final pH was measured. The saturated solution was diluted at 1:100 in 0.1 M-*N,N*-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid at pH 8.0, a non-complexing buffer for anaerobic electrochemical measurements with a nitrogen purge. Molar metal concentration was determined from the cathodic wave height (0.1 M-manganese sulfate standard). $E_{1/2}$ was determined in polarography with a hanging mercury-drop electrode (Ag/AgCl reference electrode, Potentiostat/Galvanostat model 283; EG & G, Inc.) and was used to calculate the shift in half-wave reduction potential ($\Delta E_{1/2}$) of a Mn source ($\Delta E_{1/2} = E_{1/2}$ (free Mn^{2+}) - $E_{1/2}$ (Mn complex)). Q_f is calculated from $\log(Q_f) = (n)(\Delta E_{1/2})/0.05916$, where $n = 2$, the number of electrons accepted by Mn^{2+} .

Animals, diets and treatments

All experimental procedures were approved by the Animal Care and Use Committee of Chinese Academy of Agricultural Sciences. Male broilers (Arbor Acres) were managed according to the guidelines for broiler management⁽¹⁶⁾. Birds were housed in electrically heated and thermostatically controlled cages and were allowed *ad libitum* access to the diet and tap water containing no detectable Mn.

In Expt 1, 300 1-d-old chicks were fed a maize-soyabean basal diet supplemented with 100 mg Mn/kg diet (adequate $\text{Mn}^{(17)}$) as MnSO_4 for the first 21 d. For the following 7 d, the

birds were fed a Mn-unsupplemented maize-soyabean meal basal diet (containing 12.45 mg Mn/kg diet by analysis; Table 1), in order to deplete Mn stores in the body^(8,18) (liver Mn concentration was <10 ng/g, fresh basis). The basal diet was formulated based on the National Research Council⁽¹⁹⁾ recommendations for broiler starter or grower except for Mn. On day 29, 220 Mn-deficient broilers were selected by mean body weight and randomly assigned to one of twenty-two treatments (ten birds each) in one control plus 3 (Mn sources) \times 7 (Mn concentrations) factorial arrangement. These broilers were used to prepare the ligated duodenal loops following the *in situ* ligation procedure as described below. The duodenal loops (n 10 per treatment) were infused with Mn^{2+} -free solution (control) or one of the following solutions containing 0.13, 0.27, 0.54, 1.09, 2.18, 4.37 and 8.74 mmol Mn/l (based on analysis) from either MnSO_4 , or OM or OS, respectively. Each ligated duodenal loop was considered as a replicate.

In Expt 2, 1-d-old birds were fed a maize-soyabean meal basal diet with no supplemental Mn for 13 d (containing 13.47 mg Mn/kg diet by analysis; Table 1), to deplete Mn stores in the body^(8,18) (liver Mn concentration was <6 ng/g, fresh basis). At 14 d of age, 256 Mn-deficient chicks were randomly divided into four treatment groups with eight replicate cages (eight chicks per cage) for each treatment. The four dietary treatments included a Mn-unsupplemented basal control diet (control, low Mn, containing 12.45 mg Mn/kg

Table 1. Composition and nutrient levels of the basal diets

Periods	Starter (days 1–21)	Grower (days 22–28)
Ingredients (%)		
Maize	54.51	60.65
Soyabean meal	34.00	29.93
Fishmeal	5.20	3.10
Soyabean oil	3.50	3.50
Monocalcium phosphate	0.80	1.10
Limestone	1.20	1.05
Iodised salt	0.30	0.30
DL-Met	0.15	0.10
Premix*	0.34	0.27
Calculated contents†		
Metabolisable energy (MJ/kg)	12.84	13.00
Crude protein (g/kg)†	224.7	198.6
Digestible Lys (g/kg)	13.0	11.1
Digestible Met (g/kg)	0.54	0.44
Digestible Met + Cys (g/kg)	0.90	0.77
Ca (g/kg)†	1.04	0.97
Available P (g/kg)	0.46	0.47
Mn (mg/kg)†	13.47	12.45

* Premix supplied the following amounts of vitamins and minerals (per kg diet): for the diet of days 1–21 post-feeding – retinol, 4 mg; cholecalciferol, 0.075 mg; α -tocopherol acetate, 34.5 mg; menadione, 2.0 mg; thiamin, 1.6 mg; riboflavin, 6.0 mg; niacin, 30 mg; pyridoxine, 3.0 mg; cyanocobalamin, 0.0014 mg; pantothenate, 20 mg; folic acid, 0.8 mg; biotin, 0.12 mg; choline, 500 mg; Cu, 8 mg; Zn, 40 mg; Fe, 80 mg; I, 0.35 mg; Se, 0.15 mg; for the diet of days 22–28 post-feeding – retinol, 5 mg; cholecalciferol, 0.08 mg; α -tocopherol acetate, 35 mg; menadione, 3.0 mg; thiamin, 2.4 mg; riboflavin, 9.0 mg; pyridoxine, 4.5 mg; cyanocobalamin, 0.021 mg; pantothenate, 30 mg; niacin, 45 mg; folic acid, 1.2 mg; biotin, 0.18 mg; choline, 700 mg; Cu, 8 mg; Zn, 40 mg; Fe, 80 mg; I (KI), 0.35 mg; Se, 0.15 mg.

† Analysed values; the basal diets for starter and grower broilers contained 175.32 mg Fe/kg, 23.30 mg Cu/kg, 73.5 mg Zn/kg; and 167.63 mg Fe/kg, 20.41 mg Cu/kg, 70.12 mg Zn/kg, respectively.

diet by analysis) and the control diet supplemented with 100 mg Mn/kg diet (adequate Mn⁽¹⁷⁾) as either MnSO₄, or OM or OS, respectively. Each Mn source was premixed with maize starch to the same weight, and then was added to the respective experimental diet based on its analysed Mn concentration. Variable small amounts of L-lysine monohydrochloride or DL-methionine were added to the respective experimental diets according to the amounts of lysine and methionine from supplemental organic Mn sources so as to balance lysine and methionine in each experimental diet.

Ligated duodenal loop procedure

In situ ligated intestinal loop is useful to maintain an intact intestinal morphology and functions up to 6 h in chicks⁽²⁰⁾; therefore, the *in situ* ligated small-intestinal loop was adopted based upon studies on Mn transport⁽⁹⁾ and Se^(20,21). The *in situ* ligation procedure of duodenal loops was performed as described previously^(9,20). Briefly, the feed-deprived birds (28-d-old; Mn-deficient) were anaesthetised by intravenous injection of sodium pentobarbital (20 mg/kg body weight) and the small intestine was exposed by means of longitudinal abdominal incision. At 1 cm distal to the pylorus, an inlet polyethylene cannula (inner diameter, 1.0 mm; outer diameter, 1.6 mm) was inserted into a hole made with a 22-gauge needle through the intestinal wall into the lumen. The cannula was secured with suture tied around the intestine and tubing. A 10 cm section of the intestine was isolated and an outlet cannula was inserted in a similar way at the opposite end of the loop. The loops were flushed with PBS (125 mM-NaCl, 15.9 mM-Na₂HPO₄, 1.2 mM-NaH₂PO₄, pH 7.4; 37°C) to remove any chyme that might be present, and then the distal loops were tied off to prevent the solutions out. The duodenal loop was injected with 3.5 ml of perfusion solution with a calibrated syringe through the inlet cannula, and then the inlet cannula was covered by a removable stopper. The perfused loop was placed carefully back into the abdominal cavity for the duration of the experiment. Adequate moisture levels were maintained by spraying the intestine with warm 0.9% saline and covering the abdomen with plastic wrap. All chicks were stable throughout anaesthesia, with no significant changes in heart rate, respiratory rate or body temperature during the perfusion periods.

Constituents of the intestinal perfusate

The perfusion solution injected into the duodenal loops consisted of 135.0 mM-NaCl, 20 mg/l of phenol red⁽⁹⁾, and 15.5 mM-4-morpholineethanesulfonic acid buffer (pH 6.0)⁽²²⁾. Inorganic (MnSO₄) and two organic Mn sources (OM and OS) were added to the saline medium based on their analysed Mn concentrations to obtain the desired Mn concentrations. Phenol red was a non-absorbable marker for correcting changes in the volume of the perfused solution in the intestinal loops^(9,20). Morpholineethanesulfonic acid buffer had no effect on Mn transport in the ligated intestinal loops of broilers⁽¹⁸⁾. In the present preliminary study, *in vitro* incubation of 2.18 mM-manganese sulfate in the

morpholineethanesulfonic acid buffer (pH 6.0) did not affect the concentration of phenol red up to 1 h, which indicated that phenol red remained stable during the experiment.

Sample collections

In Expt 1, the perfusates in the ligated duodenal loops were harvested through the inlet cannula at 30 min after perfusion, and then all chicks were killed by cervical dislocation. Within 30 min after perfusion, Mn uptake (evaluated by its disappearance from the intestinal lumen) increased linearly in Mn-deficient birds⁽⁹⁾, and therefore this time point was adopted to investigate the uptake kinetics of Mn. Mn contents of the perfusates were too low to be detected in the duodenal loops perfused with Mn-free solution, so there was no need to deduct the endogenous Mn in intestinal secretions. Mucosa samples of the ligated duodenal loops perfused with solutions containing 0 (control) and 2.18 mmol Mn/l from one of the Mn sources were collected at 30 min after perfusion. Our previous study has shown that the concentration of 2.18 mmol Mn/l in the perfused solution was comparable with the Mn content in the duodenal chyme of chicks fed the diet containing the Mn requirement of 120 mg Mn/kg⁽¹⁷⁾. The ligated duodenal loops were excised, flushed with ice-cold saline solution and slit lengthwise. Mucosa was scraped with an ice-cold microscope slide, immediately frozen in liquid N₂ and stored at -70°C until further analysis.

In Expt 2, at 7 and 14 d post-feeding, twenty-four chicks (three birds per cage) from each treatment were selected based on mean body weight and anaesthetised by intravenous injections of sodium pentobarbital (20 mg/kg body weight) via a wing vein. Blood was collected from the hepatic portal vein, and plasma was separated for Mn determination. The samples were pooled from each cage. After blood collection, one bird per replicate cage from each treatment was killed and the small intestine was exposed via a longitudinal abdominal incision. The duodenum (about 10 cm distal to the pylorus), jejunum (about 10 cm preceding the yolk stalk) and ileum (10 cm preceding the ileocaecal valve) were dissected, and mucosa was collected and stored as described in Expt 1.

Measurements of manganese and manganese uptake in ligated duodenal loops

Concentrations of Mn in Mn sources, diets, water, perfusion solutions, perfusates, plasma and liver were determined by inductively coupled plasma emission spectroscopy (Model IRIS Intrepid II; Thermo Jarrell Ash), as described by Li *et al.*⁽¹⁴⁾. Approximately 0.5 g of the solid sample were placed in a Teflon vessel, and 10 ml HNO₃ (70%) was added. The 2 ml liquid sample was mixed with 8 ml HNO₃ (70%) in a Teflon vessel. Then, the vessel was closed and the sample was digested using a microwave digestion unit (MARS-5; CEM Corporation). The microwave programme was run for 45 min at 2000 W and 180°C, and then was cooled by air to room temperature. Then, the vessel was opened and the solution was quantitatively transferred into a 50 ml volumetric flask. The solution was made to a total

volume of 50 ml with deionised water and mixed well for analysis. The poplar leaves and bovine liver standard reference materials (National Institute of Standards and Technology, Beijing) were used to verify the accuracy of the assays. Mn uptake was evaluated by its disappearance from the ligated duodenal loop⁽⁸⁾. The concentrations of phenol red in perfusion solutions were determined by measuring absorbency at 520, 560 and 600 nm, respectively, with a UV-visible spectrophotometer (Model Cary-100; Varian, Inc.). These wavelengths were used to correct the overestimate of phenol red that possibly occurred when measured at 560 nm wavelength only⁽²³⁾. In the ligated duodenal loops, the initial rate of Mn uptake (A ; mmol/cm per min) was calculated according to the following formula: $A = ((P_i W_i - P_f C_i W_f / C_i) \times 30 \text{ min} / 10 \text{ cm})$, where P_i and P_f are concentrations of Mn in perfusates at the beginning and at the end of the experimental period, respectively; C_i and C_f are the concentrations of phenol red in the solution before and after perfusion; and W_i is the initial weight (g) of the solution before perfusion.

Divalent metal transporter 1 mRNA expression analysis

Total RNA was extracted from intestinal mucosa using TRIzol reagent (Invitrogen). RNA quality (intact rRNA 28S/18S) was evaluated by agarose gel electrophoresis and RNA concentrations were quantified by a spectrophotometer (Varian, Inc.). First-strand complementary DNA was reverse transcribed from 2 μg of total RNA using oligo(dT)₂₀ and a SuperScript III First-Strand Synthesis system for RT-PCR (Invitrogen). Quantitative real-time PCR was performed in triplicate on an ABI 7000 apparatus (Applied Biosystems) according to optimised PCR protocols⁽⁹⁾. PCR contained 50 ng complementary DNA, 500 nmol/l forward and reverse primers, respectively, and 1 \times SYBR Green Master Mix (Applied Biosystems). The cycling programme was conducted at 50°C/2 min and 95°C/10 min, followed by forty cycles (95°C/15 s and 60.5°C/30 s) and melting curve analysis. The primers used for DMT1 (GenBank EF635922 and EF635923)⁽¹²⁾ and β -actin (GenBank L08165) included: DMT1 – forward 5'-AGCCGTTCCACTTATTTTCG-3', reverse 5'-GGTCCAAATAGGCGATGCTC-3'; β -actin – forward 5'-GAGAAATTGTGCGTGACATCA-3', reverse 5'-CCTGAACCTCTCATTGCCA-3'. Gene-specific amplification was determined by melting curve analysis and agarose gel electrophoresis. The standard curve method was used to quantify gene expression, as described previously^(9,13). Data are presented in arbitrary units as relative DMT1 mRNA levels normalised to endogenous gene β -actin⁽²⁴⁾ mRNA level. In Expt 1, the average expression of DMT1 mRNA in the control was used as a calibrator, and in Expt 2, the average expression of duodenal DMT1 mRNA in the control was used as a calibrator. All data are presented as means with their standard errors.

Kinetic and statistical analyses

In Expt 1, the kinetic analysis of Mn uptake in the ligated duodenal loops was carried out by fitting the following equations:

non-saturable diffusion (equation 1), saturable process (equation 2) or the sum of both equations mentioned above (a saturable process plus a non-saturable diffusion, equation 3)⁽²⁵⁾.

$$J_{\text{Mn}} = PA, \quad (1)$$

$$J_{\text{Mn}} = \frac{J_{\text{max}} A}{K_m + A}, \quad (2)$$

$$J_{\text{Mn}} = \frac{J_{\text{max}} A}{K_m + A} + PA, \quad (3)$$

where J_{Mn} and its maximum rate of Mn uptake (J_{max}) are given in nmol/min per cm; K_m is the Michaelis-Menten constant in mmol/l; P is the diffusivity coefficient in cm^2/min ; and A is the concentration of Mn in perfusate in mmol/l.

The fits of equations were performed using a non-linear least-squares regression program (Sigmaplot version 4.0; Jandel Scientific). The Akaike information criterion (AIC) was adopted⁽²⁶⁾ to select the best kinetic model for Mn absorption under the experimental conditions. The model with the smallest AIC was regarded as the 'best' model since it minimised the difference of the given model from the 'true' model.

The data were processed using Statistical Analysis Systems version 8.2 (SAS Institute). In Expt 1, analysis of the data of Mn uptake was performed by two-way ANOVA using the general linear model, which included Mn concentration, Mn source and their interaction. The data of DMT1 mRNA levels in the ligated duodenal loops perfused with solutions containing 0 (control) and 2.18 mmol Mn/l from one of the Mn sources were subjected to one-way ANOVA using the general linear model⁽²⁷⁾, with each loop as the experimental unit. The differences in the kinetic parameters obtained from different Mn sources were analysed by the t test. In Expt 2, analysis of the data was performed using two-way ANOVA with the general linear model⁽²⁷⁾. The model for plasma Mn contents in the hepatic portal vein included the effects of Mn source, age and their interaction. The model for DMT1 mRNA levels included the effects of Mn source, intestinal segment and their interaction. The replicate cage or individual chick served as the experimental unit. For all data, when ANOVA was significant, *post hoc* comparisons of treatment means were made using the least-squares mean test. Statistical significance was detected at $P < 0.05$.

Results

Effect of manganese source on manganese uptake in the ligated duodenal loops of chicks in Expt 1

Mn uptake in the ligated duodenal loops of chicks was affected ($P < 0.001$) by Mn source, Mn concentration and their interaction (Table 2). Mn uptake increased with increasing Mn concentrations, regardless of Mn source. Higher Mn uptakes were observed ($P < 0.05$) for the OM and OS treatments than for the MnSO_4 treatment at any Mn concentration, and for the OS treatment than for the OM treatment at Mn concentrations of 2.18, 4.37 and 8.74 mmol/l in the ligated duodenal loops of chicks.

Table 2. Effects of manganese source and manganese concentration on manganese uptake in the ligated duodenal loops of chicks (Expt 1)

(Mean values with their standard errors (*n* 10))

Mn concentration (mmol/l)	MnSO ₄ (nmol/cm)		OM (nmol/cm)		OS (nmol/cm)	
	Mean	SEM	Mean	SEM	Mean	SEM
0.13	24.5 ^a	1.61	32.4 ^b	0.78	35.5 ^b	0.32
0.27	51.7 ^a	1.93	65.0 ^b	1.54	67.4 ^b	0.98
0.54	92.3 ^a	3.01	122 ^b	1.32	121 ^b	3.32
1.08	173 ^a	5.41	253 ^b	4.03	235 ^b	4.81
2.18	274 ^a	10.2	400 ^b	20.0	442 ^c	18.0
4.37	391 ^a	23.8	566 ^b	13.9	650 ^c	22.0
8.74	499 ^a	23.0	773 ^b	22.2	808 ^c	20.5
<i>P</i>						
Mn concentration			< 0.001			
Mn source			< 0.001			
Interaction			< 0.001			

OM, organic chelate of Mn and amino acids with moderate chelation strength ($Q_1 = 16.85$ between 10 and 100); OS, organic chelate of Mn and amino acids with strong chelation strength ($Q_1 = 147.00$ between 100 and 1000).

^{a,b,c} Mean values within a row with unlike superscript letters were significantly different ($P < 0.05$).

Manganese uptake kinetics of different manganese sources in the ligated duodenal loops of chicks in Expt 1

Results of the regression analysis showed that the best models for Mn uptake kinetics of MnSO₄, OM and OS were the saturable process equations (AIC = 2.2, 2.3 and 2.3, respectively; Table 3) in the ligated duodenal loops of chicks. The kinetic analysis of Mn uptake from different Mn sources was carried out by graphical means (Fig. 1). These findings suggested that Mn uptake would be a saturable process in the duodenum of broilers, regardless of Mn source. The J_{max} values were higher ($P < 0.001$) for OM and OS (37.52 (SEM 1.65) and 40.06 (SEM 1.55) nmol/cm per min, respectively) than for MnSO₄ (23.04 (SEM 1.23) nmol/cm per min). Similarly, the K_m values were higher ($P < 0.01$) for OM and OS (4.09 (SEM 0.38) and 4.02 (SEM 0.33) mmol/l, respectively) than for MnSO₄ (3.35 (SEM 0.40) mmol/l). However, there was no significant difference ($P > 0.66$) in the K_m values and J_{max} values between the OM and OS treatments.

Effect of manganese source on divalent metal transporter 1 mRNA levels in the ligated duodenum of chicks in Expt 1

DMT1 mRNA levels were 81.0% lower ($P < 0.001$) in the ligated duodenal loops perfused with solutions containing different Mn sources than in those perfused with the Mn-free control solution (Fig. 2). In comparison with the MnSO₄ group, increased DMT1 mRNA levels were observed ($P < 0.01$) for the OM and OS groups; however, there was no significant difference ($P > 0.40$) between the OM and OS groups.

Effect of manganese source on manganese absorption in intact chicks in Expt 2

The Mn contents of plasma from the hepatic portal vein were affected ($P < 0.001$) by Mn source and chick age, but not ($P > 0.05$) by their interaction (Table 4). At 7 and 14 d of age post-feeding, plasma Mn contents were greater ($P < 0.01$) in

the Mn-supplemented groups than in the control, and in the OM and OS groups than in the MnSO₄ group, and also ($P < 0.001$) in the OS group than in the OM group at 14 d of age post-feeding. In addition, plasma Mn contents decreased ($P < 0.001$) in chicks from day 7 to 14 post-feeding.

Effect of manganese source on divalent metal transporter 1 mRNA levels in the small intestine of chicks in Expt 2

Intestinal segment, Mn source and their interaction affected ($P < 0.001$) DMT1 mRNA expression in the small intestine of chicks. DMT1 mRNA levels were much higher ($P < 0.01$) in the proximal intestine (the duodenum and jejunum) than in the ileum, and also in the duodenum than in the jejunum (Fig. 3). In the duodenum and jejunum of broilers, Mn supplementation increased ($P < 0.001$) DMT1 mRNA levels compared with the control, and the OM and OS groups increased ($P < 0.01$) DMT1 mRNA levels compared with the MnSO₄ group. Duodenal DMT1 mRNA level was higher ($P < 0.05$) for the OS treatment

Table 3. Kinetic and statistical parameters obtained after fitting Michaelis–Menten equations to the experimental data of manganese uptake in the ligated duodenal loops of chicks (Expt 1)

(Mean values with their standard errors (*n* 9))

Mn source	Kinetic parameters				
	J_{max} (nmol/cm per min)		K_m (mmol/l)		AIC
	Mean	SEM	Mean	SEM	
MnSO ₄	23.04 ^a	1.23	3.35 ^a	0.40	2.2
OM	37.52 ^b	1.65	4.09 ^b	0.38	2.3
OS	40.06 ^b	1.55	4.02 ^b	0.33	2.3

J_{max} , maximum absorption rate; AIC, Akaike information criterion; OM, organic chelate of Mn and amino acids with moderate chelation strength ($Q_1 = 16.85$ between 10 and 100); OS, organic chelate of Mn and amino acids with strong chelation strength ($Q_1 = 147.00$ between 10 and 100).

^{a,b} Mean values within a column with unlike superscript letters were significantly different ($P < 0.01$).

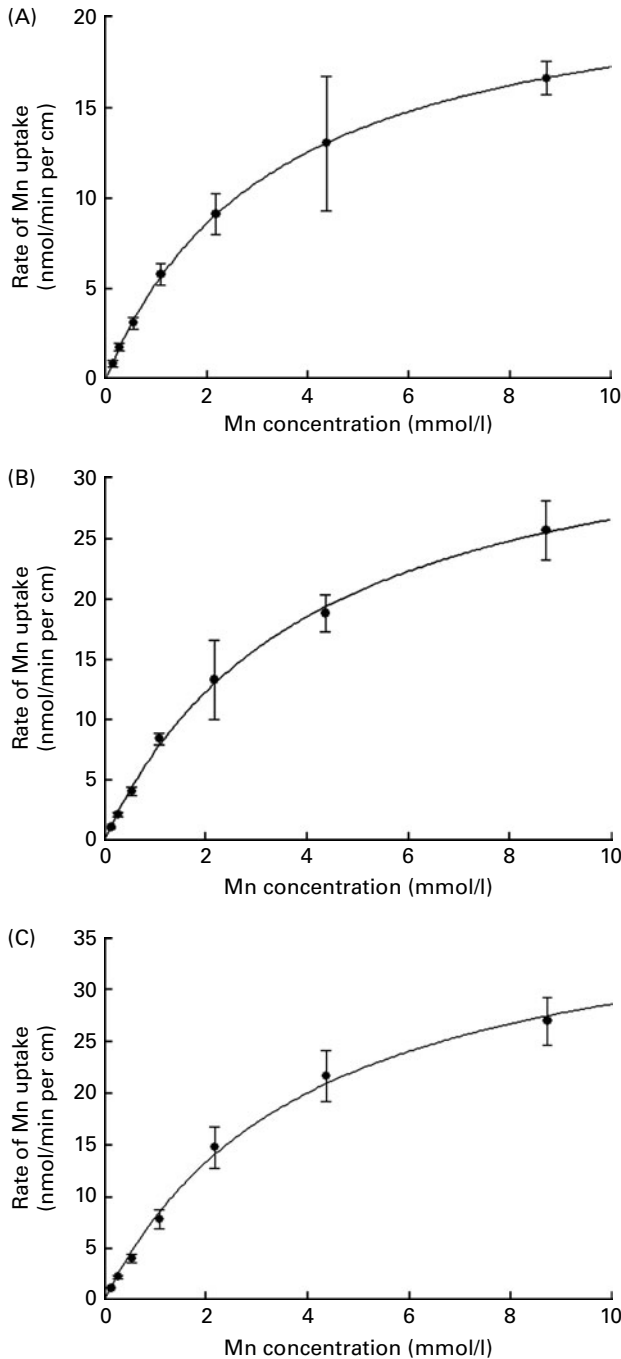


Fig. 1. Effect of manganese source on the kinetic curves of manganese uptake in the ligated duodenal loops of manganese-deficient chicks (Expt 1). The ligated duodenal loops (n 10) were perfused with solutions containing 0.13–8.74 mmol manganese/l from either (A) MnSO_4 , or one of two organic chelates of manganese and amino acids with (B) moderate ($Q_f = 16.85$ between 10 and 100) and (C) strong ($Q_f = 147.00$ between 100 and 1000) Q_f , respectively. At 30 min after perfusion, manganese transport (disappearance of manganese from the ligated duodenal loop) was determined and the initial rate of manganese transport was calculated. Values of manganese transport rates are means, with their standard deviations represented by vertical bars. All kinetic curves of manganese transport from different manganese sources in the duodenum are described by the Michaelis–Menten equation (a saturable process).

than for the OM treatment, while there was no significant difference ($P > 0.60$) in jejunal DMT1 mRNA level between the OM and OS groups. However, both Mn supplementation and Mn source had no significant effects ($P > 0.18$) on DMT1 mRNA expression in the ileum of broilers.

Discussion

Recent studies have indicated that organic Mn sources were more bioavailable than inorganic sources^(4,7,28). Chemical characteristics are considered to be important in predicting the bioavailability of complexed or chelated metals. Several studies have shown that the bioavailabilities of organic Mn sources were closely related to their Q_f ^(6,29,30). The results of the present study showed that Mn uptakes for OM and OS in the ligated duodenum of broilers were greater than that for MnSO_4 . In the intact chick model, Mn absorbed from the intestinal lumen is transported to the liver through the hepatic portal vein⁽³¹⁾. Therefore, the rapid change of plasma Mn contents in the hepatic portal vein of chicks after feeding might be an indicator reflecting Mn transport and absorption from the intestinal lumen. Results from the present study indicated that Mn transport and absorption were higher for OM and OS than for MnSO_4 , and higher for OS than for OM. All these findings are in accordance with our earlier studies using *in vitro* inverted gut sacs⁽⁸⁾ and feeding trials^(6,7). In chicks, organic Mn sources with higher Q_f were more effective than inorganic manganese sulfate in enhancing Mn absorption in the small intestine, and the transport of organic Mn was greater with strong Q_f than with moderate Q_f ^(8,18). The present study provided further support for the nutritional importance of organic Mn sources with higher Q_f .

There are two hypotheses regarding the absorption and utilisation mechanisms of mineral complexes⁽³²⁾. The first hypothesis is that the organic mineral complex or chelate with optimal Q_f could resist interference from dietary and nutritional factors in the digestive tract and directly reach the intestinal brush border, where it is hydrolysed and absorbed as ions into the blood, resulting in a higher bioavailability of the complexed or chelated than the inorganic form of the metal⁽³³⁾. The second hypothesis is that the organic mineral complex or chelate with optimal Q_f could maintain its structural integrity in the digestive tract and arrive at absorptive sites in the small intestine as the original intact molecules⁽³⁴⁾. However, until now, there has been no direct evidence supporting either of these hypotheses, mainly because of a lack of effective methods to test the organic mineral complexes or chelates. Results of the kinetic study suggested that Mn transport from different Mn sources would be a saturable process in the ligated duodenum of broilers. However, the J_{max} and K_m values for OM and OS were higher than those for MnSO_4 , which suggested that the saturable transport system for OM and OS would have a greater capacity and lower affinity than that for manganese sulfate in the duodenum of broilers. The differences in J_{max} and K_m values between inorganic and organic Mn sources indicated that there was at least one saturable transport pathway involved in organic Mn transport which differed from that for inorganic

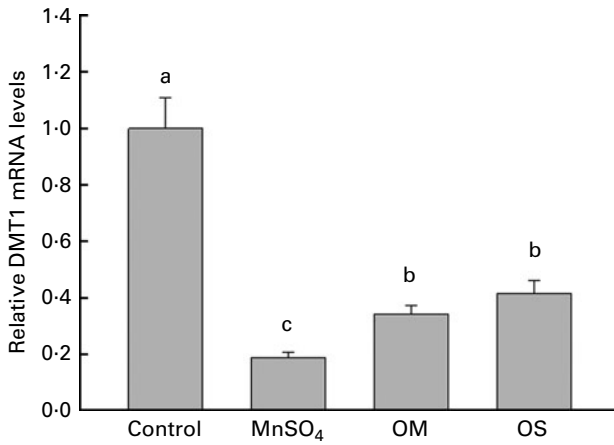


Fig. 2. Effect of manganese source on divalent metal transporter 1 (DMT1) mRNA levels in the ligated duodenal loops of manganese-deficient chicks at 30 min after perfusion as determined by real-time quantitative PCR (Expt 1). The treatments included a manganese-free basal solution (control) and the basal solution supplemented with 2.18 mmol manganese/l (close to the dietary requirement of 120 mg manganese/kg for broilers) from either MnSO₄, or one of two organic chelates of manganese and amino acids with moderate (OM; $Q_f = 16.85$ between 10 and 100) and strong (OS; $Q_f = 147.00$ between 100 and 1000) Q_f , respectively. Data are presented in arbitrary units as relative mRNA abundance normalised to β -actin transcript abundance, and the average expression of DMT1 mRNA in the control was used as a calibrator. Values are means, with their standard errors represented by vertical bars (n 8). ^{a,b,c} Mean values with unlike letters were significantly different ($P < 0.01$).

Mn in the small intestine of broilers. Inorganic Mn was absorbed by means of the dissociation of inorganic Mn in the small intestine of animals^(9,12). Conversely, several studies have suggested that organic minerals such as the chelates of Mn and amino acids could be absorbed intact, with the metal atoms remaining safely bound or protected within organic molecular structures or ligands^(32,35). Additionally, Zn-EDTA is transported from the intestinal lumen to the portal circulation as an intact complex because the Q_f of Zn-EDTA is particularly strong^(36,37). In the present study, it remained unclear whether organic Mn with higher Q_f was absorbed in the small intestine of chicks as ions, or as the intact complex, or both. However, these findings provided indirect evidence for the differences between inorganic and organic Mn transports in the small intestine of chicks.

Several recent studies have suggested that DMT1 could transport Mn across the microvillus into the enterocyte in

the small intestine of rats⁽³⁸⁾ and pigs⁽³⁹⁾. The Belgrade rat, which suffers from a spontaneous mutation of DMT1 that renders the protein inactive, has provided supporting evidence for the role of DMT1 in cellular Mn uptake⁽³⁸⁾. Belgrade rats are not only anaemic because of limited absorption of dietary Fe, but also they have decreased tissue Mn concentrations compared with their wild-type counterparts, suggesting the importance of DMT1 in Mn absorption as well. Up-regulated DMT1 expression caused by Fe deficiency increased Mn absorption in the proximate intestine of pigs⁽³⁹⁾. In the present study, dietary Mn supplementation (100 mg/kg) increased DMT1 mRNA expressions in the duodenum and jejunum, as well as plasma Mn contents in the hepatic portal vein of intact chicks. It is clear that DMT1 facilitated the transport and absorption of Mn across the intestine of chicks.

Previous research⁽²⁰⁾ has confirmed that an intact intestinal morphology and functions were well maintained in the ligated intestinal loops of chicks. To a larger extent, it relieves the concern on the viability of enterocytes after ligation. The ferritin mRNA level was found to be up-regulated 2 h after perfusion in the ligated duodenal loops of rats, and the measured changes in DMT1 mRNA levels might reflect differences in gene transcription in duodenal loops⁽⁴⁰⁾. The most direct measurement on DMT1's role in Mn transport is the evaluation of changes in DMT1 expressions due to Mn exposure. In the present study, adequate Mn exposure (2.18 mmol Mn/l) down-regulated DMT1 transcription in the ligated duodenal loops of chicks at 30 min after perfusion, compared with the control (Mn-free solution). However, on day 14 post-feeding, the adequate dietary Mn addition (100 mg Mn/kg) increased DMT1 mRNA level in the proximal intestine of intact birds, compared with the control (about 13 mg Mn/kg). The results revealed that the regulation of duodenal DMT1 mRNA expression might be time-associated. The *SMF3* (DMT1 orthologue, a major Mn transporter in the worm) mRNA level decreased remarkably 5 h after Mn exposure and was restored after a 24 h recovery period in the nematode *Caenorhabditis elegans*⁽⁴¹⁾. In addition, this correlation is supported by *in vitro* data where exposure to Mn for 24 or 48 h has been shown to increase Mn store and DMT1 mRNA expressions in the immortalised choroidal epithelial Z310 cell line by 45 and 78%, respectively⁽⁴²⁾. In the present study, at 30 min after perfusion, Mn exposure might cause high mucosal Mn accumulation in the duodenal loops of

Table 4. Effect of manganese source on plasma manganese contents in the hepatic portal vein of chicks on days 7 and 14 post-feeding (Expt 2) (Mean values with their standard errors (n 8))

Items	Control		MnSO ₄		OM		OS		<i>P</i>		
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Treatment	Day	Treatment × day
Day 7 ($\mu\text{g/l}$)*	67.2 ^a	6.1	107.3 ^b	5.5	142.7 ^c	10.7	156.6 ^c	10.9	<0.001	<0.001	0.099
Day 14 ($\mu\text{g/l}$)*	52.3 ^a	2.9	68.5 ^b	2.9	93.2 ^c	3.0	119.5 ^d	7.6			

OM, organic chelate of Mn and amino acids with moderate chelation strength ($Q_f = 16.85$ between 10 and 100); OS, organic chelate of Mn and amino acids with strong chelation strength ($Q_f = 147.00$ between 100 and 1000).

^{a,b,c,d} Mean values within a row with unlike superscript letters were significantly different ($P < 0.01$) on day 7 or 14 post-feeding.

* Mn-deficient chicks were fed Mn-supplemented diets at 14 d of age post-hatching.

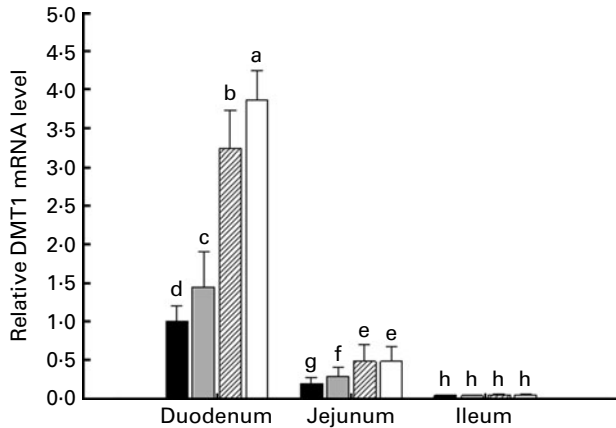


Fig. 3. Effect of manganese source on divalent metal transporter 1 (DMT1) mRNA levels in different small-intestinal segments of intact chicks. The manganese-deficient chicks (14-d-old) were fed the basal diet (control; containing about 13 mg manganese/kg) or the basal diet supplemented with 100 mg manganese/kg from either MnSO_4 , or one of two organic chelates of manganese and amino acids with moderate (OM; $Q_f = 16.85$ between 10 and 100) and strong (OS; $Q_f = 147.00$ between 100 and 1000) Q_f for 14 d (Expt 2), respectively. DMT1 mRNA levels were determined by real-time quantitative PCR. Data are presented in arbitrary units as relative mRNA abundance normalised to β -actin transcript abundance, and the average expression of duodenal DMT1 mRNA in the control was used as a calibrator. Values are means, with their standard errors represented by vertical bars. Manganese source, intestinal segment and their interaction all had significant effects ($P < 0.001$). ^{a,b,c,d,e,f,g,h} Mean values ($n = 8$) with unlike letters within the same intestinal segment were significantly different ($P < 0.05$). ■, Control; ▣, MnSO_4 ; ▨, OM; □, OS.

chicks, although mucosal Mn concentrations were not determined. Garcia-Aranda *et al.*⁽⁴³⁾ found that high mucosal Mn accumulation in the duodenal loops of rats within 90 min after perfusion was derived from the slow translocation of Mn across the mucosa and its removal in the circulation. Duodenal DMT1 expression was down-regulated by excess Mn (500 mg/kg diet) in Cu-deficient calves⁽⁴⁴⁾. The decreased DMT1 mRNA level in the ligated duodenal loops of chicks at 30 min after Mn exposure might be partially due to high mucosal Mn accumulation, which signalled to the down-regulation of duodenal DMT1 mRNA expression to prevent the toxic accumulation of Mn in the mucosa. On day 14 post-feeding, the chicks maintained stable tissue Mn levels, and an adequate Mn diet increased the duodenal Mn store of chicks compared with the Mn-deficient diet^(7,8). The increased duodenal Mn store might enhance DMT1 mRNA expression in the proximal intestine of broilers. The *in vivo* study of Garcia *et al.*⁽⁴⁵⁾ showed that DMT1 expression increased by about 35% in the brains of rat pups nurtured by dams fed on a high-Mn diet, and this elevation in DMT1 expression was not region-specific. Nonetheless, the data directly related the effect of an enhanced Mn diet to augmentation in DMT1 expression⁽⁴⁵⁾. Therefore, stably increased mucosa Mn level might up-regulate DMT1 expressions in the small intestine of intact chicks.

In the present study, the DMT1 mRNA expression profile in the small intestine of the intact broiler model showed that dietary Mn exposure significantly affected DMT1 mRNA levels in the duodenum and jejunum of broilers, while little influence

was observed in the ileum. The results of unchanged DMT1 mRNA levels in the ileum of broilers fed the Mn-supplemented diets were supported by the findings that the role of DMT1 in the process of Mn transport in the ileum of broilers was limited^(9,18). Either in the ligated duodenal loops or in the duodenum and jejunum of intact chicks, higher DMT1 mRNA levels were shown for organic Mn than for inorganic Mn, and in the duodenum of intact chicks for OS than for OM. These results indicated that organic Mn might partially be dissociated to ionised Mn in solution and cross the microvilli to the enterocyte via inorganic Mn transport. Similarly, it has been reported in early studies that Fe from iron glycine was, at least partially, dissociated from the glycine complex and entered the common non-haem Fe pool within the gastrointestinal tract⁽³⁴⁾. The dissociation of organic Mn sources depends heavily on Q_f of the compound⁽⁶⁾, which suggests that ionised Mn concentrations in the ligated duodenal loops or proximal small-intestinal lumens of intact chicks might be lower for the OM and OS groups than for the manganese sulfate group, and also lower for the OS group than for the OM group. The lower ionisation of Mn might partially explain why DMT1 mRNA levels differed among the Mn sources. To our knowledge, this is the first study to show that DMT1 mRNA expression was affected by organic Mn in animals, and this finding will open a new way to investigate the mechanism of organic Mn transport. However, much more work needs to be done to explore the role of the Mn transporter, DMT1, in organic Mn transport in the small intestine of chicks.

In conclusion, organic Mn sources with higher Q_f showed increased Mn transport and absorption in the small intestine of chicks. Kinetic data indicated that the transport of organic Mn with higher Q_f was a saturable process and that there was at least one pathway involved in organic Mn transport which differed from the transport system for inorganic Mn in the duodenum of broilers. The Mn treatment affected DMT1 mRNA expressions, and organic Mn with higher Q_f increased the expression of DMT1 mRNA in the *in situ* ligated duodenum and the proximal intestine of the intact broiler model. It will be interesting to focus more closely on the molecular mechanisms involved in organic Mn transport in the small intestine of chicks in the future study.

Acknowledgements

The present study was supported by the Earmarked Fund for Modern Agro-Industry Technology Research System, Beijing, China (project no. nycytX-42-G2-04), the Program of the National Natural Science Foundation of China, Beijing, China (project no. 30771575) and the Research Program of the Key Laboratory of Animal Nutrition, Beijing, China (project no. 2004DA125184G0812). X.-G. L. was responsible for all issues related to this paper. S.-P. B. was responsible for the planning of the study, sample analyses, collections and statistical analyses of all data, as well as the manuscript writing. L. L., R.-L. W. and L.-Y. Z. were involved in the sample analyses, and collections and statistical analyses of all data. L. X. was involved in the final expressions and interpretations of the

data. All authors participated in the writing of the final draft of the manuscript and agreed with the final content. The authors declare that there are no conflicts of interest.

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