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# Kinetic Characterization of Apical D-Fructose Transport in Chicken Jejunum

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**Abstract.** In mammals, D-fructose transport takes place across the brush-border membrane of the small intestine through GLUT5, a member of the facilitative glucose transporter family. In the present paper, we describe and characterize for the first time the apical transport of D-fructose in chicken intestine. Brush-border membrane vesicles (BBMV) were obtained from jejunum of 5- to 6-wk-old chickens. D-Fructose uptake by BBMV from chicken jejunum comprises a saturable component and a simple diffusion process. The maximal rate of transport ($V_{max}$) for D-fructose was 2.49 nmol · (mg prot)$^{-1}$ · s$^{-1}$, the Michaelis constant ($K_m$) was 29 mM, and the diffusion constant ($K_d$) was 25 nl · (mg prot)$^{-1}$ · s$^{-1}$. The apical transport of D-fructose was $Na^+$-independent, phlorizin-, phloretin-, and cytochalasin B-insensitive, and did not show cis-inhibition by D-glucose or D-galactose. These properties, together with the detection of specific GLUT5 mRNA, indicate the presence of a low-affinity high-capacity GLUT5-type carrier in the chicken jejunum, responsible for the entry of D-fructose across the brush-border membrane of enterocytes.

**Key words:** Brush-border membrane — Cytochalasin B — GLUT5 — Phloretin — Phlorizin

## Introduction

In mammal intestine, aldohexoses (D-glucose and D-galactose) are transported through the apical membrane by the $Na^+$-dependent, high-affinity and low-capacity SGLT1 (Wright et al., 1993). Ketohexose (D-fructose) is taken up by the $Na^+$-independent, low-affinity and high-capacity system GLUT5 (Kayano et al., 1990). At the basolateral membrane, all three monosaccharides are transported by a low-affinity and high-capacity system known as GLUT2 (Thorens et al., 1990).

In chickens, like in mammals, D-glucose and D-galactose use SGLT1 and GLUT2 mechanisms to cross the apical (Ferrer et al., 1994) and basolateral (Kimmich & Randles, 1975; Garriga, Moretó & Planas, 1997) membranes, respectively. Although chickens have a remarkable ability to absorb D-fructose (Obst & Diamond, 1992), it is not known how this transport takes place in the brush-border membrane of the enterocytes. In contrast, it is known that the main exit pathway for D-fructose across the basolateral membrane of chicken intestine is mediated by a GLUT2 isoform (Kimmich & Randles, 1975; Garriga et al., 1997), as is the case for mammals (Thorens, 1993).

The aim of the present study was to establish the kinetic characteristics of the apical D-fructose transport using brush-border membrane vesicles. Our results demonstrate the existence of a $Na^+$-independent, phlorizin-, phloretin-, and cytochalasin B-insensitive transport system compatible with a low-affinity, high-capacity GLUT5-type carrier in the chicken jejunum. The detection of a specific GLUT5 mRNA enables us to suggest that the apical transport of D-fructose is mediated by a GLUT5 isoform. We can conclude that a GLUT5 isoform is responsible for the transport of D-fructose through the brush-border membrane of the enterocytes of chicken.

## Materials and Methods

### Animals

Male White Leghorn chickens (*Gallus gallus domesticus* L.) were obtained from a commercial farm (Gibert, Tarragona, Spain) the day of hatch and housed in stainless steel cages at standard temperature and humidity conditions, and with an 18:6 h light-dark cycle. A commercial diet (Gallina Blanca Purina, Barcelona, Spain)

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and water were offered for *ad libitum* intake. At 5–6 weeks of age, chickens were killed in the morning, without previous withdrawal of food, by cervical dislocation followed by exsanguination. The jejunum (from the end of the duodenal loop to the Meckel diverticle) was removed, immediately flushed with ice-cold saline containing 0.2 mM phenylmethanesulfonyl fluoride (PMSF), 0.41 μM $LiN_3$ and 0.1 mM benzamidine, and opened lengthwise. The mucosa was scraped, frozen in liquid nitrogen and stored at −80°C.

The Ethical Committee of the University of Barcelona, in accordance with Spanish regulations for the use and handling of experimental animals, approved the animal manipulation and experimental procedures.

## Preparation of Brush-border Membrane Vesicles

Brush-border membrane vesicles (BBMV) were prepared according to a $MgCl_2$ precipitation method (Vázquez et al., 1997). For each BBMV preparation, 2 chickens were used. After successive centrifugations, the final pellet containing purified BBMV was resuspended in a medium containing 300 mM mannitol, 0.1 mM $MgSO_4$, 0.41 μM $LiN_3$ and 20 mM HEPES-Tris (pH 7.4), with a protein concentration of 20 to 25 $mg \cdot ml^{-1}$.

## Enzyme and Protein Determinations

The sucrase activity (α-D-glucohydrolase, EC 3.2.1.48) was assayed as a marker of the apical membrane in accordance to the routine method by Messer and Dahlqvist (1966). Ouabain-sensitive $Na^+$-$K^+$-activated ATPase ($Na^+$-$K^+$-ATPase), the marker of the basolateral membrane, was assayed following the method suggested by Colas and Maroux (1980), based on the measurement of the ouabain-sensitive $K^+$-activated phosphatase (EC 3.6.1.3). The protein concentration was determined by the method described by Bradford (1976) using bovine serum albumin as standard.

## Orientation of Vesicles

Membrane orientation was studied from sucrase activity according to Del Castillo & Robinson (1982). Sucrase activity was determined in intact vesicles and in vesicles incubated for 30 min with a mixture of 3 μM deoxycholate and 15 mM EDTA. The relationship between the sucrase activity measured in intact vesicles and the enzymatic activity determined after treatment with deoxycholate and EDTA is attributable to the outside-right-oriented vesicles.

## Transport Assays

The uptakes of D-glucose, L-glucose and D-fructose were measured at 37°C by a rapid filtration technique, as described elsewhere (Vázquez et al., 1997). For the time-course studies, D-glucose uptakes were determined in a medium containing 100 mM NaCl or KCl, 20 mM HEPES/Tris (pH 7.4), 0.1 mM $MgS0_4$, 0.41 μM $LiN_3$ and an aliquot of the labelled D-glucose.

For the kinetic analysis of D-fructose and L-glucose uptakes, the substrate concentrations used were 0.01, 0.5, 1, 5, 10, 50, 100, 150 and 200 mM. The BBMV were incubated in a medium containing an aliquot of radiolabelled D-fructose or L-glucose, 20 mM HEPES/Tris (pH 7.4), 0.1 mM $MgS0_4$, 0.41 μM $LiN_3$, the concentration of hexose required and mannitol enough to adjust the final osmolality at 320 $mosmol \cdot kg^{-1}$ (Osmomat 30 cryoscopic osmometer, Gonotec, Berlin, Germany).

All the transport assays were carried out in triplicate, using different BBMV preparations.

## Cell Isolation

Each enterocyte isolation was performed using the jejunum of 3 chickens. The pooled segments were incubated in a medium containing (in mM) 80 NaCl, 3 $K_2HP0_4$, 20 Tris-HCl, 37 mannitol, 0.1 EGTA, 27 tri-sodium citrate and 1 $mg \cdot ml^{-1}$ BSA at pH 7.4 (Ferrer, Planas & Moretó, 1986). Incubation was held for a period of 100 min at 25°C in order to obtain enterocytes isolated from the entire villus. Cell viability was assessed by Trypan blue exclusion. Enterocytes obtained were used to extract total RNA.

## RNA Extraction and Northern Blot Analysis

Total RNA was isolated from enterocytes as described previously (Chomczynski & Sacchi, 1987). Each sample was obtained from three separate chickens pooled. In every blot, total RNA obtained from rat jejunal enterocytes was added as a control of hybridization. RNA was quantified by spectrophotometric analysis at 260 nm. Samples were loaded in a formaldehyde-agarose gel (45 μg total RNA per lane) and transferred to a nylon membrane (Nytran 0.45, Schleicher and Schuell, Dassel, Germany). Specific mRNA was detected using a 2.2 kb *Eco*RI fragment encoding a rat jejunal GLUT5 transporter. Probes were labelled with α-$^{32}$P-dCTP by random priming (Random primer DNA labelling mix, Biological Industries, Kibbutz, Israel).

The Northern blot analysis was done 4 times, using different samples, and one representative experiment is shown in Fig. 4 (*see* Results).

## Chemicals

All unlabelled reagents were from Sigma Chemical (St. Louis, MO), except the reagents used to determine enzymatic activity, which were from Boehringer (Mannheim, Germany). D-[U-$^{14}$C]-Glucose (specific activity 251 $mCi \cdot mmol^{-1}$), L-[U-$^{14}$C]-glucose (specific activity 223 $mCi \cdot mmol^{-1}$) and D-[U-$^{14}$C]-fructose (specific activity 248 $mCi \cdot mmol^{-1}$), were purchased from New England Nuclear Research Products (Dreieich, Germany). The final activity of labelled substrates in the incubation medium was 2 $\mu Ci \cdot ml^{-1}$. α$^{32}$P-dCTP (specific activity 3000 $mCi \cdot mmol^{-1}$) was purchased from Amersham Ibérica (Madrid, Spain).

## Kinetic Analysis

Total D-fructose fluxes from at least three independent experiments were analyzed by nonlinear regression, using Enzfitter program (Biosoft, Cambridge, UK). As errors associated with experimental fluxes were roughly proportional to flux values, it was considered appropriate to apply a proportional weighting to the data. Kinetic constant evaluation was made by systematically testing different model equations corresponding to one or two Michaelian components plus a linear nonspecific component. The criteria used to decide the best fitting of experimental points by two different kinetic models were the following: 1) the adequacy of fitting, according to the SE deviation from constants and 2) the average deviation of the curve from experimental points calculated as the square root of $SS/df$, where $SS$ was the sum of the squares and $df$ was the degrees of freedom, as recommended by Motulsky and Ransnas (1987). Additionally, the kinetic constants were calculated subtracting the initial rates of L-glucose from total D-fructose fluxes.

## Statistical Analysis

Kinetic constants and uptakes were compared using the Student's $t$-test ($P < 0.05$).

## Results

### Characterization of the Membrane Vesicles

The results for enzymatic and functional characterization tests of BBMV are summarized in Table 1. Sucrase activity in the final BBMV preparation was 12-fold higher than that of the original homogenate. The overall recovery of this sucrase enzymatic activity was higher than 85%. There was no enrichment in the activity of the $Na^+$-$K^+$-ATPase in the final BBMV preparation and only 1.1% of the original homogenate $Na^+$-$K^+$-ATPase activity was recovered in the BBMV.

Membrane orientation was studied according to Del Castillo and Robinson (1982), and it was observed that more than 90% of the vesicle population were outside-out oriented. This result strongly indicates that the vesicles mainly consist of membrane material from the apical domain of the jejunal epithelium. The intravesicular volume, calculated at equilibrium conditions of 0.1 mM D-fructose, was $0.65 \pm 0.15$ $\mu l \cdot (mg\ prot)^{-1}$ ($n = 10$). The time-course study of $Na^+$-dependent D-glucose uptake exhibited a typical transient increase (under a negative gradient) at 5 s incubation (Table 1). This early D-glucose uptake increase was not observed in the absence of $Na^+$ (replacing NaCl by KC1; *results not shown*), which indicates the correct functioning of our BBMV preparations.

### Transport of D-Fructose across BBMV

The time course of the uptake of D-fructose was measured in the presence and absence of $Na^+$ (Fig. 1). D-Fructose uptake was always linear up to the first 10 s of incubation. After 30 s, the rate of entry slowed down considerably and reached equilibrium after 30 min. No differences were found between D-fructose uptake in presence or absence of $Na^+$. The maximal D-fructose uptake was determined at equilibrium, indicating that the mediated transport of D-fructose occurs without a transient increase of D-fructose into BBMV under a negative gradient.

Figure 2*A* shows the relationship between the external D-fructose concentration (ranging from 0.01 to 200 mM) and uptake (in pmol D-fructose $\cdot$ (mg prot)$^{-1} \cdot s^{-1}$). The best fitting of total uptake was an equation comprising the sum of a saturable and a diffusion component. Assay of more complex models yielded absurd values for kinetic constants or $[SS/df]^{1/2}$ values (Motulsky & Ransnas, 1987) far higher than those estimated with the Michaelis plus the diffusion equation. Furthermore, the Eadie-Hofstee transformation of the saturable uptake data (*not shown*) yields a straight line (correlation coefficient: 0.983; $P < 0.005$), suggesting the existence of only one component for carrier-mediated uptake. The kinetic constants that result from the Eadie-Hofstee transformation do not differ from estimates made by nonlinear regression. The initial rates of D- fructose transport were measured at 5 s of incubation time. Kinetic analysis yielded a Michaelis constant ($K_m$) of $29 \pm 3$ mM, a maximal transport capacity ($V_{max}$) of $2491 \pm 182$ pmol $\cdot$ (mg prot)$^{-1} \cdot s^{-1}$, and a diffusion constant ($K_d$) of $25 \pm 8$ nl $\cdot$ (mg prot)$^{-1} \cdot s^{-1}$. The kinetic constants calculated by subtracting the diffusional component (L-glucose fluxes) from the total D-fructose uptakes coincided with the constants estimated by nonlinear regression analysis. The self-inhibition constant (Fig. 2*B*) was $30 \pm 3$ mM which does not differ from the $K_m$.

**Table 1.** Enzymatic and functional characterization of BBMV of chicken jejunum

| Parameter | Mean ± SE (*n*) |
|---|---|
| Protein recovery (%) | 1.57 ± 0.22 (10) |
| Sucrase activity | |
| Enrichment factor | 11.7 ± 1.8 (10) |
| Overall recovery (%) | 87.6 ± 2.4 (10) |
| $Na^+$-$K^+$-ATPase activity | |
| Enrichment factor | 0.8 ± 0.1 (10) |
| Overall recovery (%) | 1.1 ± 0.3 (10) |
| % Outside-out oriented BBMV | 92.1 ± 2.4 (15) |
| Intravesicular volume ($\mu l \cdot (mg\ prot)^{-1}$) | 0.65 ± 0.15 (10) |
| Initial Rate of $Na^+$-dependent D-glucose uptake (pmol D-glucose $\cdot$ (mg prot)$^{-1} \cdot s^{-1}$) | 653 ± 15 (6) |
| D-Glucose Accumulation Ratio | 10.1 ± 0.9 (6) |

The intravesicular volume was calculated under equilibrium conditions (30 min) of 0.1 mM D-glucose uptake. Initial rate of $Na^+$-dependent D-glucose uptake was measured at 5-s incubation time in a medium containing 100 mM NaCl, using 0.1 mM D-glucose. The accumulation ratio was calculated as the relationship between the $Na^+$-dependent D-glucose uptake at 5-s incubation and under equilibrium conditions (30 min). In all cases, the values represent means ± SE for *n* experiments, carried out using different BBMV preparations.

### Effects of Different Compounds on D-Fructose Uptake

Figure 3 shows the effect of D-glucose (100 mM), D-galactose (100 mM), phlorizin (50 μM), phloretin (100 μM) and cytochalasin B (1 mM) on the uptake of 0.1, 10 and 100 mM D-fructose, and the effect of unlabelled D-fructose (100 mM) on the uptake of 0.1 and mM radiolabelled D-fructose. The results indicate that neither aldohexoses are able to inhibit the apical D-fructose transport nor do the specific ligands of SGLT1 (phlorizin) and GLUT2 (phloretin and cytochalasin B) produce a significant effect on apical D-fructose flux.

### Specific mRNA Abundance

The ratio of absorbance at 260 and 280 nm was higher than 1.8 (*data not shown*), indicating a high

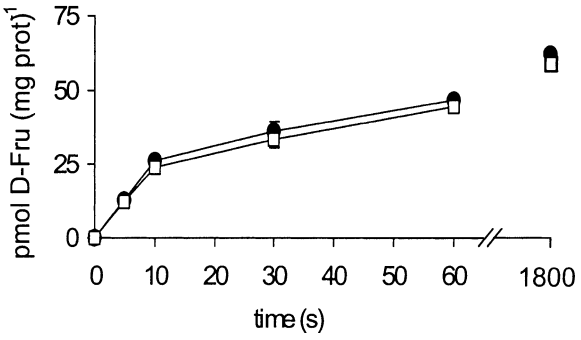


**Fig. 1.** Time course of D-fructose (D-Fru) uptake by jejunal BBMV. Transport of 0.1 mM D-fructose was measured in an isosmotic medium containing 100 mM NaCl (●) or 100 mM KCl (□). Each point represents means ± SE for 3 separate experiments.

purity and low contamination by protein fractions. Hybridization with a specific rat GLUT5 probe showed a single band of about 2.7 kb in the jejunum of both chicken and rat (Fig. 4).

## Discussion

D-Fructose is found naturally as a free sugar or combined with other carbohydrates, and as an additive in many processed foods (Burant & Saxena, 1990). In the small intestine of mammals, two members of the family of facilitative sugar transporters are involved in the transepithelial uptake of D-fructose: GLUT5 and GLUT2 (Gould & Bell, 1990). Classically, it has been reported that in the brush-border membrane, GLUT5 was responsible for the entry of D-fructose into the enterocyte (Rand et al., 1993; Miyamoto et al., 1994), and in the basolateral membrane, GLUT2 translocates D-fructose from the cytoplasm to the bloodstream (Thorens et al., 1990).

Recently, Kellet and Helliwell (2000) proposed the presence of GLUT2 in the brush-border membrane of rat jejunum immediately after a meal, which could also contribute to the apical absorption of D-fructose. It is possible to differentiate the D-fructose-mediated transport components (GLUT2 and GLUT5) by selectively inhibiting GLUT2 with phloretin (Corpe et al., 1996). However, since GLUT2 is lost from the brush-border membrane as soon as the intestine is excised, it is difficult to detect GLUT2 in most in vitro preparations, such as BBMV (Helliwell et al., 2000).

In birds, the main studies on D-fructose absorption were focused in the basolateral transport (Kimmich & Randles, 1975; Ferrer et al., 1994; Garriga et al., 1997), indicating that the exit of this hexose from the enterocytes takes place through the same isoform of GLUT that is used by D-glucose, the GLUT2. In contrast, no studies have been carried out to establish the mechanisms of apical D-fructose absorption.

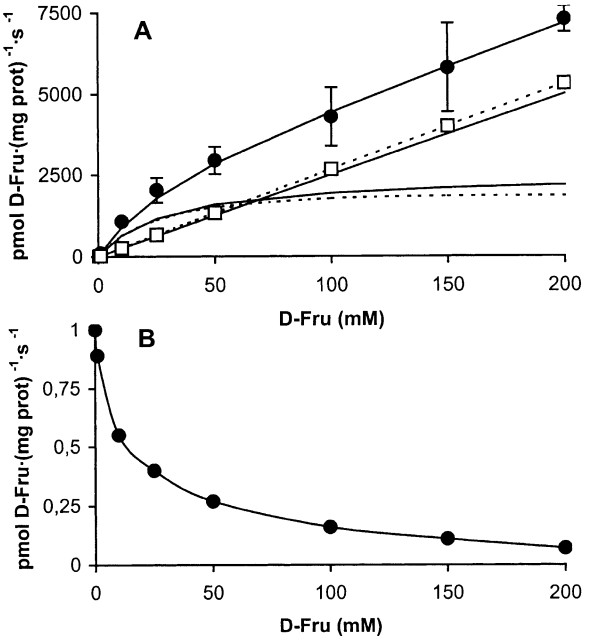


**Fig. 2.** Kinetics of D-fructose (D-Fru) uptake by jejunal BBMV of 5–6 wk-old chickens. (*A*) Total uptake of D-fructose (●) and L-glucose (□) in vesicles incubated in concentrations ranging from 0.01 to 200 mM for 5 s. Values are means ± SE of 4 independent experiments. Kinetic constants were calculated by two different ways: a) by nonlinear regression analysis (*solid lines*) and b) by subtracting the total L-glucose uptake (□ and stippled straight line; diffusion component) from total D-fructose uptake (●), giving a saturable component (stippled curve). The calculated constants were: $K_m$ 29 ± 3 mM; $V_{max}$, 2491 ± 182 pmol · (mg prot)$^{-1}$ · s$^{-1}$; and $K_a$ 25 ± 8 nl · (mg prot)$^{-1}$ · s$^{-1}$. (*B*) Inhibition of D-fructose uptake by *cis* D-fructose. The self-inhibition constant was 30 ± 3 mM.

The D-fructose $V_{max}$ (2491 pmol · (mg prot)$^{-1}$ · s$^{-1}$) obtained in the present work for BBMV of chicken jejunum is similar to that previously reported by Crouzoulon and Korieh (1991) for BBMV of rat jejunum (2675 pmol · (mg prot)$^{-1}$ · s$^{-1}$).

The Michaelis constant obtained for chicken jejunum in the present study is lower than that of 120 mM reported for rat jejunum (Corpe et al., 1996). In contrast, our $K_m$ values are very close to the $K_m$ values obtained for rabbit (Sigrist-Nelson & Hopfer, 1974), guinea-pig (Mavrias & Mayer, 1973) and hamster (Honneger & Semenza, 1973). The slight differences between the present results and those already reported can be explained by the use of different animal species and different transport preparations.

In a previous paper (Garriga et al., 1997) we described the presence of GLUT2 in the basolateral membrane of the chicken jejunum. The affinity of D-fructose for GLUT5 ($K_m$ = 29 mM) is higher than that reported for GLUT2 ($K_m$ = 40 mM), while the maximal rate of transport is higher in the case of GLUT2 (3790 *vs.* 2491 pmol · (mg prot)$^{-1}$ · s$^{-1}$). The

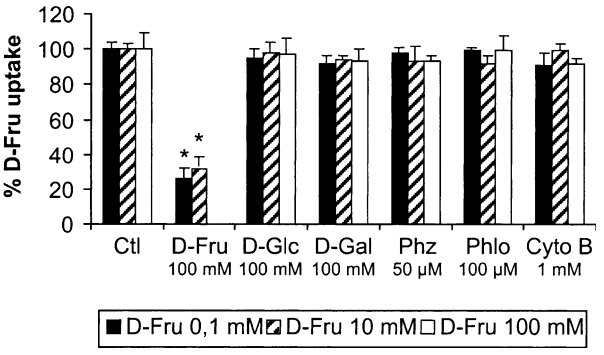


**Fig. 3.** Effect of different hexoses and inhibitors on D-fructose (*D-Fru*) uptake by jejunal BBMV. Uptakes of 0.1, 10 and 100 mM D-fructose (5 s incubation) were determined in the presence of 100 mM D-fructose; 100 mM D-glucose; 100 mM D-galactose; 50μM phlorizin; 100 μM phloretin and 1 mM cytochalasin B. Results are means of 3–6 experiments and are expressed as a percentage of control. Error bars represent SE of the mean. Statistical analysis (Student's *t*-test): *, significant difference from control ($P < 0.05$).

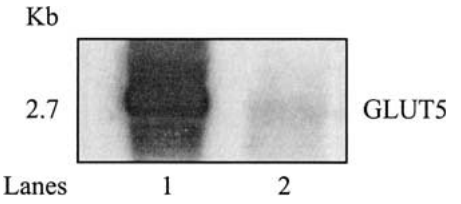


**Fig. 4.** Detection of chicken GLUT5 mRNA by Northern blot. Lane *1* corresponds to 15 μg of total rat RNA used as a control of hybridization. Lane *2* corresponds to 45 μg of total chicken RNA. The calculated molecular weight for both transcripts is 2.7 kb.

combined presence of these transporters favors the absorption of D-fructose from the intestinal lumen to the bloodstream.

The study of the effects of other hexoses added at high concentration in the medium show that jejunal D-fructose apical transport is highly specific, since none of these sugars were successful at inhibiting D-fructose uptake. This is in agreement with previous works (Sigrist-Nelson & Hopfer, 1974; Crouzoulon & Korieh, 1991) where ratios of 100:1 and 50:1 of D-glucose to D-fructose had no effect on transport of 1 mM D-fructose in BBMV. In our experiments even a molar ratio of 1000:1 (100 mM D-glucose or D-galactose to 0.1 mM D-fructose) does not show any effect on D-fructose transport in BBMV. However, the use of high concentrations of D-fructose reduced its transport by self-inhibition, confirming the high specificity of the mechanism.

Phlorizin, a specific and competitive inhibitor of $Na^+$-dependent D-glucose transport (Garriga et al., 1999) failed to inhibit D-fructose uptake. Moreover, cytochalasin B, a compound that inhibits the transport capacity of all GLUT-type transporters with the exception of GLUT5 (Carruthers, 1990), is unable to exert an inhibitory effect in the apical fructose uptake. Likewise, no inhibitory effect was observed incubating BBMV in the presence of phloretin, an inhibitor of GLUT2 (Corpe et al., 1996).

Taking into account the effects observed by different concentrations of hexoses as well as those of specific inhibitors of transporters, we can conclude that the transport of D-fructose across the brush-border membrane is mediated by a GLUT5-type transporter and not by SGLT1 or GLUT2.

The molecular characterization of chicken GLUT5 depends on the cloning and sequencing of this transporter, which has not yet been achieved. In the present work, we have used a rat GLUT5 cDNA to detect chicken GLUT5 and obtained a single transcript of the same molecular weight in both rat and chicken jejunum. With the aim of discarding a cross-hybridization between rat GLUT5 and chicken GLUT2 mRNA, an alignment of the mRNA sequences was performed using the Nucleotide-Blast search (http://www.ncbi.nlm.nih.gov/BLAST), without finding significant similarities. This suggests that the hybridization band obtained in the chicken cannot correspond to GLUT2 but corresponds to GLUT5.

The reasonably high homology between rat and chicken GLUT2 sequences (73%) and the detection of chicken SGLT1 using a rabbit SGLT1 probe (Barfull et al., 2002) demonstrate that cross-hybridization between mammalian probes and chicken mRNA is feasible when detecting message for proteins of comparable function in the two species, as is the case in the present study.

These results suggest that the apical transport of D-fructose in the chicken jejunum characterized in the present study should be attributed to a GLUT5-type transporter. Only the cloning and sequencing of this transporter in chickens and the subsequent obtainment of specific antibodies will provide powerful tools to achieve a better understanding of the expression and regulation of this transporter.

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