Biochimica et Biophysica Acta, 507 (1978) 305—321

BBA 77921

SUGAR UPTAKE INTO BRUSH BORDER VESICLES FROM DOG KIDNEY

I. SPECIFICITY

R. JAMES TURNER and M. SILVERMAN

Department of Medicine, University of Toronto, Toronto, Ontario (Canada)

(Received July 1st, 1977)

Summary

This study describes the specificity of uptake of sugars into osmotically active vesicles derived from a purified membrane fraction from dog kidney cortex. [^{3}H]Phlorizin binding experiments have also been carried out in vesiculated and non-vesiculated membrane preparations.

1. We demonstrate the existence of a sodium-dependent, phlorizin-sensitive D-glucose transport system in the vesiculated membrane preparation.

2. This transport system has similar specificity characteristics to those observed in vivo for the glucose transporter in the brush border membrane of the proximal tubule.

3. We also observe a sodium-dependent, glucose-sensitive phlorizin receptor in the same preparation with a K_d for phlorizin $\simeq 0.3$ μM and K_I for glucose $\simeq 3$ mM at 37°C, pH 7.4, 100 mM NaCl.

4. Detailed results relating to the specificity of inhibition of high affinity phlorizin binding are obtained using non-vesiculated brush border membrane fragments in the presence of D-glucose, α-methyl-D-glucoside, D-galactose, β-methyl-D-galactoside, 2-deoxy-D-glucose and D-mannose.

5. Uptake studies using vesiculated membrane fragments from newborn dog kidney indicate that the brush border D-glucose transporter is already at an advanced stage of development at birth.

Our results demonstrate that the D-glucose transport system from the dog proximal tubule brush border membrane together with its phlorizin receptor moiety is preserved intact in our membrane vesicle preparation.

Introduction

Previous in vivo work from our laboratory using the multiple indicator dilution technique, has demonstrated the existence of three apparently distinct sugar transport systems localized to the brush border membrane of the dog

proximal tubule [1]. In addition there is at least a fourth sugar carrier at the basal or antiluminal membrane of the tubular cell [2]. These "carrier" proteins act in an integrated manner to regulate the net transepithelial reabsorption of sugar molecules. We have mainly focussed our attention on the glucose transport system at the brush border membrane (G-transporter). The specificity characteristics of this transporter have been determined in some detail [1]. It is shared by the following substrates in order of decreasing affinity: α-methyl-D-glucoside, D-galactose, 2-deoxy-D-glucose, D-fructose and myoinositol.

Using in vivo methodology, a set of specific phlorizin binding sites has been localized exclusively to the brush border membrane of the dog kidney proximal tubule [3]. In vitro binding studies of [^{3}H]phlorizin to isolated brush border membrane fragments were also carried out [4] and a high affinity sodium-dependent site with $K_d \simeq 0.3$ μM at pH 7.45 and temperature 37° C was identified. Almost identical results had been obtained previously for the rat [5–7]. The specificity of inhibition of high affinity [^{3}H]phlorizin binding in vitro by an homologous series of monosaccharides and monosaccharide derivatives correlates well with the ability of these same compounds to wash off [^{3}H]phlorizin from the brush border in vivo [4], and with their apparent affinity for the G-transporter in vivo. It has been concluded, therefore, that the high affinity, Na^+-dependent phlorizin receptor (P-receptor) forms a part of the G-transporter exposed at the urine face of the brush border membrane [1,8].

As the next stage in our investigations we have extended our work on dog kidney to an in vitro brush border membrane vesicle preparation in order to study transport in the absence of metabolic and other perturbations. In this and a second paper in preparation we report on an extensive series of experiments characterizing the specificity and kinetics of sugar transport in this brush border membrane vesicle system. Sugar uptake into brush border membrane vesicles has previously been studied in rat intestine [9] and in rat [10] and rabbit [11] kidney by other authors as well as in human kidney [12] by ourselves. Our purpose here is to expand and extend some of these observations using both sugar uptake and phlorizin binding measurements.

In the present paper we focus principally on the question of specificity. We have made a concerted effort to compare and correlate results obtained from in vivo experiments and from two different in vitro brush border membrane preparations from dog kidney, namely, our vesicle system and the non-vesiculated brush border membrane fragments used for phlorizin binding studies [4].

Methods and Materials

Preparation of brush border vesicles

Vesiculated membrane fragments are prepared in two stages. First a "crude membrane fraction" is obtained from an initial kidney cortex homogenate by differential centrifugation. A fraction enriched in brush border membranes and containing closed vesicles is then prepared from this crude membrane fraction using a modification of the method of Schmitz et al. [13]. All steps are carried out at 4°C unless otherwise noted.

The renal cortex of mongrel dogs is removed by scraping with a razor blade and suspended in "isolation medium" (10 mM Triethanolamine-HCl with

250 mM sucrose, pH 7.6) to a final dilution of 10 ml/g cortex. The cortex suspension is then homogenized using a tight fitting glass-teflon homogenizer to obtain the "initial homogenate". This is filtered successively through a double layer of coarse fiberglass screen and a double layer of fine gauze and then centrifuged twice for 10 min at 190 × *g*. The resulting pellets are discarded. The supernatant is centrifuged for 20 min at 16000 × *g* and the entire pellet is resuspended in a suitable volume of fresh "isolation medium" (typically, approx. 2 ml/g original cortex mass). This "crude membrane fraction" is stored at −20°C. Any material remaining unused after two weeks is discarded.

On the day of the uptake experiment an aliquot of the crude membrane fraction (typically 10—15 ml) is thawed and centrifuged for 20 min at 48000 × *g*. The pellet is resuspended in 80 ml of Buffer A (1 mM Tris - HEPES * with 100 mM D-mannitol, pH 7.4) and recentrifuged for 20 min at 48000 × *g*. This pellet is resuspended in a small volume of fresh Buffer A and passed once through a 25 gauge needle and twice through a 30 gauge needle avoiding excess pressure and aeration. The material is then diluted with Buffer A to a final volume of 80 ml and sufficient $MgCl_2$ to give a final concentration of 10 mM is added. The diluted membranes are allowed to stand on ice with occasional stirring for 10 min, then the contents of the beaker are centrifuged for 10 min at 2000 × *g* yielding a solid brownish pellet which is discarded. The supernatant is centrifuged for 20 min at 48000 × *g* yielding a small white pellet which is resuspended in 1 ml of the appropriate buffer for the experiment (see below) and recentrifuged for 20 min at 30000 × *g*. The final pellet is resuspended in the appropriate buffer for the experiment and passed twice through a 30 gauge needle. This "final vesicle fraction" is incubated at 37°C for 15 min and stored on ice until used.

To ensure for bacteria-free solutions all buffers are prepared from autoclaved distilled water and routinely passed through 0.22 μm Millex disposable filter units (Millipore, Bedford, Mass.) before use.

Preparation of brush border membrane fragments

As previously reported [4] large non-vesiculated brush border membrane fragments for phlorizin binding studies were prepared according to the procedure of Wilfong and Neville [14].

Criteria of purity

The fractions are routinely assayed for enzymes known to be characteristic of brush border microvilli, basal lateral membranes and mitochondria, namely, alkaline phosphatase [15], Na^+ and K^+-dependent ATPase ($(Na^+ + K^+)$-ATPase) [16], and succinic dehydrogenase [17]. Protein was determined by the Lowry method [18] after precipitation with 10% trichloracetic acid using bovine serum albumin as the standard.

The preparations are monitored with phase microscopy and, in addition, thin sections of the final vesicle fraction were examined periodically with the electron microscope.

* 1 mM Tris-HEPES: 1 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) buffered with Tris to pH 7.4.

Sugar transport studies

Two types of transport studies are presented in this paper. These are "timed uptake studies", where the uptake of various radioactively-labelled sugars are measured as a function of time, and "initial uptake studies", where the initial rate of uptake of a given sugar is estimated from a 10 or 15 s sample. All the uptake experiments reported here were carried out at 25°C. Unless otherwise noted the "final vesicle fraction" is suspended in Buffer A in the case of the "timed uptake studies" and in Buffer A/300 (1 mM Tris-HEPES containing 300 mM D-mannitol) in the case of the "initial uptake studies". The protocols for the two types of experiments are identical to those in ref. 12.

Radioactivity was measured in a Nuclear Chicago Mark II liquid scintillation counter. Appropriate ^{14}C and ^{3}H standards were used to calculate efficiency and crossovers. Since all samples were prepared identically no correction for quenching was found to be necessary. The specific activity of radiolabelled substances was determined by preparing and counting aliquots of incubation medium under the same conditions as samples from the uptake studies.

Experiments carried out using the above protocols, but with no membranes present, indicate that there is no significant binding or retention of radioactive sugars by the millipore filters.

All experiments were carried out in triplicate. The errors indicated are the S.E.M.

Phlorizin binding studies

[^{3}H]phlorizin binding measurements to large non-vesiculated brush border membrane fragments were carried out at 37°C and pH 7.45 in a buffer containing 20 mM Tris · HCl, 140 mM NaCl and 5 mM EDTA, using the same procedures described in ref. 4. In order to determine the efficacy of different sugar substrates in inhibiting phlorizin binding two types of experiments were carried out:

(*a*) Phlorizin binding over a range of phlorizin concentrations in the "high affinity" region (0.05—1.0 μM) was measured as a function of sugar concentration. In order to correct for unspecific phlorizin binding we have operationally defined the residual phlorizin bound to the membranes in the presence of 100 mM D-glucose as the unspecific component. For phlorizin concentrations in the "high affinity" region the unspecific binding defined in this way is found to be consistently approximately 10% of the total phlorizin bound. Consequently, where data on phlorizin binding in the presence of 100 mM D-glucose were not available, 10% of the total phlorizin bound was subtracted to correct for unspecific effects.

(*b*) In "wash off" experiments the amount of bound [^{3}H]phlorizin which could be "washed off" the membrane fragments by adding various concentrations of a given sugar was measured relative to an appropriate control [4]. These experiments were carried out at a single phlorizin concentration in the "high affinity" region (from 0.016 to 0.05 μM). Maximal "wash off" for the purposes of this experiment was taken to be that resulting from the addition of 1 μM phlorizin.

In order to correlate these data obtained from intact brush border membrane fragments with our vesicle data we have repeated some of our previous phlor-

izin-binding measurements using the vesicle system. These experiments were carried out in Buffer A at 25 and 37°C using a protocol identical to the "initial uptake studies".

Chromatography

Membrane vesicles were incubated and filtered as described above. The intravesicular contents were extracted by soaking the filters in distilled water and the extract was concentrated. Thin-layer chromatography was carried out on the concentrated extract using the solvent system *n*-butanol/ethanol/water (50 : 32 : 18, v/v).

Prior to each phlorizin-binding assay the [^{3}H]phlorizin was chromatographed using the solvent system chloroform/methanol/water (65 : 25 : 4, v/v) to verify its purity.

Materials

All materials used were of the highest chemical grade available. Unlabelled L-glucose, D-glucose, α-methyl-D-glucoside, D-galactose, D-mannose, 2-deoxy-D-glucose and 3-*O*-methyl-D-glucose were purchased from Sigma Chemical Co. (St. Louis, Mo.). HEPES, Tris and phlorizin were also from Sigma. D-Fructose was from Nutritional Biochemical Corp. (Cleveland, Ohio), 5-thio-D-glucose from Aldrich Chemical Co. (Milwaukee, Wisc.), β-methyl-D-galactose from Schwartz-Mann (Orangeburg, N.Y.) and phloretin from K and K Laboratories Inc. (Plainview, N.Y.). D-Mannitol was a product of the Fisher Chemical Co. (Montreal, Quebec). All radioactively-labelled materials were purchased from New England Nuclear Corp. (Boston, Mass.) and were of the highest specific activity available. The [^{3}H]phlorizin used was from lot numbers 674—169 and 929—221.

Results

Enzymatic characterization of the membranes

Table I shows the average enrichment of enzyme markers for the "crude membrane fraction" and the "final vesicle fraction" measured relative to the "initial homogenate" and to each other. Although there was some variation in both absolute and relative enzyme activities from preparation to preparation we generally find roughly a 10-fold enhancement of alkaline phosphatase activity (the brush border membrane marker); a 4-fold enhancement of ($Na^+ + K^+$)-ATPase activity (the antiluminal membrane marker) and a 4-fold reduction of succinate dehydrogenase activity (the marker for mitochondria) in the final vesicle fraction measured relative to the initial homogenate. These data indicate that the "final vesicle fraction" used for uptake studies contains a significant antiluminal membrane contamination. However, as we will show shortly, uptake into antiluminal membrane vesicles is usually unimportant in the experiments we are concerned with here.

The yield of our procedure is typically 2.0—2.5 mg of "final vesicle fraction" per g of original kidney cortex.

TABLE I

ENZYMATIC CHARACTERIZATION OF MEMBRANE FRACTIONS *

	Initial homogenate	Average enrichments		
	Specific activity **	Crude membrane fraction / Initial homogenate	Final vesicle fraction / Initial homogenate	Final vesicle fraction / Crude membrane fraction
Alkaline phosphatase (mU/mg protein)	(475—1980) 1060 ± 516	1.82 ± 0.60	9.9 ± 3.2	5.4 ± 1.3
$(Na^+ + K^+)$—ATPase (μmol P_i/h per mg protein)	(1.20—6.03) 2.9 ± 1.5	1.67 ± 0.67	3.9 ± 1.2	2.9 ± 1.6
Succinic dehydrogenase (μmol/min per mg protein)	(0.025—0.059) 0.044 ± 0.011	2.36 ± 0.52	0.24 ± 0.10	0.11 ± 0.06

* Values represent averages ± S.E.M. for 35 vesicle preparations from a total of 12 initial homogenates.

** Range of specific activities is given in brackets.

Sugar uptake studies

A. Binding vs. uptake

It is crucial to the interpretation of our results to be able to distinguish between membrane transport and membrane binding effects. Fig. 1 shows the results of an experiment in which the uptakes of D- and L-glucose were measured as a function of extravesicular osmolarity. The osmolarity of the incubation medium was varied by changing its sucrose concentration. Since sucrose is a relatively impermeant solute which is not hydrolyzed in the kidney [20,21] increasing its concentration in the incubation medium should lead to osmotic shrinkage of any vesiculated membrane fragments. From Fig. 1 it is clear that the uptake of both D- and L-glucose are linear functions of the inverse osmolarity indicating that they represent transport into an osmotically active space. The small non-specific component of uptake seen when the results are extrapolated to infinite osmolarity may be due to a slow leak of sucrose into the vesicles or to non-specific binding or trapping by the membranes or filters. In any case, whatever accounts for this small component, the fact that it is not stereo-specific indicates that it is of no functional significance.

B. Metabolism vs. uptake

No metabolic conversion of D- or L-glucose could be detected following uptake into the membrane vesicles. When the intravesicular contents were submitted to thin-layer chromatography no significant component of radioactivity could be detected outside the glucose spot. Moreover, the uptake of α-methyl-D-glucoside, a non-metabolizable sugar, was found to be very similar to that of D-glucose (see below), again indicating that metabolism does not play a role in uptake in our vesicle system.

C. The time dependent uptake of D-glucose

(i) Sodium dependence. The effect of sodium on the uptake of D- and L-glucose is illustrated in Fig. 2. Inspection of this diagram reveals that the initial uptake of D-glucose is enhanced 3-fold when 100 mM NaCl is present in equi-

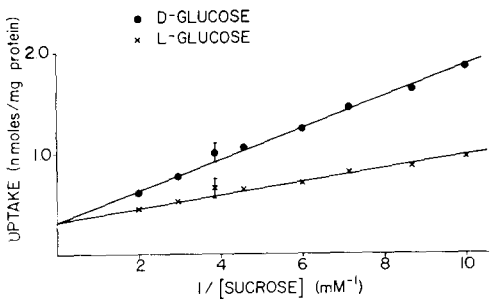

Fig. 1. Uptake of 1 mM D- and L-glucose as a function of the extravesicular osmotic pressure. The membranes were preincubated in Buffer SN (1 mM Tris-HEPES, pH 7.4, with 100 mM sucrose and 100 mM NaCl). The incubation medium was Buffer SN containing sufficient labelled D- and L-glucose to give a 1 mM final concentration and additional sucrose as indicated. Uptake was measured by taking a 100 μl sample after 20 min. A "stop solution" containing 10 mM Tris-HEPES, 450 mM NaCl and 0.2 mM phlorizin was used.

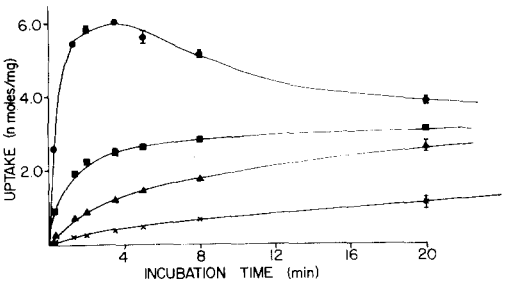

Fig. 2. The timed uptake of 1 mM D- and L-glucose at 25°C. The uptake of D-glucose is shown in the presence of a 100 mM NaCl gradient (●), in the presence of 100 mM NaCl in equilibrium inside and outside the vesicles (■) and in the absence of sodium (▲). The vesicles were preincubated either in Buffer A or Buffer A containing 100 mM NaCl. The incubation medium was Buffer A containing sufficient labelled D- and L-glucose, NaCl and phlorizin to give the final concentrations indicated. The uptake of L-glucose (X) is the same in each case.

librium inside and outside the vesicles and 8-fold when an initial 100 mM NaCl gradient (outside > inside) is imposed. In addition, in the "Na^+ gradient" experiment the intravesicular D-glucose concentration is seen to temporarily rise to twice its equilibrium value indicating a concentrative uptake in the presence of an initial sodium gradient. Similar curves have been published for brush border membrane vesicle preparations from rat [10], rabbit [11] and human [12] kidney as well as from rat intestine [9]. These results suggest that D-glucose and sodium are co-transported by the glucose transporter in the brush border membrane.

Fig. 3 represents an experiment similar to the one just described which was done on very pure antiluminal membrane obtained by pooling the cleanest fractions from a number of free-flow electrophoresis runs carried out using the method of ref. 19. The figure demonstrates quite conclusively that the uptake of D-glucose by the antiluminal membrane occurs by sodium independent-facilitated diffusion. This experiment, together with the fact that very pure brush border membrane preparations, likewise obtained from free-flow electrophoresis, show similar behavior to Fig. 2, strongly implies that the sodium-dependent component of D-glucose uptake seen in our vesicle system is entirely due to brush border membranes. The much slower sodium-independent uptake is then a combination of facilitated diffusion by the antiluminal membrane and simple diffusion (cf. L-glucose uptake).

(ii) Phlorizin sensitivity. The effect of phlorizin on glucose uptake is shown

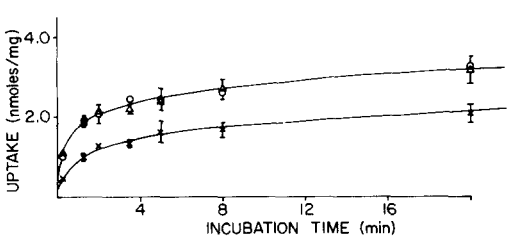

Fig. 3. The timed uptake of 1 mM D- and L-glucose into purified antiluminal membranes at 25°C. The uptake of D-glucose is shown in the presence of initial 100 mM NaCl (○) and KCl (△) gradients. The uptake of L-glucose (X) is the same in each case.

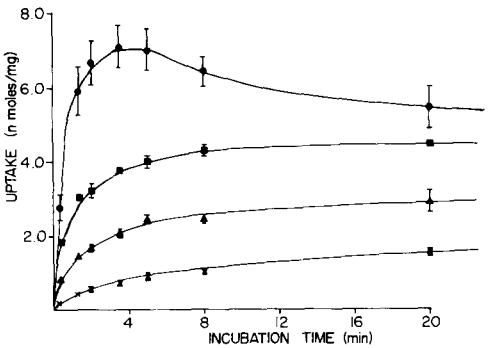

Fig. 4. The timed uptake of 1 mM D-glucose in the presence of a 100 mM NaCl gradient along with 100 μM phlorizin (▲), 10 μM phlorizin (■) and no phlorizin (●). The uptake of 1 mM L-glucose (X) is the same in each case.

in Fig. 4. Phlorizin concentrations of 10 and 100 μM reduce the initial uptake to 0.65 and 0.29 of that found in the absence of the drug. The overshoot is abolished at 10 μM. The fact that 100 μM phlorizin has not caused the D-glucose curve to superimpose on the L-glucose curve is a reflection of residual glucose uptake into antiluminal membrane vesicles in our preparation. No effect of phlorizin on the uptake of L-glucose could be detected.

D. Uptake of other sugars

In Fig. 5 we show the timed uptake of several other sugars. Of all the substrates tested (see Table II) only D-glucose and α-methyl-D-glucoside were found to overshoot their equilibrium intravesicular concentration in the presence of an initial sodium gradient.

Table II compares the initial velocity of uptake of various monosaccharides and monosaccharide derivatives at 1 mM concentration in the presence and absence of an initial 100 mM sodium chloride gradient (outside > inside) and in the presence of 10 μM phlorizin as well the sodium chloride gradient. With the exception of D-mannose, these ligands seem to fall naturally into two groups. The first group contains D-glucose, α-methyl-D-glucoside and D-galactose. These

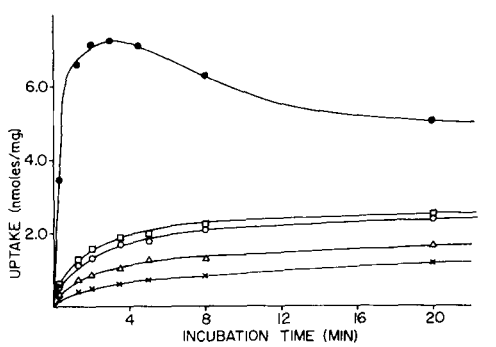

Fig. 5. The timed uptake of various sugars at 1 mM concentration in the presence of a 100 mM sodium gradient. The uptake of α-methyl-D-glucoside (●), D-galactose (□), D-mannose (○), 3-*O*-methyl-D-glucose (△) and L-glucose (X) are shown. The data represent the results of separate experiments which have been normalized so that the uptake of L-glucose at 20 min is the same in each case.

TABLE II

COMPARISON OF SODIUM DEPENDENCE AND PHLORIZIN SENSITIVITY FOR SIX MONOSACCHARIDES AND MONOSACCHARIDE DERIVATIVES

Sugar	Initial sugar uptake less L-glucose uptake (nmol/mg per 10 s)				
	Control (no sodium)	100 mM sodium gradient	Sodium gradient / control	100 mM sodium gradient + 10 μM phlorizin	Sodium + phlorizin / sodium gradient
D-Glucose	0.200 ± 0.028	2.82 ± 0.53	14 ± 5	0.92 ± 0.16	0.33 ± 0.12
α-Methyl-D-glucoside	0.032 ± 0.009	2.24 ± 0.43	70 ± 33	0.61 ± 0.12	0.27 ± 0.10
D-Galactose	0.054 ± 0.013	0.68 ± 0.06	13 ± 4	0.17 ± 0.04	0.25 ± 0.08
D-Mannose	0.135 ± 0.005	1.10 ± 0.14	8 ± 1	0.47 ± 0.05	0.43 ± 0.10
2-Deoxy-D-glucose	0.230 ± 0.032	0.54 ± 0.06	2.3 ± 0.6	0.38 ± 0.01	0.70 ± 0.10
3-O-Methyl-D-glucose	0.197 ± 0.011	0.42 ± 0.02	2.1 ± 0.2	0.25 ± 0.04	0.60 ± 0.12

sugars show a marked increase in uptake upon addition of sodium to the incubation medium and a significant inhibition by 10 μM phlorizin. The second group made up of 2-deoxy-D-glucose and 3-*O*-methyl-D-glucose shows considerably less sodium dependence and phlorizin inhibition. The behavior of D-mannose is intermediate between these two groups in that it is moderately stimulated by sodium and partially inhibited by phlorizin.

E. Inhibition of initial D-glucose uptake

The initial velocity of 1 mM D-glucose uptake was also measured in the presence of various inhibitors. The data are shown in Table III. The following sugars are seen to inhibit D-glucose uptake (in order of decreasing inhibitory capacity): α-methyl-D-glucoside, D-galactose, 5-thio-D-glucose and 2-deoxy-D-glucose. Those sugars exhibiting no significant inhibition include L-glucose, D-mannose, 3-*O*-methyl-D-glucose, and D-fructose. These data correlate almost perfectly with the in vivo specificity characteristics of the G-transporter of the brush border membrane [1]. Table III also shows that phlorizin has more than 10 times the inhibitory capacity of its aglycone phloretin, a finding in good agreement with the in vivo and in vitro results [4—7,22].

F. Ontogenic development of the glucose transporter

Timed uptake studies similar to those described above have also been carried out on renal vesicle preparations from embryo (approximately two weeks before term), newborn and four- and six-week old dogs. The data for the newborn dog is shown in Fig. 6. Since very little tissue was available for this experiment only the "Na^+ gradient" curve was done. The presence of the characteristic overshoot of the intravesicular D-glucose concentration observed in the adult dog (Fig. 2) indicates that the brush border membrane G-transporter is already at an advanced stage of development at birth. In a second manuscript, now in preparation, we also demonstrate that the newborn G transporter is competitively inhibited by phlorizin concentrations in the micromolar range.

In the case of the embryo tissue no sodium-dependent overshoot was seen although the initial uptake of D-glucose was enhanced by a factor of 2 over that of L-glucose. However, since the embryonic kidneys were not sufficiently well developed that the cortex could be distinguished, the entire organs were homo-

TABLE III

THE INHIBITORY EFFECTS OF VARIOUS SUBSTANCES ON THE INITIAL (15 s) STEREOSPECIFIC UPTAKE OF 1 mM D-GLUCOSE AT 25°C

The incubation medium was Buffer A containing sufficient NaCl and D- and L-glucose to give initial extravesicular concentrations of 100 mM and 1 mM, respectively, and sufficient inhibitor to give the initial extravesicular concentration indicated.

Inhibitor	Uptake (nmol/mg protein per 15 s)	Relative uptake *
None	1.68 ± 0.01	1.00
KCl replacing NaCl	0.50 ± 0.01	0.30 ± 0.01
5 mM α-Methyl-D-glucoside	1.10 ± 0.08	0.65 ± 0.05
25 mM D-Galactose	1.23 ± 0.03	0.73 ± 0.02
50 mM D-Galactose	1.09 ± 0.04	0.65 ± 0.03
25 mM 5-Thio-D-glucose	1.37 ± 0.01	0.82 ± 0.01
50 mM 5-Thio-D-glucose	1.17 ± 0.04	0.70 ± 0.03
25 mM 2-Deoxy-D-glucose	1.51 ± 0.11	0.90 ± 0.07
50 mM 2-Deoxy-D-glucose	1.29 ± 0.02	0.77 ± 0.02
25 mM 3-*O*-Methyl-D-glucose	1.62 ± 0.01	0.96 ± 0.01
50 mM 3-*O*-Methyl-D-glucose	1.57 ± 0.01	0.93 ± 0.01
25 mM D-Mannose	1.67 ± 0.03	0.99 ± 0.02
50 mM D-Mannose	1.77 ± 0.06	1.05 ± 0.04
25 mM D-Fructose	1.62 ± 0.03	0.96 ± 0.02
50 mM D-Fructose	1.62 ± 0.04	0.96 ± 0.03
25 mM L-Glucose	1.67 ± 0.07	0.99 ± 0.05
50 mM L-Glucose	1.84 ± 0.04	1.10 ± 0.03
1 μM Phlorizin	1.35 ± 0.03	0.80 ± 0.02
10 μM Phlorizin	0.81 ± 0.03	0.48 ± 0.02
10 μM Phloretin	1.37 ± 0.07	0.82 ± 0.05
100 μM Phloretin	1.00 ± 0.03	0.60 ± 0.02

* Uptake is given relative to that with no inhibitor present.

genized for this particular vesicle preparation. Thus the resulting final vesicle fraction must have contained a large amount of medullary and other non-brush border membranes and the overshoot, if present, might have been swamped by non-brush border membrane uptake.

The sodium dependence and phlorizin sensitivity of the D-glucose uptake into brush border membrane vesicles from four- and six-week old dog kidneys were essentially identical to those observed in the adult.

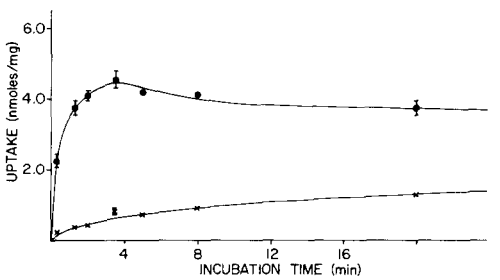

Fig. 6. The timed uptake of 1 mM D-glucose (●) and 1 mM L-glucose (X) into brush border membrane vesicles prepared from newborn dog kidney cortex. An initial 100 mM NaCl gradient was present.

Phlorizin binding

A. Vesicle system

In order to be able to compare and correlate the results presented in the preceeding section with our phlorizin-binding studies on large intact brush border membrane fragments we have repeated several of our previous phlorizin-binding assays in the vesicle system using the protocol of the "initial uptake studies". Fig. 7 shows a Scatchard plot of the binding of [^{3}H]phlorizin to our brush border membrane vesicles as a function of phlorizin concentration at 37°C. The inhibitory effect of 5 and 50 mM D-glucose is also shown. The competitive nature of the D-glucose inhibition is obvious. We estimate from the figure that the K_I for glucose is approx. 3 mM. Fig. 8 (a) and (b) illustrate the binding of phlorizin in the vesicle preparation at 37°C and 25°C, respectively. In this case we have taken the residual binding when NaCl is replaced by KCl as a measure of the phlorizin unspecifically bound. The sodium-dependent component of the phlorizin binding found by subtraction is also shown. These results indicate the existence of a single sodium-dependent phlorizin binding site in the μM range in our vesicle system. The dissociation constant of this site is ≈ 0.3 μM at 37°C and ≈ 0.6 μM at 25°C (pH 7.4, 100 mM NaCl). The K_d value at 37°C is in good agreement with the value ($Kd \approx 0.3$ μM at pH 7.45, 140 mM NaCl) obtained previously in our laboratory using intact brush border membrane fragments [4]. It is interesting that the vesicle system seems to contain a considerably larger number of receptor sites than are typically found in preparations containing large brush border membrane fragments. In the latter, values of 12—40 pmol of receptor/mg protein have been found by ourselves [4] and other workers [5—7], while in the vesicle system we find approx. 200 pmol of

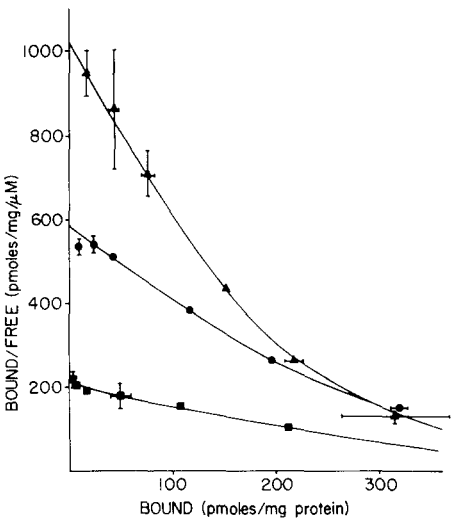

Fig. 7. A Scatchard plot of phlorizin binding to brush border membrane vesicles at 37°C. The vesicles were preincubated in Buffer A/300. The incubation medium was Buffer A containing sufficient NaCl to give a final concentration of 100 mM as well as [^{3}H]phlorizin and unlabelled glucose as indicated. Binding was measured after 1 min according to the protocol of the "initial uptake studies". No phlorizin was present in the stop solution. The phlorizin concentration range was 0.018—2.4 μM. Binding is shown in the absence of glucose (▲) and in the presence of 5 mM (●) and 50 mM (■) D-glucose.

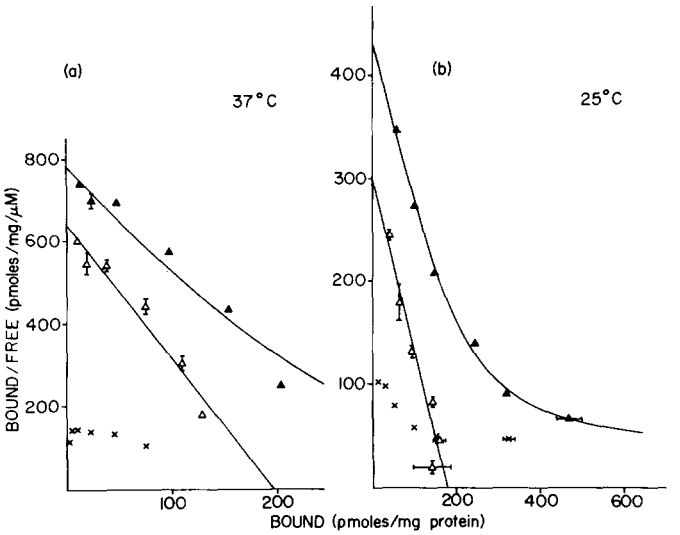

Fig. 8. Scatchard plots of phlorizin binding to brush border membrane vesicles at (a) 37°C and (b) 25°C. Binding is shown in the presence of 100 mM NaCl (▲) and 100 mM KCl (X). The sodium-dependent component of phlorizin binding found by subtracting these results at each concentration is also shown (△). The linear fits to the sodium-dependent data were found using the method of least squares.

receptor/mg protein. This could be due to the relative inaccessability of some receptor sites in more intact brush border preparations. Also, the vesicle system contains substantially less mitochondrial contamination than the membrane preparations used in previous phlorizin binding studies [4]. Based on our earlier estimates we found the number of high affinity P-receptor sites to be about 30 times smaller than the number of glucose transporters estimated in vivo by Diedrich (0.6 μmol/100 g kidney, ref. 23). Repeating these calculations on our newer figure of 200 pmol of receptor/mg protein we find 0.2 μmol of P-receptor (or glucose carrier)/100 g dog kidney. Considering the uncertainties involved in this type of computation this is a very acceptable correlation. This agreement between in vivo and in vitro results gives further credence to the hypothesis that the sodium-dependent, glucose sensitive P-receptor is part of the G-transporter at the brush border membrane.

B. Intact membrane fragments

Fig. 9 summarizes the data for inhibition of phlorizin binding by different sugars. At each sugar concentration the percentage inhibition of phlorizin binding was calculated for 4 phlorizin concentrations in the "high affinity" region. Corrections for unspecific binding were made as previously described. These 4 values were then averaged and plotted against the logarithm of the sugar concentration to give the logarithmic dose response-type curves shown in the figure.

Since the linear portion of these dose-response curves for α-methyl-D-glucoside, β-methyl-D-galactose and D-galactose are parallel to that of D-glucose we conclude that these sugars are competitive inhibitors along with D-glucose for the same "high affinity" phlorizin binding site. The concentration of each sugar that gives 50% inhibition of phlorizin binding is a measure of its K_I for the site.

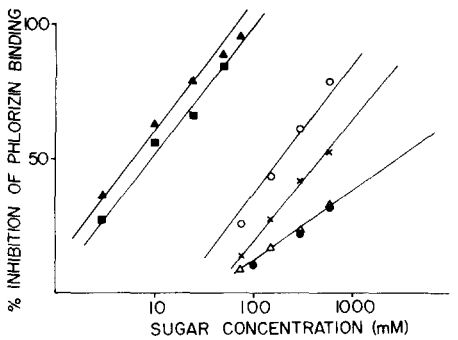

Fig. 9. Inhibition of phlorizin binding at 37°C by α-methyl-D-glucoside (▲), D-glucose (■), β-methyl-D-galactose (○), D-galactose (X), 2-deoxy-D-glucose (△) and D-mannose (●). This experiment was carried out on non-vesiculated brush border membrane fragments as described in the text.

These concentrations are 9.5 mM, 6 mM, 190 mM and 500 mM for D-glucose, α-methyl-D-glucoside, β-methyl-D-galactose and D-galactose, respectively. The curves for 2-D-glucose and D-mannose are not parallel to those discussed above indicating that these sugars inhibit the high affinity phlorizin site by a different mechanism from D-glucose.

The capacity of these and other sugars to inhibit high affinity phlorizin binding was estimated independently by a second method. The percentage of bound [^{3}H]phlorizin which could be displaced or "washed off" by a given sugar was measured relative to that "washed off" by 1 μM phlorizin (see Materials and Methods). The results are shown in Table IV. These data agree quite well with the results summarized in Fig. 9. The estimated sugar concentration giving 50% wash-off are approx 5—10 mM for both D-glucose and α-methyl-D-glucoside, 150—200 mM for β-methyl-D-galactose and 450—500 mM for D-galactose. There is negligible wash off with 2-deoxy-D-glucose, D-mannose and D-fructose at 600 mM concentration. The fact that 5-thio-D-glucose yields 50% wash off at a concentration of approx. 300—350 mM is consistent with our previous specificity data on the G transporter showing that, although the oxygen atom in the pyranose ring participates in the substrate interaction with the G transporter, it is nevertheless not an absolute requirement for this interaction to take place.

As shown by Levitski [24], under conditions where radioactive substrate concentration (in this case [^{3}H]phlorizin) exceeds its receptor concentration, the dissociation constant, K_I, of a displacing ligand for the same receptor is given by

$$K_I = \frac{I_{0.5}}{\frac{S^*}{K_d} + 1}$$

where $I_{0.5}$ is the concentration of ligand required to displace 50% of the bound substate, S^* is the radioactive substrate concentration and K_d is the substrate-receptor dissociation constant. The results shown in Table IV were obtained using S^* values from 0.016 to 0.05 μM. Since the K_d of phlorizin for its receptor is ≈0.3 μM the ratio S^*/K_d in the wash-off experiments ranged from 0.05 to 0.17. Thus the $I_{0.5}$ values obtained from our wash-off experiments provide a

TABLE IV

'WASH OFF' OF BOUND [^{3}H]PHLORIZIN BY VARIOUS SUGARS

	Sugar concentration (mM)	Wash-off (%)
D-Glucose	5 (3) *	47 **
	10	53
α-Methyl-D-glucoside	5	39
	10	50
D-Galactose	450	11
	500	61
β-Methyl-D-galactose	200	81
5-Thio-D-glucose	350	59
2-Deoxy-D-glucose	600 (2)	0
D-Fructose	600 (2)	0
D-Mannose	600 (2)	0

* The brackets indicate the number of times the same experiments was repeated.

** The values represent the average of 3 or 4 phlorizin concentrations in the high affinity region (see text).

good measure of the dissociation constants of these sugars for the P-receptor (G-transporter).

Discussion

We have presented results on two kidney membrane preparations in this paper. In the first, a vesicle system, we have demonstrated the existence of a sodium-dependent, phlorizin-sensitive D-glucose transport system with similar specificity characteristics to those observed in vivo for the glucose transporter in the brush border membrane of dog kidney. In the presence of a sodium gradient the sodium-dependent component of D-glucose uptake in our vesicle system represents the major part of the total initial uptake observed. Since D-glucose uptake in a very pure antiluminal vesicle preparation showed no sodium dependence whatsoever we conclude that the sodium-dependent D-glucose carrier is localized to the brush border membrane. We have also established that there is a sodium-dependent, glucose-sensitive phlorizin receptor in our vesicle system with essentially the same K_d for phlorizin and K_I for glucose as the P-receptor previously identified in our laboratory using purified non-vesiculated membrane fragments.

Results which further characterize the specificity of this P-receptor were also presented. In this case the earlier non-vesiculated brush border membrane preparation was used. These results further strengthen the case that the P-receptor is identical with the high affinity phlorizin site observed in vivo in the proximal tubule and that this receptor forms a part of the glucose transporter exposed at the urine face of the brush border membrane.

The data from each of our membrane preparations indicate that the phlorizin-sensitive brush border membrane glucose transporter observed in vivo has been preserved in a reasonably intact form.

As already mentioned, on the basis of in vivo work, the G-transporter in the brush border membrane is shared, in order of decreasing affinity, by D-glucose, α-methyl-D-glucoside, D-galactose, 2-deoxy-D-glucose and D-fructose. Our new

data largely confirm these earlier results [4] and emphasize the excellent correlation between those sugars which share the glucose transporter and those which competitively inhibit high affinity phlorizin binding in vitro. D-Fructose and 2-deoxy-D-glucose are two possible exceptions. In vivo data show that D-fructose shares both the D-glucose and D-mannose transporter [1]. In our vesicle system, however, 50 mM D-fructose shows no significant inhibition of D-glucose uptake (Table III) nor does 600 mM D-fructose seem to inhibit high affinity phlorizin binding (Table IV). Agreement between the in vivo and in vitro results is possible if we assume that the affinity of D-fructose for the G-transporter is very low ($K_I > 600$ mM). In our indicator dilution experiments fractional reabsorption of tracer radiolabelled sugars are measured at D-glucose plasma levels of approx. 70—90 mg% (approx. 4 mM) making it very likely that the sugar reabsorbing sites in the more distant portions of the proximal tubule are totally unsaturated. In this sense our in vivo method is very "sensitive" and it is not surprising that we would discern a detectable brush border membrane G transporter interaction even for a sugar with very low affinity.

The situation for 2-deoxy-D-glucose is not completely clear. It has been established that 2-deoxy-D-glucose shares the glucose transporter in the dog renal brush border membrane in vivo [25]. We have also shown that 2-deoxy-D-glucose inhibits the uptake of D-glucose in our vesicle system (Table III). However, 2-deoxy-D-glucose uptake shows little sodium dependence or phlorizin sensitivity (Table II), and its mode of inhibiting phlorizin binding is clearly not the same as that of D-glucose (Fig. 9). Recent results from our laboratory (manuscript in preparation) help to clarify these points. Briefly stated, using the indicator dilution technique we have found a previously overlooked 2-deoxy-D-glucose transporter at the antiluminal membrane. Thus, given the very low affinity of 2-deoxy-D-glucose for the glucose transporter at the brush border membrane, contamination of our vesicle preparation by antiluminal membrane could account for the relatively low sodium dependence and phlorizin sensitivity of its uptake.

Our results on newborn dogs suggest that the brush border membrane transport mechanism for D-glucose has already reached a relatively mature stage at birth, certainly from the point of view of its sodium dependence and phlorizin sensitivity. This finding is consistent with the clearance experiments of Baker et al. [26]. Since our cumulative evidence in dogs is so strong that α-methyl-D-glucoside shares the glucose transporter at the brush border membrane, it is surprising that other workers have observed very low uptake values for this sugar into cortical slices from newborn rat kidney compared to adult rats [27]. This discrepancy remains to be clarified.

Acknowledgements

R.J. Turner is a Centennial Fellow. This work was supported by grant MT4590 from the Medical Research Council. The authors wish to acknowledge the secretarial assistance of Mrs. S. McGugan and the technical assistance of Miss R. Rutherford and Mrs. O. Grafstein.

References

1 Silverman, M. (1976) Biochim. Biophys. Acta 457, 303—351
2 Silverman, M. (1977) Am. J. Physiol. 1, F455—F460
3 Silverman, M. (1974) Biochim. Biophys. Acta 339, 92—102
4 Silverman, M. and Black, J. (1975) Biochim. Biophys. Acta 394, 10—30
5 Frash, W., Frohnert, P.T., Bode, F., Baumann, K. and Kinne, R. (1970) Pflugers Arch. 320, 265—283
6 Bode, F., Baumann, K. and Diedrich, D.F. (1972) Biochim. Biophys. Acta 290, 134—150
7 Glossmann, H. and Neville, Jr., D.M. (1972) J. Biol. Chem. 247, 7779—7789
8 Vick, H., Diedrich, D.F. and Baumann, K. (1973) Am. J. Physiol. 224, 552—557
9 Hopfer, U., Sigrist-Nelson, K. and Murer, H. (1975) Ann. N.Y. Acad. Sci. 264, 414—427
10 Kinne, R., Murer, H., Kinne-Saffran, E., Thees, M. and Sachs, G. (1975) J. Membrane Biol. 21, 375—395
11 Beck, J.C. and Sacktor, B. (1975) J. Biol. Chem. 250, 8674—8680
12 Turner, R.J. and Silverman, M. (1977) Proc. Natl. Acad. Sci. U.S. 74, 2825—2829
13 Schmitz, J., Preiser, H., Maestracci, D., Ghosh, B.K., Cerda, J.J. and Crane, R.K. (1973) Biochim. Biophys. Acta 323, 98—112
14 Wilfong, R.F. and Neville, Jr., D.M. (1970) J. Biol. Chem. 245, 6106—6112
15 Bessey, O.A., Lowry, O.H. and Brock, M.J. (1946) J. Biol. Chem. 164, 321—329
16 Post, R.A. and Sen, A.K. (1967) Methods Enzymol. 10, 762—769
17 Pennington, R.J. (1961) Biochem. J. 80, 649—654
18 Lowry, O., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265—275
19 Chant, S. and Silverman, M. (1977) Kidney Int. 11, 348—356
20 Sacktor, B. (1968) Proc. Natl. Acad. Sci. U.S. 60, 1007—1014
21 Silverman, M. (1973) J. Clin. Invest. 52, 2486—2494
22 Chan, S.S. and Lotspeich, W.D. (1962) Am. J. Physiol. 203, 975—979
23 Diedrich, D.F. (1966) Am. J. Physiol. 211, 581—587
24 Levitski, A. (1976) in Hormone and Antihormone Action at the Target Cell (Clark, J.H., Klee, W., Levitski, A. and Wolff, J., eds.), pp. 79—86, Dahlem Konferenzen
25 Silverman, M., Aganon, M.A. and Chinard, F.P. (1970) Am. J. Physiol. 218, 735—742
26 Baker, J.T. and Kleinman, L.I. (1974) J. Physiol. 243, 45—61
27 Segal, S., Rea, C. and Smith, I. (1971) Proc. Natl. Acad. Sci. U.S. 68, 372—376