Biochimica et Biophysica Acta, 394 (1975) 10–30

BBA 76973

HIGH AFFINITY PHLORIZIN RECEPTOR SITES AND THEIR RELATION TO THE GLUCOSE TRANSPORT MECHANISM IN THE PROXIMAL TUBULE OF DOG KIDNEY

MELVIN SILVERMAN* and JUDITH BLACK

Department of Medicine, University of Toronto and Division of Nephrology, Toronto General Hospital, Toronto, Ontario (Canada)

(Received November 18th, 1974)

SUMMARY

A set of high affinity phlorizin receptors in a brush border membrane preparation from dog kidney cortex is described. The dissociation constant, K_d is $\approx$ 0.3 μM (20 mM Tris · HCl, 140 mM Na^+, 5 mM EDTA pH 7.45, 37 °C). The number of receptor sites is $\approx 12 \cdot 10^{-12}$ mol/mg membrane protein. Preincubation with sugar substrates shows that the high affinity phlorizin binding is completely abolished by D-glucose (100 mM), 3-deoxy-3-fluoro-D-glucose (125 mM) and α-methyl-D-glucopyranoside (125 mM), while 40–50 % inhibition is observed with glucose concentrations as low as 5 mM. D-Galactose and β-methyl-D-galactopyranoside inhibit 20–40 % at 125 mM while 2-deoxy-D-glucose and 2-deoxy-D-galactose inhibit minimally ($\approx$ 25 %) at the same concentration. L-Glucose, D-mannose, D-xylose, myoinositol, D-fructose and 3-*O*-methyl-D-glucose do not inhibit significantly in concentrations up to 600 mM. Unlabelled phlorizin (1 μM) and D-glucose (125 mM) completely wash off bound [^{3}H]phlorizin from the high affinity site. In contrast, phloretin (100 μM) is only about 50 % as effective in displacing bound [^{3}H]phlorizin. Binding decreases with decreasing sodium concentration and is abolished by *N*-ethylmaleimide (7 mM). No inhibition is observed with ouabain (0.125 mM), cytochalasin B (0.1–42 μM) and concanavalin A (10–10 000 μg/ml). The specificity of inhibition of phlorizin binding in vivo to the luminal membrane of the proximal tubule in dog kidney has also been investigated. α-Methyl-D-glucopyranoside completely washes off bound [^{3}H]phlorizin. D-Galactose is only about 10 % as effective at equivalent doses. There is no observable wash off of bound [^{3}H]phlorizin with D-fructose, myoinositol, D-mannose or 2-deoxy-D-glucose. The relative affinity of monosaccharides for the glucose transport receptor at the brush border was investigated in vivo using the multiple indicator dilution technique to determine their fractional reabsorption under identical conditions of phlorizin blockade. The relative affinities are in the order D-glucose $\approx$ α-methyl-D-glucopyranoside > D-galactose > 2-deoxy-D-glucose > D-fructose $\approx$ myoinositol. It is concluded (i) that phlorizin receptors on the brush border of the proximal tubule in vivo are identical to the high affinity phlorizin bind-

* Scholar of the Medical Research Council of Canada.

ing sites in the brush border membrane fraction in vitro and (ii) that these phlorizin receptor sites are either in close proximity, or identical, to the glucose transport receptor.

INTRODUCTION

Previous studies [1–3] have indicated that the reabsorption process for D-glucose across the proximal tubule probably involves synergistic behaviour of at least two transport systems, one located at the brush border and one at the antiluminal surface. As the next step in dissecting out the components of this sugar reabsorptive mechanism, we have focussed our attention on the glucose transport system in the brush border membrane. This mechanism (referred to as the G or glucose "transport receptor") has well-defined specificity characteristics. We have determined the chemical and steric determinants of its interaction with an homologous series of monosaccharides and monosaccharide derivatives [2, 3]. More recently the glucose transport mechanism at the brush border has also been studied in vivo using tritiated phlorizin of high specific activity as a probe of the glucose transport receptor [4]. These latter results confirm the presence of a set of binding sites for phlorizin located exclusively on the brush border surface of the proximal tubule in dog kidney.

By means of an in vitro approach, Frash et al. [5] demonstrated stereospecific, Na^+-dependent phlorizin binding to a brush border membrane fraction from rat kidney. This work was followed up by other investigators [6–8]. However, the precise relation of the in vitro high affinity phlorizin receptor to the in vivo glucose transport mechanism has remained unclear (see ref. 9). Because of this controversy there seems to be a real need to tie together the in vivo and in vitro results in a single animal species.

In this paper we extend our work in dog kidney and demonstrate the presence of a high affinity receptor site for phlorizin in a brush border membrane preparation. The in vitro specificity of inhibition of [^{3}H]phlorizin binding by sugars is correlated with the ability of these same sugar substrates to wash off bound [^{3}H]phlorizin from the brush border in vivo. Also, we compare the specificity of sugar inhibition of [^{3}H]phlorizin binding in vitro and in vivo to the relative affinity of these sugars for the glucose transport mechanism located at the brush border of the proximal tubule in vivo.

The results show that the isolated brush border membrane fraction prepared from dog kidney cortex contains the intact high affinity phlorizin receptor defined in vivo and that the phlorizin receptor forms part of (or is identical to) the glucose (G) transport receptor located in the brush border of the proximal tubule.

MATERIALS AND METHODS

Preparation of brush border membrane fractions

Brush border membrane fractions were prepared by two methods. In our initial studies we followed the procedure of Heidrich et al. [10]. The homogenate and final pellet in sucrose triethanolamine buffer (P5 in the terminology of ref. 10) was used for enzymatic assays. This pellet was then washed twice in buffer (20 mM

Tris · HCl, 140 mM sodium chloride, 5 mM EDTA pH 7.4) and stored at −20 °C for [^{3}H]phlorizin binding experiments. Homogenization was carried out by means of a motor driven teflon pestle (modified Potter-Elvehjem system).

More recently we have adopted the method of Wilfong and Neville [11], in which homogenization is carried out by hand with a loose fitting Dounce glass homogenizer. The membranes are stored in buffer (see above) at −20 °C. All enzyme determinations and binding assays are carried out within 5 days of preparation.

Each membrane preparation is characterized in three ways.

(i) *By determination of enzyme marker activity.* The following enzymatic activities were measured. Alkaline phosphatase [12] and maltase [13] were used as markers for the brush border membrane, succinic dehydrogenase [14] for mitochondrial contamination, glucose-6-phosphatase [15] for microsomal contamination and Na^+- and K^+-dependent ATPase [16] for assessment of basal (antiluminal) membrane content. All measurements were carried out in duplicate. To ensure that the assays were performed under enzyme saturation conditions, increasing volumes of homogenate and membrane pellet were shown to yield a linear increase in enzymatic activity. Protein determinations were made using either the Biuret [17] or Lowry [18] methods, depending upon the amount of protein in the final membrane pellet. Bovine serum albumin was used as the standard for the protein determination.

(ii) *By morphological studies.* All preparations were monitored by phase contrast microscopy. Electron microscopy was carried out on thin sections prepared as follows. Fixed in 3 % glutaraldehyde in cacodylate buffer, washed in cacodylate with 6.84 % sucrose, post-fixed in 1 % Palade's osmic acid pH 7.2 for 1 h, stained in block with uranyl acetate in water for 1 h, dehydrated in graded acetone, embedded in epon, sections on grid stained with aqueous uranyl acetate for 15 min followed by lead citrate (Reynolds) for 15 min.

(iii) *By determination of [^{3}H]phlorizin binding.* The binding assay was carried out using the millipore filtration technique described by Glossmann and Neville [6]. Briefly, 100–300 μg of membrane protein in 50 μl of buffer (20 mM Tris · HCl, 140 mM sodium chloride, 5 mM EDTA, pH 7.45) was diluted with another 50 μl of buffer and incubated for 2 min at 37 °C. At 2 min, a pulse of 20 μl of tritiated phlorizin in appropriate concentration (0.014 → 10 μM) was added. After a further period of 2.5 min of incubation at 37 °C, the membrane protein was rapidly diluted with 2 ml of ice cold buffer (4 °C) and immediately filtered over a 45 μm millipore membrane. It was verified that no protein passed through the millipore filter. The residue was subject to two successive 2 ml washes of ice cold buffer, placed in a liquid scintillation vial containing 1 ml of 5 % sodium dodecylsulphate and incubated in a shaking water bath for 1 h. To this was added 10 ml of a liquid scintillation mixture as previously described [1]. The vials were allowed to stand for 24 h in the dark and counted in a Nuclear Chicago Mark II liquid scintillation spectrometer for 40 min, to ensure good counting statistics. Quench correction was made using an external standard ratio technique. At each phlorizin concentration tested, measurements were carried out at least in triplicate. Controls were performed (also in triplicate) in an identical manner leaving out the membrane protein. Bound phlorizin, (b), in pmol/mg membrane protein was calculated by first subtracting ^{3}H-counts (dpm) bound to millipore filters (controls) from dpm bound to the membrane protein. For each [^{3}H]phlorizin solution used, 20 μl was added directly to liquid scintillation fluid and

the dpm determined. In this way the total, (T), of phlorizin added in pmol was experimentally measured. Free phlorizin, (f) was calculated from ($T-b$).

The in vivo experiments and associated analytical procedures were carried out according to the protocol of the multiple indicator dilution technique as previously described [1, 4, 19].

Materials

Tritiated phlorizin was purchased from New England Nuclear. The specific activity was 5 Ci/mM (1 pmol = 11 000 dpm). The purity was verified using thin-layer chromatography with the solvent system chloroform/methanol/water in proportions of 65 : 25 : 4. The radioactive sugars used in the in vivo studies consisted of D-[^{14}C]glucose (234 Ci/mmol), D-[1-^{14}C]galactose (52.9 Ci/mmol), D-[^{14}C]fructose (185 Ci/mmol), D-[6-^{3}H]galactose (168 Ci/mmol), D-[6-^{3}H]glucose (8.45 Ci/mmol), all from New England Nuclear Corporation, and [2-^{3}H]myoinositol (4.5 Ci/mmol) from Amersham-Searle. Phloretin and phlorizin were purchased from K & K Laboratories, Plainview, New York. D-Glucose was purchased from the J. T. Baker Chemical Company, Phillipsburg, New Jersey. Xylose, 2-deoxy-D-glucose, L-glucose, D-galactose, 3-*O*-methyl-D-glucose and D-mannose were all purchased from Sigma Chemical Company, St. Louis. α-Methyl-D-glucopyranoside, β-methyl-D-glucopyranoside, 2-deoxy-D-galactose and inositol were purchased from Schwartz-Mann, Orangeburg, New York and fructose from Nutritional Biochemical, Cleveland, Ohio. Ouabain and bovine serum albumin were purchased from Sigma Chemical Company, *N*-ethylmaleimide from Schwartz-Mann, concanavalin A from Cal Biochem, San Diego, Ca. and cytochalasin B from I. C. I. Research Laboratories, Alderly Park, Cheshire, England. All other chemicals used were purchased commercially from standard sources, in reagent grade wherever possible. The alkaline phosphatase assay was carried out using a colorometric kit (Boehringer Mannheim, Mannheim, Germany).

3-Deoxy-3-fluoro-D-glucose was a generous gift from Dr A. Foster of the Chester Beatty Research Institute, Institute of Cancer Research, Royal Cancer Hospital, London, England.

RESULTS

I In vitro phlorizin binding to brush border membranes

(*A*) *Enzyme marker characterization.* Table I shows the specific activities of enzyme markers comparing starting homogenate with membrane pellets prepared by the method of Heidrich et al. [10]. In our hands, this method yielded roughly 4–5-fold enrichment in brush border enzyme markers with an average 8–9 times enrichment in antiluminal fragments. There was some contamination from microsomes and mitochondria.

In contrast, our results using the method of Wilfong and Neville [11] are shown in Table II. Inspection of Table II shows that the purity of this brush border preparation was increased, yielding an average of 12 times enrichment for alkaline phosphatase and 9 times for maltase. The maltase enrichments have special significance since maltase has been specifically located at the brush border of the proximal tubule in dog kidney, spatially superficial to the glucose transport mechanism [20]. The

TABLE I

ENZYME MARKER DATA

Brush border membranes prepared by the method of Heidrich et al. [10].

	Homogenate (H) spec. act.	Pellet (P) spec. act.	P/H
Alkaline Phosphatase (9) (mU/mg protein[a])	(376–848) 632 ± 62[b]	(1448–4568) 3039 ± 377	(3.3–6.2) 4.44 ± 0.55
Maltase (3) (μM glucose $\times 10^{-3}$/min per mg protein)	(4.06–12.75) 7.89 ± 3.14	(1538–7676) 55.99 ± 24.87	(3.8–11.09) 7.0 ± 2.6
Succinic dehydrogenase (9) (μM/min per mg protein)	(0.023–0.037) 0.032 ± 0.003	(0.011–0.035) 0.022 ± 0.003	(0.46–1.2) 0.67 ± 0.08
Glucose-6-phosphatase (5) (μM P_i/h per mg protein)	(0.98–5.4) 2.23 ± 0.90	(1.33–3.44) 2.45 ± 0.44	(0.25–2.6) 1.66 ± 0.49
Na^+ and K^+ ATPase (8) (μM P_i/h per mg protein)	(1.14–2.46) 1.84 ± 0.16	(12.61–17.34) 15.21 ± 0.55	(5.6–12.95) 8.76 ± 0.91

[a] Protein determinations were made using biuret method [17].

[b] Results are given as range of specific activity (in brackets), followed by mean values ± S.E.

TABLE II

ENZYME MARKER DATA

Brush border membranes prepared using method of Wilfong and Neville [11].

	Homogenate (H) spec. act.	Pellet (P) spec. act.	P/H
Alkaline phosphatase (13) (mU/mg protein[a])	(496–1600) 812 ± 88[b]	(5648–18 122) 9485 ± 1138	(7.4–18.7) 11.91 ± 0.95
Maltase (7) (μM glucose $\times 10^{-3}$/min per mg protein)	(6.3–16.3) 11.3 ± 1.6	(45–242) 100 ± 27	(4.4–14.9) 9.0 ± 1.7
Succinic dehydrogenase (12) (μM/min per mg protein)	(0.023–0.059) 0.031 ± 0.003	(0.003–0.039) 0.024 ± 0.003	(0.13–1.7) 0.81 ± 0.16
Glucose-6-phosphatase (11) (μM P_i/h per mg protein)	(0.8–4.4) 2.25 ± 0.38	(0–8.6) 2.21 ± 0.88	(0–3.5) 1.00 ± 0.34
Na^+ and K^+ ATPase (10) (μM P_i/h per mg protein)	(0.48–5.56) 2.33 ± 0.58	(0.40–18.14) 6.25 ± 1.84	(0.19–9.6) 3.62 ± 0.97

[a] Protein determinations made using Lowry method [18].

[b] Results are given as range of specific activity (in brackets), followed by mean values ± S.E.

presence of antiluminal membrane fragments was reduced so that enrichments of only 3–4 times in Na^+ and K^+-dependent ATPase were recorded. There was variable contamination of mitochondria and microsomes in individual preparations but in most cases this was small. Thus the method of Wilfong and Neville gave relatively "clean" brush border preparations.

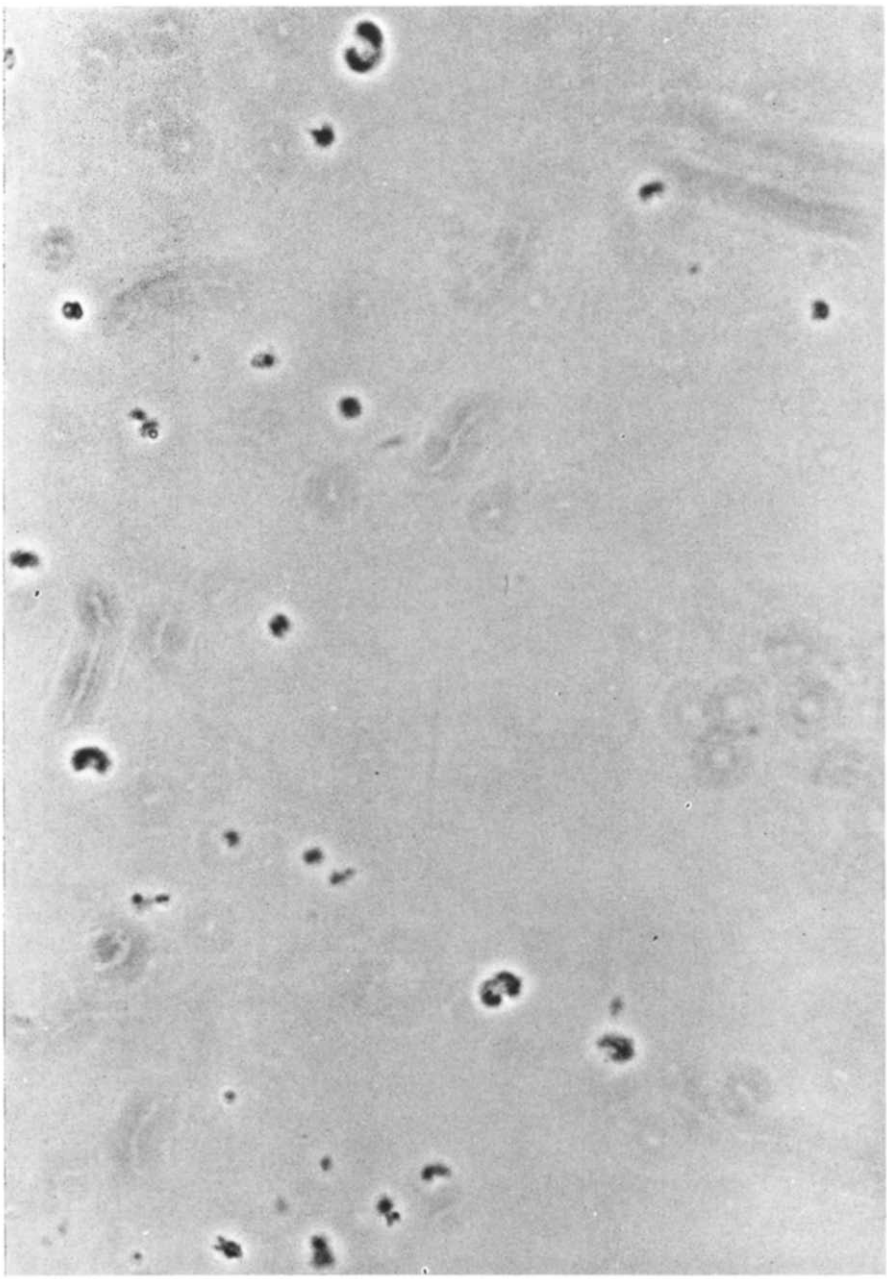

Fig. 1. Phase contrast micrograph of brush border membrane preparation from dog kidney; magnification × 500.

(*B*) *Morphology*. Fig. 1 shows a phase contrast micrograph of a typical membrane fraction prepared using the method of Wilfong and Neville. Fig. 2 is an electron micrograph of a thin section of such a membrane pellet. In contrast to Figs 1 and 2, morphologic assessment of the membrane fraction obtained from the Heidrich method showed small fragments of membranes but no intact brush borders.

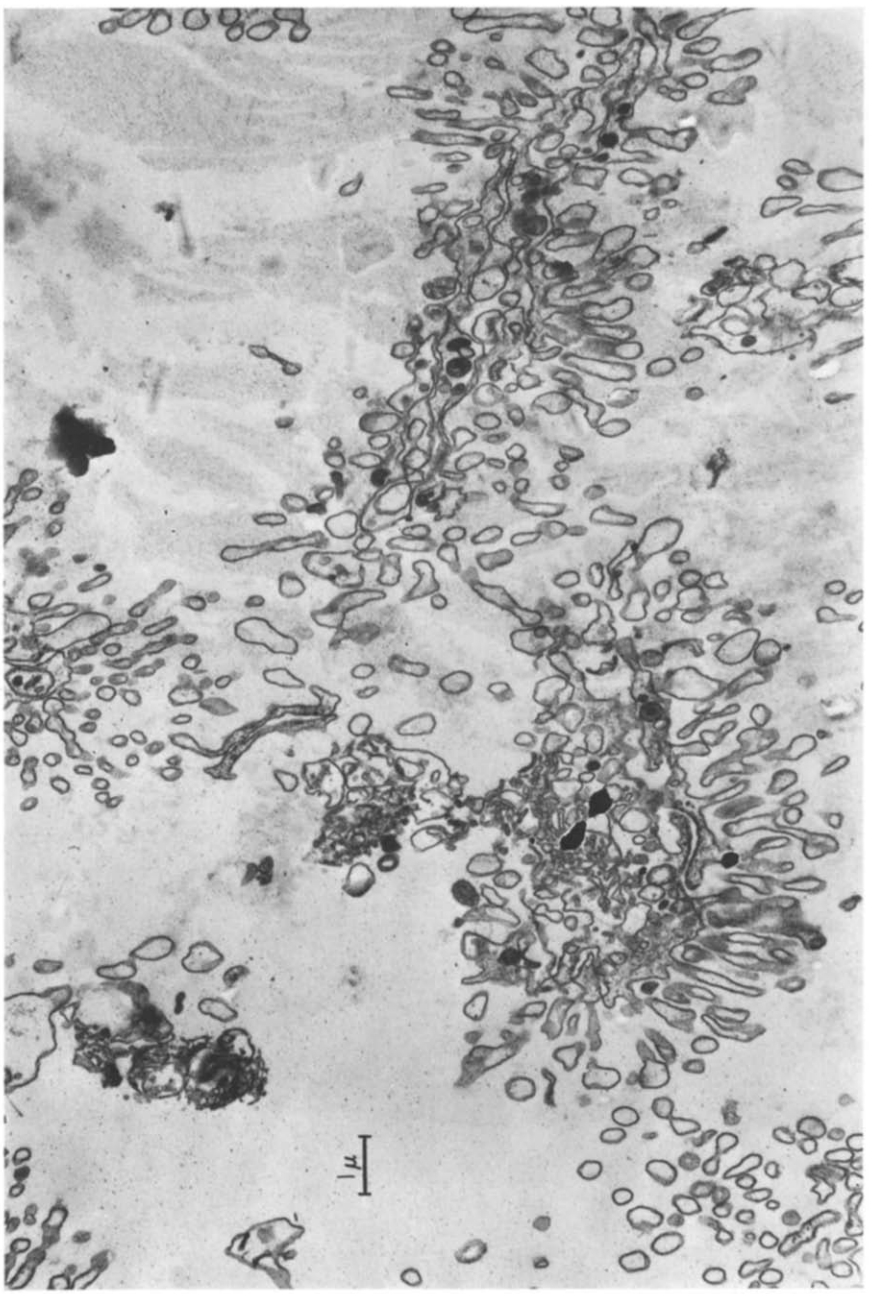

Fig. 2. Electron micrograph of brush border membrane preparation from dog kidney, fixation 3 % glutaraldehyde, thin section stained with uranyl acetate, magnification × 6840.

From the enzymatic marker determinations and from morphologic assessment, it became apparent that in our hands the purity of the brush border membrane fraction was more a function of the homogenization technique than of the method of preparation. When following the method of Heidrich, we had employed excessively rigorous homogenization which resulted in fragmentation of the brush border membranes. More gentle hand homogenization with a loose-fitting glass Dounce Homogenizer, as was used when following the method of Wilfong and Neville, enabled us to obtain large intact brush border fragments as seen in Figs 1 and 2.

(*C*) *Binding assay*. The binding of [^{3}H]phlorizin to the membrane prepared using the method of Wilfong and Neville is presented in Fig. 3a in the form of a Scatchard Plot [21]. The data points represent a mean value of measurements made in different preparations at identical [^{3}H]phlorizin concentrations. Inspection of Fig. 3 reveals that the membrane fraction exhibits a region of high affinity binding as well as one or more sets of low affinity sites. Our determinations were made over a range of phlorizin concentrations from 0.014 to 10 μM and were not carried out over the extensive range of concentrations used by previous workers (i.e. 0.1–100 μM) [6, 7]. However, the range of [^{3}H]phlorizin concentration in the present study was quite sufficient to define the region of high affinity binding. The line through the high affinity region (Fig. 3b) was fitted by eye after the contribution of the low affinity

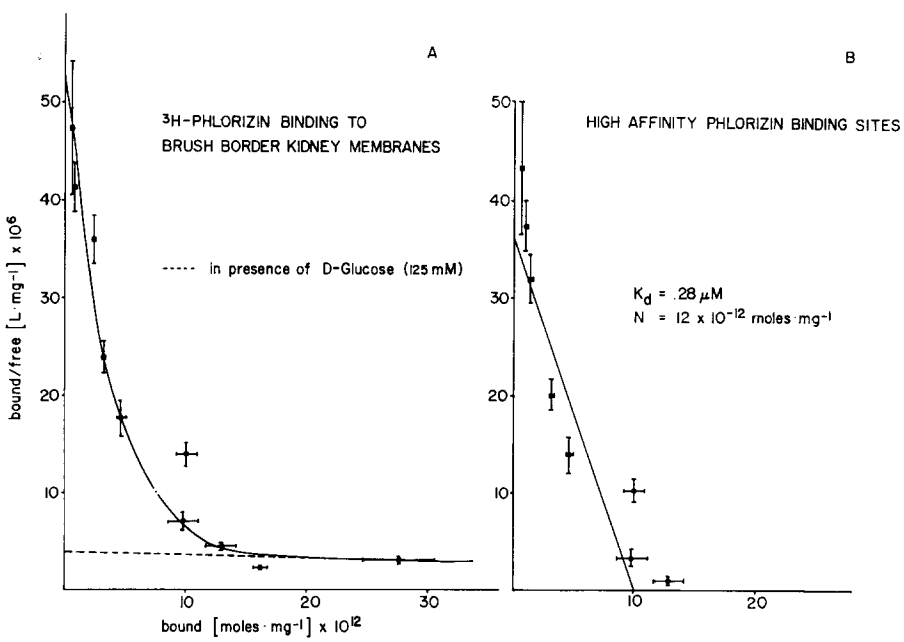

Fig. 3. Scatchard plot of [^{3}H]phlorizin binding to brush border kidney membranes. Prepared according to the method of Wilfong and Neville [10]. (A) [^{3}H]Phlorizin binding to brush border kidney membranes alone (——) and in the presence of D-glucose 125 mM (- - -). The data points represent a mean value of measurements made in different preparations at identical [^{3}H]phlorizin concentrations. The means are plotted plus or minus standard error. (B) High affinity phlorizin binding sites derived by taking the difference between phlorizin binding alone and in the presence of D-glucose. Curves have been drawn by inspection.

binding sites was subtracted (as determined after complete inhibition with D-glucose (125 mM)). Inspection of the experimental points in Fig. 3b reveals that the data are compatible with more than one set of receptor sites in the high affinity regions. We feel, however, that there is little to be gained in fitting the data with multiple receptor site models at this time. The important point is that only the region designated as "high affinity" was found to contain stereospecific sites. It is this region which we shall correlate with the glucose transport activity. For simplicity, therefore, we shall treat the high affinity region as being represented by a single set of sites. Then calculation of N, the number of binding sites per mg membrane protein and K_a, the affinity constant, assuming one binding site per receptor yields values of $N \approx 12 \cdot 10^{-12}$ mol/mg membrane protein and $K_a \approx 3.6 \cdot 10^6$ L/mol ($K_d \approx 0.3\,\mu$M). Fig. 4 shows the data of Fig. 3b replotted in the form of bound [^{3}H]phlorizin as a function of free phlorizin concentration.

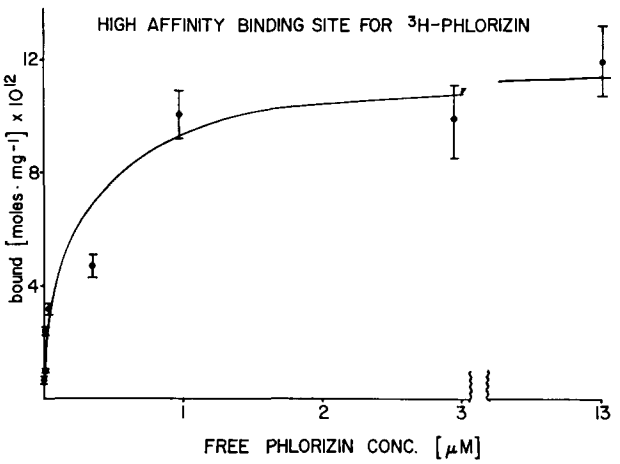

Fig. 4. High affinity phlorizin binding plotted as a function of phlorizin concentration.

Specificity of sugar inhibition of high affinity phlorizin binding

The specificity of inhibition of high affinity phlorizin binding was tested with a set of sugar substrates ranging in concentration from 5 to 600 mM (final concentration). The sugar solutions were prepared in the same buffer as was used to store the membranes. The pH of each sugar solution was adjusted to 7.45. Each sugar solution was checked for purity by thin-layer chromatography. Only 3-*O*-methyl-D-glucose was found to contain a contaminant of D-glucose. For these specificity studies, binding is measured in the presence of buffer (control) and in the presence of the test sugar at one or two different [^{3}H]phlorizin concentrations, preselected in the region of high affinity binding. Inhibition is expressed as a percentage of the control (i.e. phlorizin binding in the absence of sugar). The standard error is $\pm 20\,\%$, therefore inhibition $< 20\,\%$ is insignificant. Table III summarizes these results. Sugars which showed significant inhibition were studied over a range of concentration and the results are shown in Fig. 5. D-Glucose and α-methyl-D-glucopyranoside were equally effective, giving up to 50 % inhibition at concentrations as low as 5 mM. 3-Fluoro-3-deoxy-D-

TABLE III

SPECIFICITY OF INHIBITION OF PHLORIZIN BINDING*

Substrate	Conc. (mM)	Inhibition
D-Glucose	5–125	yes
α-Methyl-D-glucopyranoside	5–125	yes
3-Fluoro-3-deoxy-D-glucose	25–125	yes
D-Galactose	25–300	yes
β-Methyl-D-galactopyranoside	25–300	yes
2-Deoxy-D-glucose	25–600	minimal
2-Deoxy-D-galactose	25–300	minimal
L-Glucose	125–600	no
D-Xylose	25–600	no
D-Fructose	125–600	no
Myoinositol	125–600	no
3-*O*-Methyl-D-glucose	125–600	minimal**
D-Mannose	125–600	no

* Determined at final [^{3}H]phlorizin concentrations of 0.075–0.085 μM. No inhibition signifies $< 20\%$ inhibition.

** Contamination with D-glucose.

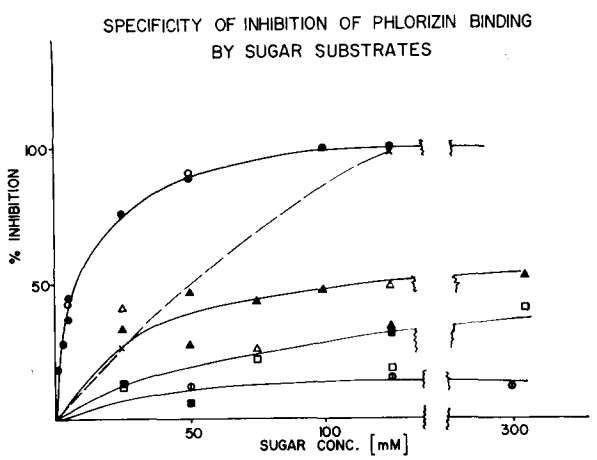

Fig. 5. The inhibition of phlorizin binding by sugar substrates expressed as a % inhibition of control binding (final phlorizin concentration of 0.075–0.085 μM) plotted as a function of sugar concentration. All values are ±20%, thus % inhibition of ≦ 20% is not significant. Each point is the mean of four determinations. Lines drawn by inspection. (○), D-glucose; (●), α-methyl-D-glucopyranoside; (×), 3-fluoro-3-deoxy-D-glucose; (△), β-methyl-D-galactopyranoside; (▲), D-galactose; (□), 2-deoxy-D-glucose; (■), 2-deoxy-D-galactose, (Φ), D-xylose.

glucose was the next most inhibitory compound giving ≈30% inhibition at a concentration of 25 mM followed by D-galactose and β-methyl-D-galactopyranoside which gave inhibition of 40–50% at concentrations of 125 mM. 2-Deoxy-D-glucose and 2-deoxy-D-galactose were minimally inhibitory (20–30%) at 125 mM. The points for 3-fluoro-3-deoxy-D-glucose are joined by a dashed line because insufficient amounts of the sugar were available to carry out enough assays to define the complete inhibition curve.

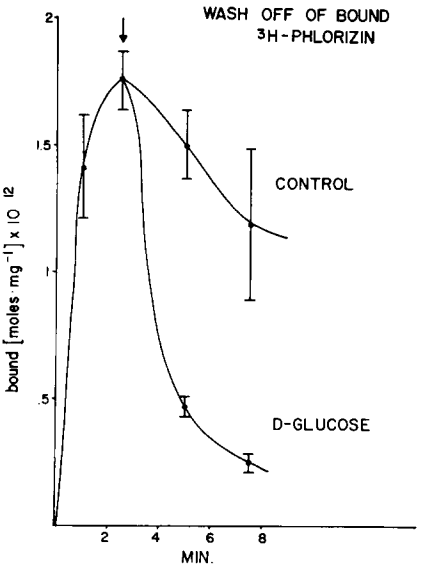

Fig. 6. Displacement of bound [^{3}H]phlorizin by D-glucose. The control curve shows the wash off of bound phlorizin after addition of 20 μl of buffer containing D-glucose 125 mM.

In Fig. 6, the uptake of [^{3}H]phlorizin was determined using a concentration of 0.07 μM. At 2.5 min, 20 μl of buffer containing 125 mM D-glucose was added and the remaining bound [^{3}H]phlorizin was measured. Inspection reveals that the [^{3}H]-phlorizin is entirely washed off by D-glucose compared to a control buffer without D-glucose. Similarly Fig. 7 compares the ability of phlorizin and phloretin to wash off bound [^{3}H]phlorizin from the high affinity site. Phloretin is only about 50 % as effective as phlorizin even at 100 × the concentration.

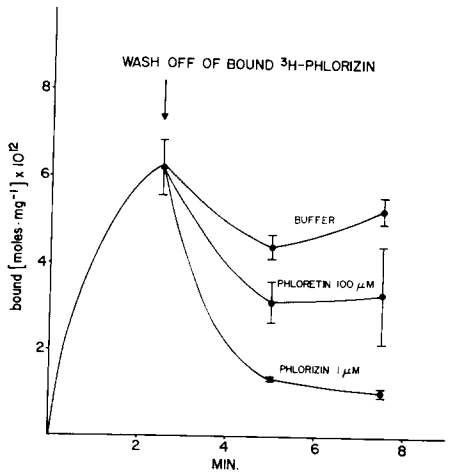

Fig. 7. Displacement of bound [^{3}H]phlorizin by phlorizin (1 μM) and phloretin (100 μM). Control represents wash off in response to addition of buffer alone.

The Na^+-dependence of high affinity phlorizin binding was confirmed by replacing Na^+ with K^+. The results are shown in Fig. 8a. Binding was measured over a limited range of phlorizin concentrations. In Fig. 8b we show that ouabain in a final concentration of 0.125 mM has no effect on high affinity phlorizin binding or on the ability of D-glucose (125 mM) to completely inhibit this binding. Cytochalasin B in concentrations of 0.1–42 μM shows no inhibition of high affinity phlorizin binding, the sulphydryl blocking agent *N*-ethylmaleimide (7 mM) completely abolishes phlorizin binding and concanavalin A at concentrations of 100–1000 μg/ml in the incubation medium does not inhibit binding.

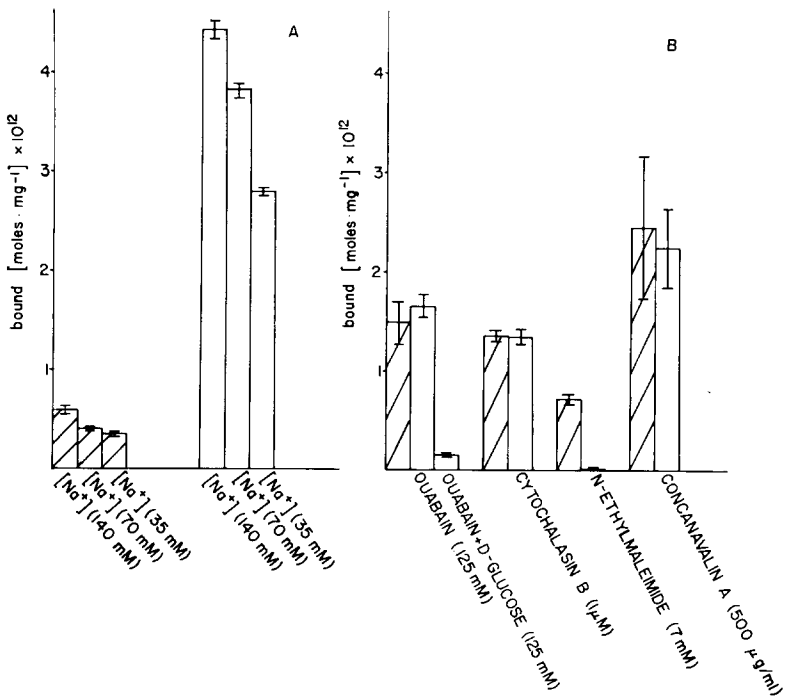

Fig. 8. (A) The effect of decreasing Na^+ concentration on high affinity phlorizin binding at 0.01 μM phlorizin (left) and 0.1 μM (right). (B) Shows the effect of ouabain, cytochalasin B, *N*-ethylmaleimide and concanavalin A on high affinity phlorizin binding. Initial phlorizin concentration 0.03–0.08 μM.

In summary, these in vitro studies demonstrate the existence of high-affinity phlorizin receptor sites in a membrane preparation from dog kidney cortex enriched in brush border membranes. The phlorizin binding can be inhibited by D-glucose, in concentrations as low as 1–3 mM, but not by L-glucose in concentrations up to 600 mM. Moreover, the inhibition of phlorizin binding by other sugar substrates has well defined specificity characteristics, i.e. in order of decreasing inhibitory potency: D-glucose = α-methyl-D-glucopyranoside > 3-fluoro-3-deoxy-D-glucose > D-galactose = β-methyl-D-galactopyranoside > 2-deoxy-D-glucose = 2-deoxy-D-galactose. The high-affinity binding site is also Na^+ dependent and inhibited by a sulphydryl blocking agent.

II. In vivo phlorizin binding to brush border surface of proximal tubule

In a recent publication [4], we showed that there exists only one set of phlorizin receptors in dog kidney in vivo and that these are located along the brush border of the proximal tubule. Moreover, these phlorizin receptor sites can be blocked by D-glucose but not by the aglycone phloretin.

In order to establish that the brush border phlorizin receptors in vivo are identical to the high affinity phlorizin receptors defined in vitro (see above) we extended our investigations to determine the specificity of inhibition of phlorizin binding in vivo by an homologous series of sugar substrates. We estimate that the maximum plasma sugar concentrations reached in this series of in vivo phlorizin "wash off" experiments is about 100 mM.

Table IV summarizes the ability of different sugar substrates to wash off bound [^{3}H]phlorizin from the brush border of the proximal tubule. The new data given in Table IV of this paper when combined with our earlier work in ref. 4 reveal that only D-glucose, α-methyl-D-glucopyranoside and D-galactose compete with the "high affinity" phlorizin receptor. In fact D-glucose, α-methyl-D-glucopyranoside and phlorizin are capable of completely washing off luminal bound [^{3}H]phlorizin if urine sampling is carried out for sufficient time. In contrast, D-galactose in comparable doses is only about 10 % as effective in washing off bound labelled phlorizin.

TABLE IV

URINE "WASH OFF" OF LUMINAL BOUND [^{3}H]PHLORIZIN

All sugars were administered via femoral vein in a dose of 50 g over 2–5 min.

Substrate	Bound phlorizin*	Fraction washed off
α-Methyl-D-glucopyranoside	0.92	0.92
D-Galactose	0.92	0.06
2-Deoxy-D-glucose	0.91	0.00
D-Mannose	0.90	0.00
D-Fructose	0.92	0.00
Inositol	0.92	0.00

* Expressed as a fraction of filtered [^{3}H]phlorizin.

Several points are worth emphasizing. (i) The ability of D-glucose and phlorizin to wash off luminal bound [^{3}H]phlorizin in vivo coincides exactly with their ability to displace [^{3}H]phlorizin from high affinity receptor sites in vitro. (ii) Phloretin is at least 100 × less effective in displacing [^{3}H]phlorizin from its binding site on the brush border than phlorizin. (iii) In both in vivo and in vitro studies D-galactose has less affinity for the phlorizin receptor site than D-glucose. All other tested sugars (in comparable doses) do not compete. (3-Deoxy-3-fluoro-D-glucose is not available in sufficient quantities to permit testing with this in vivo protocol.)

On the basis of the foregoing results, we conclude that the phlorizin receptor sites on the brush border of the proximal tubule in vivo are identical to the in vitro high affinity phlorizin receptor sites in our brush border membrane preparaton and that the specificity of inhibition of binding by an homologous set of sugar substrates is preserved during the membrane isolation procedures.

We next sought to determine the relationship between these high affinity phlorizin receptor sites and the glucose "transport receptors" located on the luminal surface of the proximal tubule.

III. Specificity of interaction of sugars with the glucose transport receptor at the brush border of the proximal tubule in vivo

In earlier work we showed that there exists a phlorizin sensitive glucose (G) transport receptor at the brush border of the proximal tubule which is shared by D-glucose, α (and β-)methyl-D-glucopyranoside, D-galactose, 2-deoxy-D-glucose, D-fructose and myoinositol. However, one of the unresolved areas in our earlier work was that we made no attempt to determine the relative affinities of these different sugars for the G receptor site. In order to do this it was necessary to carry out a new series of experiments in which unlabelled phlorizin was infused systematically in concentrations which are known to only partially inhibit the fractional reabsorption of tracer D-glucose [1]. Then, approximately 30 min after the start of the phlorizin infusion, the urine outflow curves for pairs of simultaneously injected radiolabelled sugars are determined in response to an arterial pulse injection according to the protocol of the multiple indicator dilution technique. Injection solutions are made up to contain pairs of radiolabelled sugars from the following: D-glucose, 2-deoxy-D-glucose, D-galactose and D-fructose. All experiments have been carried out in fasting animals where plasma glucose averages $\approx$ 100 mg%. Since phlorizin reduces the number of available "free" glucose transport receptors along the luminal surface of the proximal tubule, the fractional reabsorption of a radiolabelled test sugar relative to simultaneously injected labelled D-glucose under identical conditions of phlorizin blockade should also be a reflection of its relative affinity for the glucose transporter. On the other hand, if the fractional reabsorption of a radiolabelled test sugar exhibits less inhibition to phlorizin than D-glucose, we conclude that it has an alternate site of interaction with the luminal surface of the nephron, different from the G receptor.

Fig. 9a shows that under conditions where there is almost complete inhibition of D-galactose reabsorption (superposition of the D-galactose and creatinine curves), the fractional reabsorption of tracer D-glucose has only been reduced from 100 to 88 %. In another experiment using even smaller phlorizin infusion rates, 2-deoxy-D-glucose was inhibited to an even greater extent than D-galactose (Fig. 9b). Therefore the order of affinities of sugar substrates for the phlorizin sensitive glucose transport receptor is D-glucose > galactose > 2-deoxy-D-glucose. Thus we expect D-galactose to inhibit high affinity phlorizin binding to a much lesser extent than D-glucose at equal concentrations. This is consistent with our observations on D-galactose inhibition of phlorizin binding in vivo (Table IV) and in vitro (Fig. 5). Moreover, since the interaction of 2-deoxy-D-glucose with the G receptor is more easily inhibited than D-galactose under identical conditions of phlorizin blockage, we expect this sugar to have only minimal inhibitory activity on [^{3}H]phlorizin binding at equivalent concentrations. This expectation is consistent with our observation that 2-deoxy-D-glucose shows minimal inhibition of [^{3}H]phlorizin binding in vitro at 125 mM and is not able to wash off [^{3}H]phlorizin in detectable quantities in vivo (when we attain estimated concentrations of only 100 mM).

The urine outflow curves for simultaneously filtered creatinine, [^{3}H]phlorizin and D-[^{14}C]fructose under identical conditions of phlorizin infusion are shown in

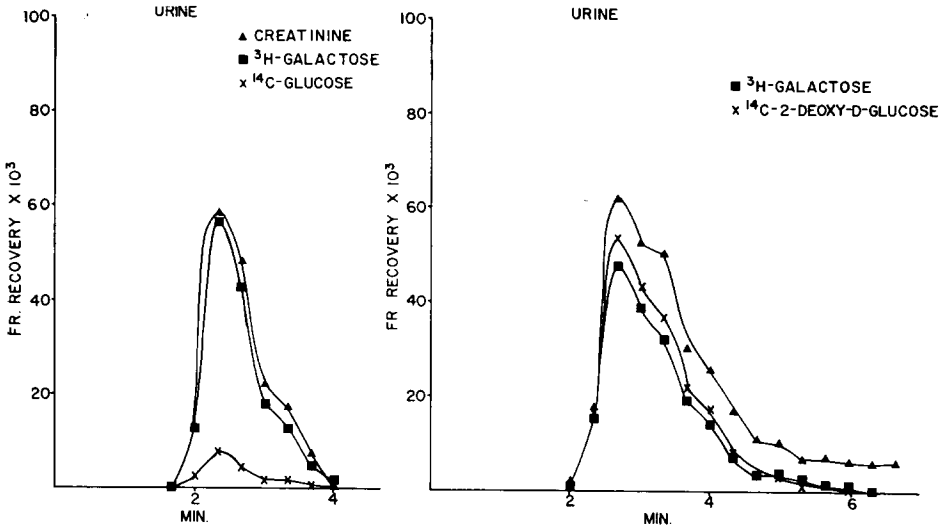

Fig. 9. Left hand panel shows urine outflow curves for simultaneously filtered creatinine, D-[^{3}H]-galactose and D-[^{14}C]glucose during systemic phlorizin infusion at 1.4 μg/kg body weight per min. Fractional reabsorption for galactose is reduced from 67 to 14 %. D-Glucose is reduced from 100 to 88 %. Right hand panel shows urine outflow curves for ^{3}H-labelled galactose and [^{14}C]2-deoxy-D-glucose relative to the glomerular marker creatinine during phlorizin infusion at 0.6 μg/kg per min. Fractional reabsorption of galactose is reduced from 67 to 40 % while that for 2-deoxy-D-glucose is reduced from 69 to 32 %.

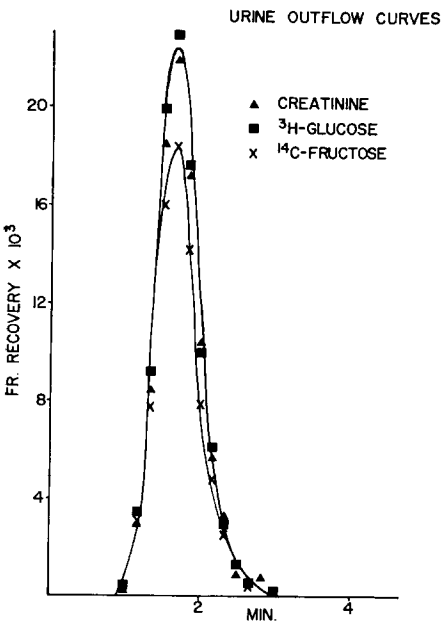

Fig. 10. Simultaneously urine outflow curves for creatinine D-[^{3}H]glucose and [^{14}C]fructose during infusion of phlorizin at 6.3 μg/kg per min. Fractional reabsorption of glucose is reduced from 100 to 0 % while fructose is reduced from 69 to 15 %, thus showing the presence of a non-phlorizin-sensitive fructose site.

Fig. 10. Inspection reveals that glucose reabsorption has been completely inhibited whereas 15–20 % of D-fructose is still extracted by the luminal surface. Thin-layer chromatography of urine effluents in the presence and absence of phlorizin shows that none of the filtered [^{14}C]fructose is recovered in urine as D-[^{14}C]glucose. We conclude from these experimental results that as in the case of myoinositol [3] fructose interaction with the luminal membrane occurs partially via the G receptor (45 % of the observed fractional reabsorption) and partially via a separate site which is insensitive to inhibition by low doses of phlorizin (accounting for 15–20 % of the observed fractional reabsorption). The existence of a fructose pathway independent of the G receptor is consistent with studies of fructose transport in the intestine [22, 23]. We conclude that the relative affinity of fructose for the G receptor is much less than for D-glucose and is similar to myoinositol. This explains why fructose does not inhibit high affinity phlorizin binding in vivo or in vitro.

If we summarize our results for the fractional reabsorption of radiolabelled sugars at the brush border G receptor sites as measured both in the presence and absence of phlorizin at the same plasma glucose concentration (100 mg%), we can list the interacting substrates in order of decreasing affinity for the glucose transport system: D-glucose = α-methyl-D-glucopyranoside > D-galactose > 2-deoxy-D-glucose > D-fructose = myoinositol.

DISCUSSION

We have demonstrated a set of high-affinity phlorizin binding sites in a brush border membrane preparation from dog kidney with a dissociation constant $K_d \approx 0.3$ μM. The number of receptor sites is $12 \cdot 10^{-12}$ mol/mg membrane protein. For a single red cell $1.3 \cdot 10^{-12}$ g dry weight [24] is distributed over a surface area of 135 μm^2 [25]. Assuming 50 % is protein we can calculate that there is $\approx 0.65 \cdot 10^{-12}$ g protein/100 μm^2 or ≈ 4800 molecules of phlorizin receptor per 100 μm^2 of brush border membrane.

The high affinity phlorizin sites have a well defined specificity of inhibition by sugar substrates. D-Glucose inhibits 40–50 % of high affinity phlorizin binding at concentrations as low as 5 mM and completely inhibits binding at 100 mM. Thus the estimated K_I for D-glucose of 5 mM is of the same order as the apparent K_m of the D-glucose transport system [27]. L-Glucose shows no inhibition up to concentrations of 600 mM. Most significant is the ability of D-glucose to displace [^{3}H]phlorizin from its high affinity sites both in vivo and in vitro. Also the fact that phloretin is at least 100 times less potent as an inhibitor of high affinity phlorizin binding than D-glucose, correlates with the relative efficacies of these two drugs in inhibiting glucose transport in vivo [28].

From the specificity data in Table III and Fig. 5 we deduce that the participation of the OH group at the carbon 1 position of D-glucose is not important in the inhibition of phlorizin binding both in vivo and in vitro, just as it is unimportant in the interaction of pyranosides with the glucose transport receptor [2, 3]. The inhibition of [^{3}H]phlorizin binding by 3-deoxy-3-fluoro-D-glucose is consistent with our hypothesis [2, 3] that pyranose interaction with the G receptor site involves hydrogen bonding at certain key hydroxyl (OH) groups, i.e. carbons 3 and 6 of D-glucose, since

replacement of OH by fluorine preserves the hydrogen bonding capacity at the carbon 3 position [29].

Phlorizin binding in vitro was found to be sodium dependent and sensitive to inhibition by a sulphydryl blocking agent. This does not necessarily mean, however, that an SH group is present at the binding site in vivo, but may simply reflect changes in the exposure of membrane proteins measured during isolation procedures [30]. Phlorizin binding was not affected by concanavalin A at concentrations varying from 100–1000 μg per 100 ml. Therefore if concanavalin A binds to renal brush border membranes this must occur at sites distant from the phlorizin receptor. Cytochalasin B in concentrations of 0.1–42 μM has no inhibitory effect on phlorizin binding. This correlates with the findings of Shin Lin et al. [31] on high affinity binding sites for cytochalasin on red cells. The cytochalasin sites seem to be identical to the phloretin-sensitive glucose transport mechanism in erythrocytes and can also be demonstrated on other mammalian cell membranes which have glucose transporting systems sensitive to phloretin but not phlorizin. In contrast to these systems, the glucose transporting system in kidney has much greater affinity for phlorizin than phloretin (by several orders of magnitude) and thus the ineffectiveness of cytochalasin in inhibiting phlorizin binding in our preparation is not surprising.

TABLE V

CORRELATION BETWEEN BRUSH BORDER PHLORIZIN RECEPTOR AND GLUCOSE TRANSPORT RECEPTOR

Sugar substrate	Inhibition of phlorizin binding in vitro[a]	Inhibition of phlorizin binding in vivo[b]	Relative affinity for phlorizin-sensitive glucose transport receptor
D-Glucose	100	100	100
α-Methyl-D-glucopyranoside	100	100	100
3-Fluoro-3-deoxy-D-glucose	80–100	N.T.	N.T.
D-Galactose	20–30	10	10–20
β-Methyl-D-galactopyranoside	20–30	N.T.	N.T.
2-Deoxy-D-glucose	0–10	0	≈ 10
2 Deoxy-D-galactose	0–10	0	N.T.
D-Fructose	0	0	<10[d]
Myoinositol	0	0	<10[d]
D-Mannose	0	0	0
L-Glucose	0	0	0
D-Xylose	0	0	0
3-*O*-Methyl-D-glucose	0	N.T.	0

[a] Sugar concentration 125 mM. Standard error of 20 % has been subtracted so that indicated values represent significant inhibition.

[b] Estimated sugar concentration ≈ 100 mM.

[c] Relative affinities have been estimated by calculating the ratio of percent decrease in fractional reabsorption of labelled glucose relative to labelled monosaccharide at equivalent dosage of phlorizin (see legends of Figs 12 and 13 and appropriate sections in text).

[d] Since fructose and myoinositol have alternate sites other than the G receptor, their relative affinity can be estimated only from the fractional reabsorption via the G receptor in the absence of phlorizin, i.e. 54 and 45 %, respectively, relative to 69 % for 2-deoxy-D-glucose.

N.T. Indicates substrates which have not been tested.

In order to emphasize the correlation between our in vivo and in vitro studies comparing the behaviour of the phlorizin receptor and glucose transport mechanism at the brush border we have compiled all of the pertinent information into a single table (Table V). It is apparent that in dog kidney the specificity of inhibition of high affinity phlorizin receptors by sugar substrates in vivo has been preserved in the isolated brush border membrane preparation in vitro. But, most important, there is an excellent correlation between the inhibition of phlorizin binding in vivo and in vitro by sugar substrates and their relative affinity for the glucose transport receptor at the brush border membrane of the proximal tubule in vivo.

At this point it is reasonable to compare our data with published studies on phlorizin binding to brush border preparations in rat and rabbit kidney. In the rat, the high affinity phlorizin binding was found in 2 studies to have a K_d of 0.2 μM [6] and 0.8 μM [7]. This compares favourably with the value of $K_d \approx 0.3$ μM for our dog preparation. As far as the specificity of inhibition by sugars of phlorizin binding in rat kidney our results coincide almost exactly with those of Glossmann and Neville [6]. The only exception is the behaviour of 2-deoxy-D-glucose and 2-deoxy-D-galactose. In our experiments, both of these sugar substrates inhibited [^{3}H]phlorizin binding only minimally at 125 mM. In ref. 6, 2-deoxy-D-galactose did not inhibit, whereas 2-deoxy-D-glucose did. We believe this discrepancy is due to the fact that the 2-deoxy derivatives have sufficiently low affinity for the glucose transport receptor that the experimentally observable inhibition of [^{3}H]phlorizin binding is variable and minimal at high concentrations $\geqslant$ 125 mM.

Chesney et al. [9] have recently studied a set of phlorizin receptor sites in rabbit kidney. These authors found a K_d of 8 μM for their phlorizin receptor, an order of magnitude greater than that found in the present study. Of even greater concern is their finding that phlorizin was displaced from its high affinity receptors in the rabbit by phloretin but not by D-glucose. We feel that these results indicate that Chesney et al. have been examining a nonspecific phlorizin binding site which is not related to the glucose transport mechanism. This fact becomes especially evident when one realizes that the range of phlorizin concentrations employed by these authors is 0.5–5000 μM. Using these concentrations, high affinity phlorizin binding was found to occur in the range $0.01 \cdot 10^{-9}$–$0.1 \cdot 10^{-9}$ mol/mg, i.e. 10–100 pmol/mg membrane protein. In contrast, examination of our data reveals that high affinity phlorizin binding occurs over the range 0-30 pmol/mg membrane protein.

Finally it is of interest to compare the amount of bound phlorizin per 100 g kidney associated with complete inhibition of glucose transport in vivo with a calculated total number of high affinity phlorizin receptor sites per 100 g kidney, using the experimental value of $12 \cdot 10^{-12}$–$40 \cdot 10^{-12}$ mol/mg membrane protein (this study and [6]). As already stated, there are $\approx 1.8 \cdot 10^5$–$7.2 \cdot 10^5$ receptor sites per proximal tubular cell. Assuming $3.5 \cdot 10^{10}$ proximal tubular cells/100 g kidney, there must be of the order of 0.03 μmol of high affinity phlorizin receptor per 100 g of kidney. Occupancy of all these sites should abolish glucose transport. Diedrich [26] has measured the amount of kidney bound phlorizin required to completely block glucose reabsorption under conditions of maximal glucose loading and has found this to be $\approx$ 0.6 μmol/100 g kidney, a factor of 30 greater than our estimate. This apparent discrepancy is really not as great as would appear at first sight. All membrane preparations contain "contaminating" protein (i.e. non brush border protein) and hence our

starting value of $10 \cdot 10^{-12}$–$40 \cdot 10^{-12}$ mol/mg membrane protein is certainly an underestimate. Moreover, there may also be some displacement of bound [^{3}H]-phlorizin during the dilution and washing steps of the millipore binding assay. This would also give us falsely low values for our estimate of the maximum number of receptor sites. On the other hand, Diedrich's value of 0.6 μmol is only an upper limit because the kidney content of phlorizin which he measured probably includes a significant portion of drug which is not bound to the specific glucose transport site. Moreover, his value for kidney bound phlorizin must be corrected for extracellular phlorizin content. Because his experiments were carried out under glucose loading conditions, i.e. with plasma glucose concentrations of $\approx$ 600–700 mg %, the amount of intratubular non-bound phlorizin (extracellular phlorizin) may be much higher than his original estimate of 2–4 % (because of competition for site occupancy).

In conclusion, on the basis of the studies presented in this paper we feel that the phlorizin receptor is either identical to or part of the glucose transporter exposed at the urine (exterior) face of the brush border membrane of the proximal tubule in dog kidney and that according to our specificity criteria it has been isolated intact in the membrane preparation. This system can now be employed in further studies to elucidate the molecular mechanisms involved in glucose transport.

ACKNOWLEDGEMENTS

This work was supported by the Medical Research Council of Canada, MA 4590, and the Atkinson Charitable Foundation. The authors would like to acknowledge the invaluable suggestions of Drs H. Glossmann and D. Diedrich as well as Dr Phil Seeman for reviewing the manuscript. The authors are also indebted to S. McGugan for excellent typing, A. Chatzilias for technical assistance and C. Lumsden for helpful discussions. Preparation of samples for electron microscopy was carried out by M. Betlem, from the Histology Department, University of Toronto.

REFERENCES

1 Silverman, M., Aganon, M. A. and Chinard, F. P. (1970) Am. J. Physiol. 218, 735–742
2 Silverman, M., Aganon, M. A. and Chinard, F. P. (1970) Am. J. Physiol. 218, 743–750
3 Silverman, M. (1974) Biochim. Biophys. Acta 332, 248–262
4 Silverman, M. (1974) Biochim. Biophys. Acta 339, 92–102
5 Frasch, W., Frohnert, P. T., Bode, F., Baumann, K. and Kinne, R. (1970) Pflugers Arch. 320, 265–283
6 Glossmann, H., Neville, Jr, D. M. (1972) J. Biol. Chem. 247, 7779–7789
7 Bode, F., Baumann, K. and Diedrich, D. F. (1972) Biochim. Biophys. Acta 290, 134–150
8 Vick, H., Diedrich, D. F. and Baumann, K. (1973) Am. J. Physiol. 224, 552–577
9 Chesney, R., Sactor, B. and Kleinzeller, A. (1974) Biochim. Biophys. Acta 332, 263–277
10 Heidrich, H.-G., Kinne, R., Kinne-Saffran, E. and Hannig, K. (1972) J. Cell Biol. 54, 232–245
11 Wilfong, R. F. and Neville, Jr, D. M. (1970) J. Biol. Chem. 245, 6106–6112
12 Bessey, O. A., Lowry, O. H. and Brock, M. J. (1946) J. Biol. Chem. 164, 321–329
13 Dahlqvist, A. (1964) Anal. Chem. 7, 18–25
14 Pennington, R. J. (1961) Biochem. J. 80, 649–654
15 Hubscher, G. and West, R. G. (1965) Nature 205, 799–800
16 Post, R. A. and Sen, A. K. (1967) Method. Enzymol. 10, 762–769
17 Gornall, A. G., Bardawill, C. J. and David, M. M. (1949) J. Biol. Chem. 177, 751–776
18 Lowry, O., Rosenbrough, N. J., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275

19 Chinard, F. P., Taylor, N. R., Nolan, F. and Enns, T. (1959) Am. J. Physiol. 196, 535
20 Silverman, M. (1973) J. Clin. Invest. 52, 2486–2494
21 Scatchard, G. (1949) Ann. N.Y. Acad. Sci. 51, 660–679
22 Gracey, M., Burke, V. and Oshin, A. (1971) Biochim. Biophys. Acta 266, 397–406
23 Guy, M. J. and Deren, J. J. (1971) Am. J. Physiol. 221, 1051–1056
24 Dodge, J. T., Mitchell, C. and Hanahan, D. (1963) Arch. Biochem. Biophys. 111, 119–130
25 Seeman, P., Kwant, W. O. and Sauks, T. (1969) Biochim. Biophys. Acta 183, 499–511
26 Diedrich, D. R. (1966) Am. J. Physiol. 211, 581–587
27 Baeyer, H. V., Von Carta, C., Haeberle, D. and Deetjen, P. (1973) Pflugers Arch. 343, 273–286
28 Chan, S. S. and Lotspeich, W. D. (1962) Am. J. Physiol. 203, 975–979
29 Barnett, J. E. G. (1967) Adv. Carbohydr. Chem. 22, 177–227
30 Ullrich, K. J., Fasold, H., Kloss, S., Rumrich, G., Salzer, M., Stao, K., Simon, B. and de Vries, J. (1973) Pflugers Arch. 344, 51–68
31 Lin, S. anb Spudich, J. A. (1975) J. Biol. Chem., in the press