Biochimica et Biophysica Acta, 339 (1974) 202–209

BBA 76566

EVIDENCE FOR A SINGLE KIND OF D-GLUCOSE BINDING SITE ON RENAL BRUSH BORDERS

ROBERT J. CHERTOK and SUZANNE LAKE

Bio-Medical Division, Lawrence Livermore Laboratory, University of California, Livermore, Calif. 94550 (U.S.A.)

(Received September 24th, 1973)

SUMMARY

The binding of D-glucose to renal brush border was studied in a preparation isolated from rabbit kidney. The data indicate only one kind of binding site for D-glucose. The initial rate of binding was too rapid to be determined either directly or by extrapolation to zero time. Estimations of the amount of D-glucose bound at equilibrium for various concentrations of D-glucose yielded a straight line in a kinetic plot. This indicated a single binding site on the brush borders with a K_{diss} of $4.8 \cdot 10^{-5}$ M and a binding capacity of 264.8 pmoles/mg of protein. This conclusion is further supported by demonstration of a requirement for Na^+ at two different concentrations of glucose, 1 and 100 μM, in the presence of Tris buffer. This binding was inhibited by other sugars and by phlorizin indicating binding to a specific site.

INTRODUCTION

It is generally accepted that glucose is accumulated in the proximal tubular cells of mammalian nephrons, but investigation of this accumulation has been difficult due to the rapid rate of glucose catabolism. Our recently published method [1] for the isolation of renal brush borders makes it possible to look for evidence of glucose binding by these cell fragments. Such binding could be the first step in glucose transport by the renal proximal tubule.

Two reports in the literature assert that there are two kinds of binding sites on the renal brush border [2, 3], defined by differences in kinetic values, Na^+ dependence, and inhibition by sugars. Our work appears to contradict these conclusions, since only one kind of binding site for D-glucose can be demonstrated in our renal brush border preparations. This binding is Na^+-dependent and is specific for D-glucose, since it can be inhibited by various sugars and phlorizin.

METHODS

Three kinds of experiments were done: (1) time studies with labeled sugars to determine the rate and specificity of glucose binding by brush border fragments,

(2) studies of Na^+ dependence of the binding, and (3) studies of inhibition of glucose binding by other sugars and phlorizin.

Rabbit renal brush borders were isolated by the method reported earlier [1] except that the last wash in 90 mM NaCl, 5 mM EDTA was omitted. The pellet was resuspended in a buffer solution composed of 0.01 M Tris, 0.032 M $MgCl_2$ and 0.1 M NaCl (pH 7.3). For the Na^+-dependence studies, the pellet was suspended and sometimes washed in the same buffer solution minus the NaCl.

The reaction mixtures for the time-course experiments contained the volume of D-[^{3}H]glucose (spec. act. 8.65 Ci/mmole) necessary to make the reaction mixture either 0.65 or 1.3 μM in D-glucose; the amount of L-[^{14}C]glucose (spec. act. 3 mCi/mole) required to give a counting ratio, ^{3}H to ^{14}C, of 2 to 4, unlabeled glucose, added if necessary to give higher concentration of substrate, the buffer solution, and the brush border suspension. The labeled L-glucose was added to check for the presence of nonspecific binding.

The reaction mixtures for the Na^+-dependence studies were the same except that the NaCl-free buffer was used, with NaCl added to give each specified concentration of Na^+; all mixtures were brought to identical salt concentrations by the addition of KCl.

For the sugar inhibition studies, the reaction mixtures were the same as for the time studies. The test sugar or phlorizin was added to a concentration 500 times higher than that of D-glucose. When D-glucose was used as the inhibitor, it was only 55 times higher than that of the substrate D-glucose.

All reactions were started by the addition of brush border suspension after the substrate mixture and the brush border suspension had been incubated separately at 37 °C. All reactions were run at 37 °C.

For each observation, a 0.5-ml aliquot was taken from the reaction mixture at the prescribed time and pipetted into a Millipore receptable containing 5 ml of the buffer solution (buffer solution minus NaCl in the Na^+-dependence studies). This solution was immediately filtered through a 3-μm Millipore filter. The filter was then washed with another 5 ml of buffer solution and placed in a counting vial. Filter standard, run with each experiment, were reaction mixtures with the brush border suspension replaced by buffer solution.

Each filter was dissolved in 10 ml of Aquasol (New England Nuclear) and analyzed for ^{3}H and ^{14}C on a Nuclear-Chicago liquid scintillation counter. Bound D-glucose in pmoles was calculated from the ^{3}H activity of the filter, i.e. from the number of ^{3}H dpm, by reference to counting standards. The counting efficiency was approximately 18% for ^{3}H and approximately 25% for ^{14}C.

Protein was estimated by the method of Lowry as presented in ref. 4.

RESULTS AND DISCUSSIONS

Several recently published reports on glucose binding to isolated renal brush borders [2, 3] assert that two kinds of binding sites of D-glucose could be detected in their brush border preparations. One site had high affinity but low capacity for glucose; the other had low affinity but high capacity for glucose. The high-affinity site was Na^+ dependent [2], the low affinity site was Na^+ independent. The Na^+ dependence at the high affinity site could not be demonstrated by Chesney et al. [3].

Our first attempts to reproduce these experiments with our renal brush border preparation appeared to give similar results. Fig. 1 shows a double reciprocal plot of D-glucose bound per mg of protein at 2 min versus initial glucose concentration. A definite break in the line indicates two binding sites, one with $K_m = 6 \cdot 10^{-6}$ M and $V = 5.8 \cdot 10^{-12}$ moles/mg protein, and the other with $K_m = 180 \cdot 10^{-6}$ M and a $V = 101 \cdot 10^{-12}$ moles/mg protein. This would seem to support the data of Busse et al. [2], although the break in the line occurred at a different concentration of glucose. In subsequent experiments, however, the points for the high-affinity site did not consistently give a straight line but rather one that tended to curve downward, suggesting that it was not the true initial velocities that were being plotted.

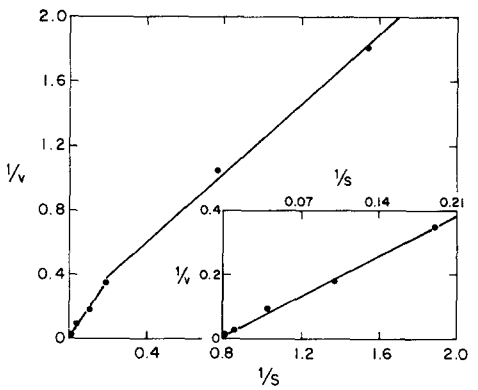
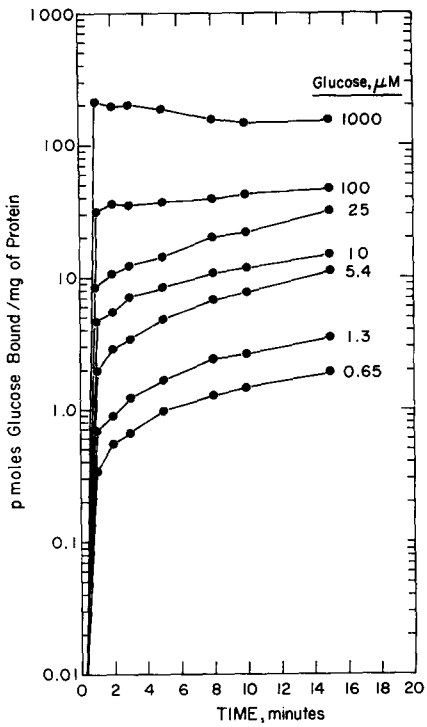


Fig. 1. Double-reciprocal plot of bound D-glucose expressed as the pmoles/mg protein per 2 min versus the substrate concentration expressed in μM; $n = 5$. Inset: expanded view of binding at high concentrations of glucose.

Several time studies at different concentrations of D-glucose gave the results shown in Fig. 2. It became obvious that the rates of binding were very rapid and that the true initial velocity for each concentration studied occurred before 1 min. The time studies were then repeated at shorter time intervals in an attempt to obtain initial velocities. Fig. 3 shows some of the results. It is clear that at concentrations of glucose higher than 1.3 μM the initial velocities are too high to be estimated with any degree of accuracy by the methods used.

An alternative method for studying the binding of D-glucose to renal brush borders is to determine binding at equilibrium. Since the amount of glucose bound appeared to approach equilibrium with time and initial velocity measurements could not be obtained over a sufficiently wide range of glucose concentrations, it seemed reasonable to estimate the amount of glucose bound for each initial concentration of glucose as time approached infinity, that is, to plot $1/t$ versus $1/GB$, GB representing the amount of D-glucose bound per mg of protein. The values obtained at $1/t = 0$ were assumed to represent equilibrium binding. Thus, if the equilibrium constant, K_{diss}, is defined by

$$K_{diss} = \frac{[G]_f\,[B]_f}{[GB]} \tag{1}$$



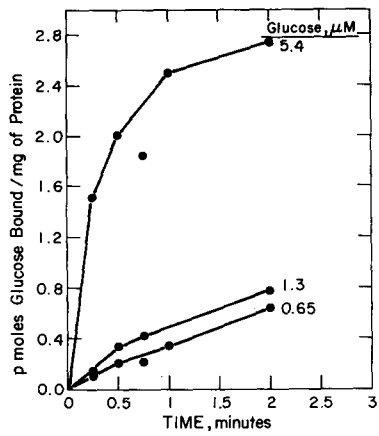


Fig. 2. Time-course of the binding of D-glucose to renal brush border at several concentrations of D-glucose; $n = 5$.

Fig. 3. Time course of the binding of D-glucose to renal brush border, showing the rapid rate of binding; $n = 3$.

where $[G]_f$ is free D-glucose, $[B]_f$ is free D-glucose binding sites on the brush borders, and $[GB]$ is the brush border–glucose complex, then both the equilibrium constant and the number of binding sites can be obtained by plotting $1/[GB]$ versus $1/[G]$ as shown in Eqn 2:

$$\frac{1}{[GB]} = \frac{K_{diss}}{[B]} \frac{1}{[G]} + \frac{1}{[B]} \tag{2}$$

This method is discussed in more detail in a review by Hughes and Klotz [5].

The data in Fig. 2 suggest that the amount of glucose bound is very small compared to the total amount of glucose present. Therefore, $[G]_f$ is considered to be equivalent to $[G]$ in the derivation of Eqn 2 from Eqn 1.

A plot of $1/[GB]$ versus $1/[G]$ is shown in Fig. 4. The straight line indicates that the binding sites on the brush borders in question are equivalent. The value for $[B]$ is 264.8 pmoles/mg protein and K_{diss} is $4.8 \cdot 10^{-5}$ M.

The K_{diss} was calculated for each concentration of glucose studied using the value of $[B]$ obtained from Fig. 4. The average K_{diss} value of $4.7 \cdot 10^{-5}$ M showed a standard deviation of $\pm 1.4 \cdot 10^{-5}$.

In a recent report, Chesney et al. [3] showed two kinds of binding sites using a Scatchard plot [5] to evaluate their data at 1 min. If our data taken at 2 min are plot-

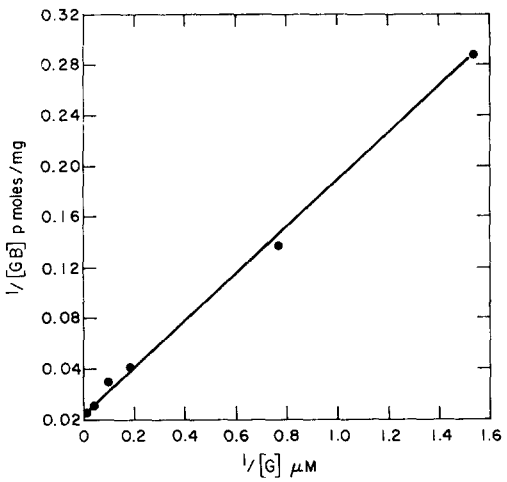


Fig. 4. Double reciprocal plot of the equilibrium binding of D-glucose (pmoles/mg of protein) at several concentrations of glucose; $n = 3$–5.

ted according to Scatchard, a very similar line is obtained, showing two apparent sites (Fig. 5). Scatchard plots and Klotz [6] plots were derived for use with equilibrium binding data. Our treatment of the data is consistent with the mathematical limitations of the equations used. A Scatchard plot of our estimated equilibrium data give a straight line (correlation coefficient = 0.903 with $K_0 = 1.9 \cdot 10^{-5}$ and

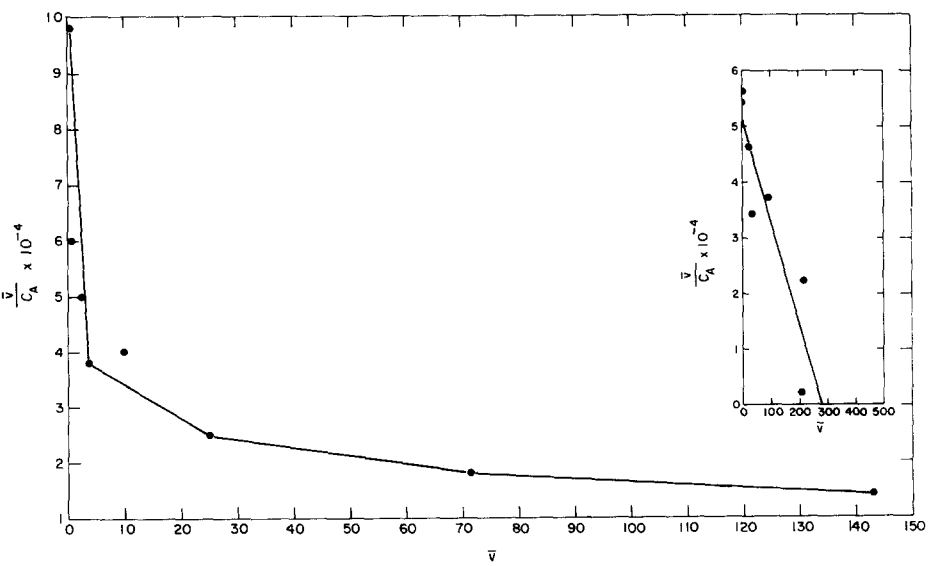


Fig. 5. A Scatchard plot showing the relationship between the concentration of D-glucose and the amount of glucose bound at 2 min; $\bar{v} = [GB]$ or pmoles of glucose bound per mg protein; $C_A = [G]$ or concentration of glucose. The inset shows the relationship between the concentration of D-glucose and the amount of glucose bound at equilibrium.

$n = 275.8$ pmoles/mg protein (Fig. 5, inset)). These data are in reasonable agreement with the values calculated from Fig. 4.

TABLE I

K_{diss} CALCULATED FOR EACH CONCENTRATION OF D-GLUCOSE AND FROM THE SLOPE OF A LINE RESULTING FROM PLOTTING THE ESTIMATED AMOUNT OF D-GLUCOSE BOUND AT EQUILIBRIUM VERSUS INITIAL CONCENTRATION OF D-GLUCOSE (FIG. 4)

Substrate glucose (μM)	K_{diss}* ($M \times 10^{-5}$)	K_{diss}** ($M \times 10^{-5}$)
0.65	4.9	4.8
1.3	4.5	
5.4	5.2	
10.0	6.8	
25	4.7	
100	2.3	

* Calculated from Eqn 2 using the value of $[B]$ obtained from Fig. 4 (264.8 pmoles/mg protein).

** Calculated from the slope of line in Fig. 4.

TABLE II

EFFECTS OF SUGARS AND PHLORIZIN ON THE BINDING OF D-GLUCOSE BY RENAL BRUSH BORDERS

Labeled glucose (substrate) was 2 μM; reaction time was 1 min; $n = 6$.

Sugar (1 mM)	% Inhibition
Phlorizin	80.7
D-Glucose (110 μM)	96.0
3-*O*-Methyl-D-glucose	85.1
D-mannose	82.8
2-Deoxy-D-glucose	80.4
D-Arabinose	25.9
D-Ribose	20.4
D-Fructose	18.8
D-Galactose	54.3

It can be concluded from these data that there is only one kind of binding site for D-glucose on the renal brush borders studied; this conclusion is consistent with the published data for intestinal brush borders [7, 8].

Frasch et al. [9] studied the inhibition of phlorizin binding by D-glucose to brush borders isolated from rat kidneys. Their data demonstrate that two receptor sites for phlorizin exist within the membrane fraction. The low-affinity site is unaffected by D-glucose; the high affinity site is inhibited by D-glucose. Their study also indicates that D-glucose affects phlorizin binding only by competing for the common receptor site but not by affecting the affinity between receptor and phlorizin through combination with a different binding site.

It is well known that brush border preparations tend to vesiculate spontaneously and that glucose transport into these vesicles can be misinterpreted as glucose binding. However, our studies clearly demonstrate glucose binding to a receptor site, as indicated by; (1) the extremely rapid initial rate of binding, (2) the kinetic data indicating only one uptake system and (3) the inhibition of D-glucose binding by phlorizin, a nonpenetrating glycoside.

Requirement for Na^+ was demonstrated at two concentrations of D-glucose, 1 μM and 100 μM. If the brush borders were not washed with Na^+-free buffer prior to the experiment, the preparation contained up to about 5 mM Na^+; the data at 1 μM glucose were obtained under these conditions as shown in Fig. 6B. When the brush border preparation was washed in the Na^+-free buffer several times with extreme precautions, the concentration of Na^+ could be reduced to 0.03–0.04 mM, the data for 100 μM glucose were obtained under these conditions (Fig. 6A). The amount of D-glucose bound at 15 mM Na^+ differs significantly from that bound at 5 mM Na^+ at $\rho \leqslant 0.05$ by the two-sample rank test [10]. The amount of D-glucose bound at 17 mM Na^+ differs significantly from that bound at 0.03–0.04 mM (zero on the abscissa) at $\rho \leqslant 0.025$. An increase in the concentration of Na^+ beyond 15–17 mM did not significantly increase the degree of binding. The Na^+ dependence demonstrated at these two concentrations of glucose supports the conclusion that there is only one binding site.

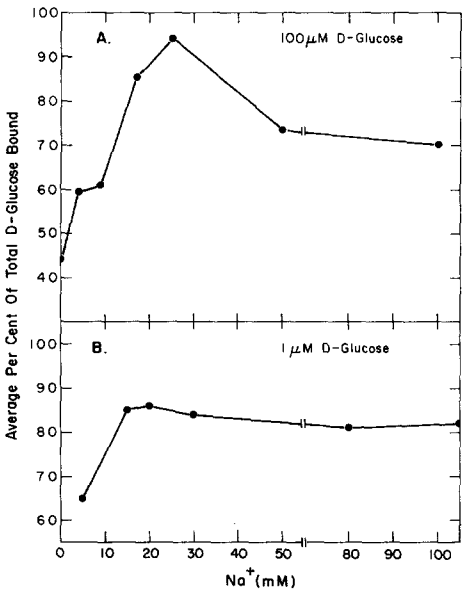


Fig. 6. Effect of [Na^+] on the binding of D-glucose to renal brush borders: (A) 100 μM glucose, brush borders washed at least two times in Na^+-free buffer, $n = 6$. (B) 1 μM glucose, brush borders were not washed in Na^+-free buffer, $n = 7$.

It was suggested by Chesney et al. [3] that the Na^+ dependence observed for the high-affinity site by Busse et al. [2] was the result of using Tris to replace the Na^+ in the medium. They could demonstrate that Tris inhibited the binding in the absence

and presence of Na^+. Tris was always present as the buffer in our reaction mixtures. However Na^+ did not exert a significant effect on the binding until the concentration was at least 15 mM which is consistent with the published data for intestinal brush borders [11].

The binding of D-glucose is inhibited by other sugars and phlorizin, (Table II), thus supporting the evidence for a specific site.

CONCLUSIONS

The initial rate of D-glucose binding to renal brush borders is too rapid to be measured by the usual technique of extrapolation to zero time. Determination of binding at equilibrium yields a straight-line slope, indicating a single kind of binding site for D-glucose on the renal brush borders studied. This conclusion is supported by a demonstration of requirement for Na^+ at two different concentrations of D-glucose. This binding was inhibited by other sugars and by phlorizin, also indicating binding to a specific site.

ACKNOWLEDGMENTS

The authors thank Dr Thomas E. Smith for his many helpful suggestions and Joseph Serpa, Jr, for his technical assistance. This work was performed under the auspices of the U.S. Atomic Energy Commission. Reference to a company or product name does not imply approval or recommendation of the product by the University of California or the U.S. Atomic Energy Commission to the exclusion of others that may be suitable.

REFERENCES

1 Chertok, R. J. and Lake, S. (1972) J. Cell. Biol. 54, 426–430
2 Busse, D., Elsas, L. J., and Rosenberg, L. E. (1972) J. Biol. Chem. 247, 1188–1193
3 Chesney, R. W., Sactor, B. and Rowen, R. (1973) J. Biol. Chem. 248, 2182–2191
4 Layne, E. (1957) in Methods in Enzymology (Colowick, S. P. and Kaplan, N. O., eds), Vol. 3, pp. 448–450, Academic Press Inc., New York
5 Hughes, T. R. and Klotz, M. (1956) in Methods of Biochemical Analysis (Glick, D., ed.), Vol. III, pp. 288–299, Interscience Publishers, Inc., New York
6 Scatchard, G., Scheinberg, I. H. and Armstrong, S. H., Jr. (1950) J. Am. Chem. Soc. 72, 540–546
7 Klotz, I. M., Walter, F. M. and Pivan, R. B. (1946) J. Am. Chem. Soc. 68, 1486–1490
8 Crane, R. K. (1962) Fed. Proc. 21, 891–895
9 Faust, R. G., Leadbetter, M. G., Plenge, R. K. and Mc Caslin, A. J. (1968) J. Gen. Physiol. 52, 482–494
10 Frasch, W., Frohnert, P. P., Bode, F., Baumann, K. and Kinne, R. (1970) Pflügers Arch. 320, 265–284
11 Goldstein, A. (1964) in Biostatistics, An Introductory Text, pp. 55–56. The Macmillan Company, New York
12 Faust, F. G., Shearin, S. J. and Misch, D. W. (1972) Biochim. Biophys. Acta 255, 685–690