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STUDIES ON THE ORGANIZATION OF THE BRUSH BORDER IN INTESTINAL EPITHELIAL CELLS

VI. GLUCOSE BINDING TO ISOLATED INTESTINAL BRUSH BORDERS AND THEIR SUBFRACTIONS

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SUMMARY

A sensitive method for the measurement of specific glucose binding to isolated hamster intestinal brush borders has been developed. The method is based on the difference in binding of L-[^{14}C]glucose and D-[^{3}H]glucose as measured by retention of radioactivity on Millipore filters. Properties of the binding have been studied. Binding appears to be localized to brush borders. It exhibits a specificity towards a variety of test sugars which is different from the specificity of intestinal active transport. The K_m of binding is 2 μM. Binding to intact brush borders shows no requirement for Na^+. Binding is inhibited by Ca^{2+}, Co^{2+}, Fe^{2+}, and Zn^{2+} and activated by Mg^{2+} and Mn^{2+}. Binding to brush borders is highest in the distal portion of the small intestine. The distribution of binding among subfractions of the brush border has been studied. Possible interpretations of this binding are discussed.

INTRODUCTION

Isolation and purification of molecules involved in membrane transport processes by the usual biochemical techniques is made difficult by the disruptive nature of the methods themselves, since the assay methods for membrane transport require maintenance of compartmentalization. Attempts to isolate transport systems have been made, however, by relying on the property of specific binding by membrane molecules of substrates themselves or their competitive inhibitors. Recently, a number of variations of the substrate binding approach have been used to study membrane-localized binding sites in isolated subcellular preparations containing both lipid and protein components of the membrane. Up to date reviews by PARDEE[1] and STEIN[2] discuss some attempts applied to bacterial and mammalian membranes. Investigators attempting to isolate carbohydrate transporting systems in mammalian tissues have largely used erythrocyte membranes, which though they possess a specific carrier system, do not accumulate sugar against the concentration gradient in a process of "active transport". Isolation of the intestinal brush border has opened the way to study the subcellular structures involved in active transport and absorption.

The brush border possesses two functions with respect to carbohydrates; namely, terminal digestion, since it is the site of localization of hydrolytic enzymes and active transport. Consequently, it can be regarded as an organelle concerned with the integration of these functions. Binding of glucose to this organelle has been recently reported by FAUST *et al.*[3,4]. Similarly, DIEDRICH[5] has reported the binding of phlorizin. Since we have separated the brush border into subfractions[6], it was of interest to us also to investigate the specific binding of D-glucose. In order to be able to do so on small amounts of material recoverable, especially from subfractions, a sensitive and reproducible binding assay system had to be developed. This paper describes the method of assay, the properties of binding, the distribution of binding among brush border subfractions, and some further purification of the binding component. Preliminary reports of some of the data have been previously presented[7,8].

METHODS

Hamster brush borders were prepared by our usual procedure[6] using Waring Blendor disruption of mucosal scrapings in 0.005 M EDTA. This procedure swells and disrupts plasma and nuclear membranes leaving the brush border as the largest cellular substructure in the homogenate. It can be subsequently easily recovered by differential centrifugation. The isolated and purified brush borders were taken up in 50 mM Tris buffer at pH 7.4 containing 5 mM $MgCl_2$, since Mg^{2+} has been shown to increase glucose binding. In a typical experiment, 0.5 mg of brush border protein in 1 ml of the buffer was added to a mixture of radioactive D-[^{3}H]glucose and L-[^{14}C]glucose and incubated at 37° for 15 min. The concentration of glucose used varied with the experiment from 5 to 0.1 μM with 100000–10000 counts/min in the sample. However, in some experiments as indicated 1 mM to 0.1 μM glucose has been used. An attempt was made to keep both the counts/min, ^{14}C and ^{3}H, and the molar concentrations of D- and L-glucose about equal in each experiment.

After completion of incubation, the sample was removed from the water bath and passed through a previously wetted Millipore filter (SM 5 μ Millipore Filter Corp., Bedford, Mass.) under suction. The filtrate was collected directly into a scintillation vial for counting. The vial was exchanged, and the filtered material was quickly washed with 1 ml of the buffer. The filter was then removed and placed on the bottom of a third scintillation vial to which 1 ml of buffer was added. All samples were then counted as described below. All experiments were done at least in duplicate. Experience showed that following 15 min of incubation about 9% of the total counts were to be found in the brush border fraction on the filter, 14% in the wash and the remainder in the filtrate. The ratio of D-glucose/L-glucose, *i.e.*, ^{3}H/^{14}C, in the wash was identical to that in the original reaction mixture, whereas this ratio in the brush border fraction on the filter was many times higher.

Counting

The method of PATTERSON AND GREEN[9] was used for scintillation counting. The scintillation mixture contained 3 g of 2,5-diphenyloxazole, 100 mg of 1,4-bis-[2-(4-methyl-5-phenyloxazolyl-2)]-benzene per l of toluene. For counting, 2 parts of this solution were mixed with 1 part of Triton X-100 (Rohm and Haas). Each 1-ml sample was shaken with 10 ml of scintillation solution prior to counting.

Determination of counts bound in a binding experiment

The number of counts bound was calculated by first determining the distribution of D-[^{3}H]glucose (D) and L-[^{14}C]glucose (L) in each sample and in the original reaction mixture. The ratios of glucose for each sample (D_S/L_S) and the reaction mixture (D_{RM}/L_{RM}) were then calculated. From these ratios, it is then possible to calculate the expected amount of D-glucose (X) to be found in each sample using L-glucose as a control to correct for non-specific binding and entrapment.

$$\frac{D_{RM}}{L_{RM}} = \frac{X}{L_S}$$

Subtracting this result from the total D-glucose determined in the sample, we obtained the counts of D-glucose specifically bound.

$D_S - X =$ D-glucose bound specifically

Radioactive compounds

Various combinations of the radioactive D- and L-glucose compounds given below were used in binding experiments with identical results. In some experiments mannitol was used in place of L-glucose. L-[^{3}H]Glucose was prepared by the Wilzbach technique and purified by paper chromatography in our laboratory. L-[1-^{14}C]Glucose (0.533 mC/mmole), D-[1-^{14}C]glucose (10 mC/mmole), D-[$^{14}C_6$]glucose (110 mC/mmole), D-[1-^{3}H]glucose (3465 mC/mg) and D-[1-^{14}C]mannitol (0.5 mC/mg) were purchased from New England Nuclear.

Column chromatography

Ion exchange columns. In order to check the identity of the radioactivity after binding, a large scale binding experiment was carried out. Brush borders were prepared from four hamsters and incubated with radioactive glucose under conditions described above. At the end of the incubation the mixture was subjected to centrifugation at 600 × *g* for 10 min and the supernatant decanted. To the precipitate, 3 ml of distilled water were added and the sample was homogenized in a Potter–Elvehjem homogenizer and placed in a boiling water bath for 10 min. The sample was then again washed, homogenized and applied to a 1 cm × 17 cm (Dowex 2-X8) column in the formate form.

The column was first eluted with water and subsequently with 1 M sodium formate as shown under RESULTS.

Sephadex G-10 columns. For chromatography on Sephadex G-10, 1 cm × 45 cm columns prepared by equilibration with 50 mM Tris buffer at pH 7.4 were used. 1 ml of the sample was applied at room temperature to the column and the column was eluted with 50 mM Tris.

Binding of glucose to everted sacs with subsequent fractionation

An everted sac approx. 5 cm in length was prepared from hamster jejunum. The sac, washed successively in three beakers containing saline, was tied at both ends to prevent the fluid from entering the serosal side. It was then placed in an incubation medium containing 9 ml of Tris buffer (pH 7.4) and 1 ml radioactive mixture with 10 μM glucose and 1 mM 2,4-dinitrophenol. The tissue was incubated for 10 min at 37°,

rinsed in saline to remove adhering medium and quickly scraped to obtain mucosa from which brush borders were then rapidly prepared.

The supernatant from the brush border precipitates was subjected to differential centrifugation at 2000 × *g*, 9000 × *g* and 19000 × *g*. Samples were withdrawn and counted.

Protein determination and enzyme assays

Disaccharidase activities were measured as described by DAHLQVIST[10]. Alkaline phosphatase was measured by the rate of splitting of *p*-nitrophenyl phosphate. Protein was estimated by the procedure of LOWRY *et al.*[11].

RESULTS

In order to establish the properties of the binding, we first undertook experiments on whole undisrupted brush borders. The binding was found to be directly related to the amount of brush border protein in the incubation mixture (Fig. 1). In routine experiments, brush borders totaling 0.5 mg of protein were used in the incubation mixture. To differentiate specific binding from solubilization in micelles or lipid membrane components, pH and temperature characteristics were investigated. As can be seen from Fig. 2, glucose binding exhibited a pH optimum between pH 7 and 8 when the binding was carried out in buffer containing acetate, imidazole,

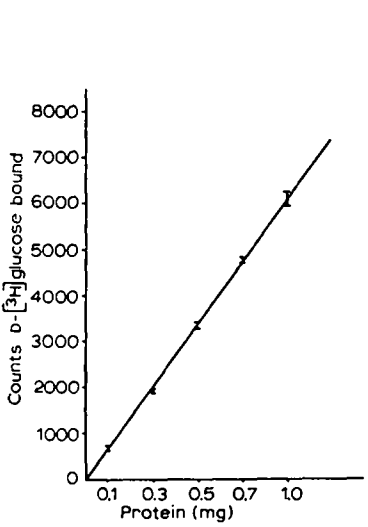


Fig. 1. Dependence of counts bound on amount of brush borders as measured by protein. Counts are determined as described within the text.

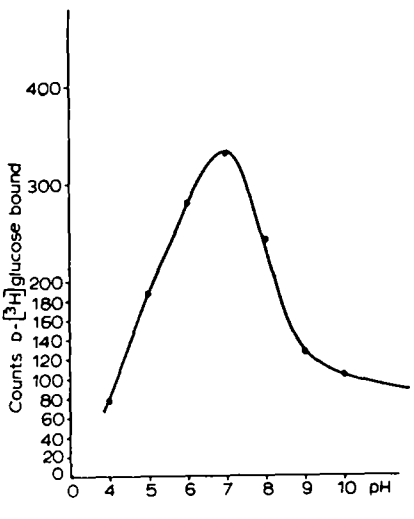
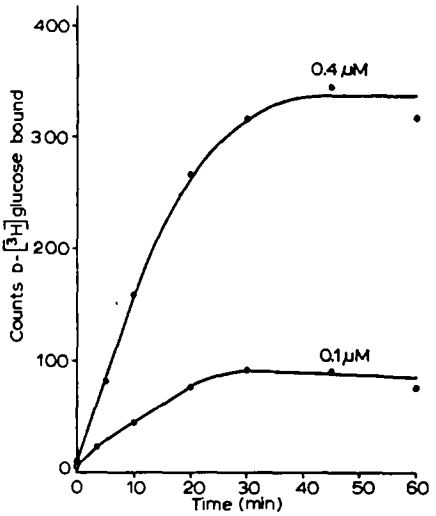


Fig. 2. Dependence of binding on pH.

Tris and glycine. Binding was also related to temperature, time and concentration of glucose. Binding occurred between 15 and 50°, with an optimum at about 37° and Q_{10} of about 2. Boiling destroyed all binding capacity. Binding increased with time and reached a maximum in about 30–40 min as seen in Fig. 3. 15-min incubation

periods were routinely used in subsequent experiments. Binding capacity was labile and Fig. 4 shows the course of inactivation of a brush border preparation stored at 0° and tested over a period of 9 days.



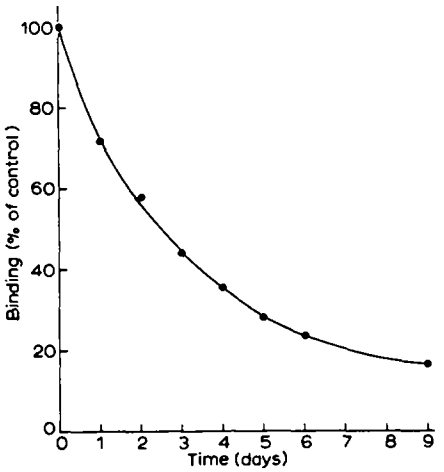
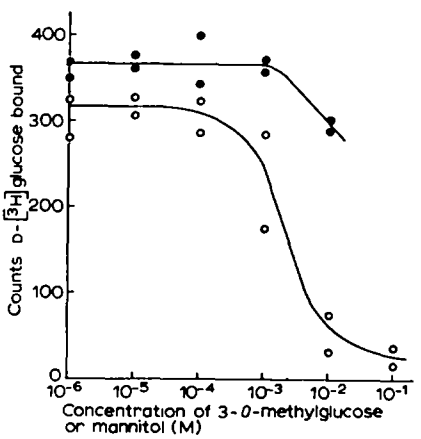


Fig. 3. Binding capacity *vs.* time of incubation.

Fig. 4. Loss of binding capacity with time. Brush border preparation was stored at 0°.

Binding appeared to be specific to the brush border. When an identical binding experiment was carried out using a (HA 0.45 μ) Millipore filter with the remainder of the homogenate after removal of the brush borders, no binding could be detected even at higher protein concentrations than those used in the experiments with brush borders. No activity was found when an insoluble protein such as casein was used for binding.



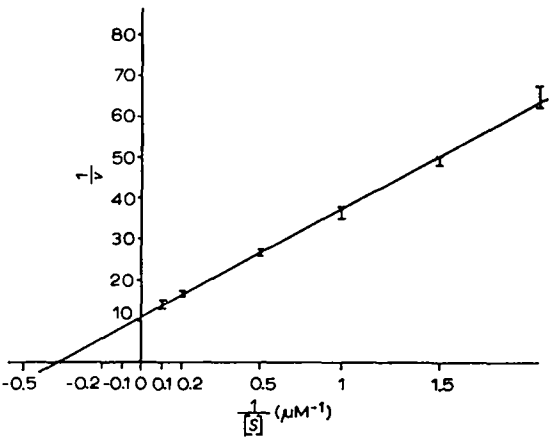


Fig. 5. Inhibition of binding of D-glucose (1 μM) by various concentrations of 3-*O*-methylglucose (O—O) and mannitol (●—●).

Fig. 6. Lineweaver–Burk plot of D-glucose binding.

Since L-glucose has been recently shown to be actively transported, although with a comparatively high K_m, namely 65 mM (ref. 12), two simultaneous binding experiments with D-glucose were carried out using D-mannitol and L-glucose as controls to exclude entrapment and non-specific binding. Within experimental error, the number of counts bound using either mannitol or L-glucose were identical. In the presence of L-glucose mannitol was also tested as an inhibitor of D-glucose binding. As can be seen in Fig. 5 there is no inhibition of binding below 0.01 M mannitol. In addition, binding of D-glucose was observed in presence of varying concentrations of L-glucose from 50 nM to 0.01 M. Over this concentration range no variation in D-glucose counts bound could be observed; L-glucose and D-glucose appear not to compete for the binding under study.

The K_m for binding was about 2 μM as seen from Fig. 6. The binding was reversible with high concentrations of cold glucose in a washout experiment, but the rate of debinding was very slow. In a typical experiment, bound brush borders were incubated for 20 min in the presence of 2 mM glucose to displace the radioactive material and 50% of activity was released. Without added unlabeled glucose, only 10% of the counts were released during the same time period.

Binding specificity

Table I shows data obtained from inhibition experiments. Duplicate runs were not averaged in order to show the variability. The glucose concentration used was

TABLE I

COMPETITION FOR BINDING WITH D-[^{3}H]GLUCOSE

D-[^{3}H]Glucose concn., 0.5 μM.

Conditions	D-[^3H]-*Glucose (D)* (*counts/min*)	L-[^{14}C]-*Glucose (L)* (*counts/min*)	*D/L ratio*	*Counts bound*	*Activity* (%)
Control	3231	1016	3.17	2181	100
	2561	608	4.20	1932	
+ phlorizin (0.1 mM)	510	487	1.04	3	1
	398	386	1.03	1	
Actively transported hexoses (50 μM)					
+ D-glucose	499	434	1.03	1	1
	602	575	1.04	9	
+ D-galactose	1747	412	4.24	1321	68
	2053	531	3.85	1503	
+ 3-*O*-methyl-D-glucose	2676	716	3.73	1936	93
	2621	710	3.68	1887	
+ α-methyl D-glucoside	1427	556	2.56	852	45
	1900	857	2.21	1015	
Nonactively transported hexoses (50 μM)					
+ D-mannose	640	401	1.59	225	10
	688	473	1.45	199	
+ D-mannitol	2817	582	4.83	2215	94
	2326	641	3.62	1664	
+ 2-deoxy-D-glucose	500	352	1.42	137	7
	780	608	1.28	151	

that close to the K_m of binding. As can be seen, among the actively transported sugars which should show inhibition if this binding is related to transport[13], cold glucose did show such inhibition while galactose and α-methyl D-glucoside showed only partial inhibition at the concentrations used. 3-*O*-Methylglucose did not inhibit at all.

The lack of inhibition with 3-*O*-methylglucose was further investigated using the sugar at higher concentration. We have found no significant decrease in binding against 0.1 μM glucose until we reached 3-*O*-methylglucose concentration of 1 mM where we had obtained 20% inhibition. Only close to its limit of solubility did the sugar show an inhibition of binding of 80% (Fig. 5).

On the other hand, some hexoses which are not actively transported in the intact gut and do not interact measurably with glucose transport[13], showed strong inhibition of glucose binding. For example, mannose and 2-deoxyglucose strongly inhibited glucose binding. Those pentoses tested had no effect.

The following amino acids were tested for inhibition of glucose binding; namely, glycine, lysine, arginine, histidine, phenylalanine and glutamic acid. None of these showed significant inhibition when used at 0.5 mM concentration.

Effects of ions on binding

Glucose transport in the hamster intestine is known to be dependent on Na^+ and it appears to be coupled to the inward downhill Na^+ gradient. It was therefore of great interest to determine the effect of Na^+ and K^+ as well as of ouabain, an inhibitor of Na^- pumping, on glucose binding to isolated brush borders. The results in Table II show that there was little effect of all monovalent ions except Li^+. Binding was 90% of the control in the presence of 1 mM ouabain.

TABLE II

EFFECT OF MONOVALENT IONS ON BINDING

Ion added	*% of control binding*		
Concn. (mM):	*10*	*50*	*100*
Na^-	93	104	105
K^-	103	100	105
Li^-	63	60	54
NH_4^-	—	92	—
Rb^+	—	93	—
Cs^+	—	84	—

Divalent ions, on the other hand, (see Table III) showed profound effects on binding. Ca^{2+}, Co^{2+}, Fe^{2+}, Zn^{2+} were inhibitory, Mg^{2+} and Mn^{2+} activated and to the same extent. When used together, Mn^{2+} and Mg^{2+} were not additive.

Effect of sulfhydryl reagents

When the disulfides were reduced and their reduction was maintained by addition of Cleland's reagent (dithiothreitol), binding was significantly activated. Sulfhydryl inhibitors such as *p*-chloromercuribenzoate and iodoacetate completely inhibited binding (see Table IV).

Effects of various cofactors on binding

Various substrates known to participate in transport processes in bacteria or to be closely coupled to metabolism of glucose have been tested as inhibitors or activators of glucose binding (Table V). Of these, ATP, ADP, AMP and PP_i inhibited binding. NAD^+ had the greatest inhibitory activity.

TABLE III

EFFECT OF DIVALENT IONS ON BINDING

Total added ion concentration was 10 mM.

	Ion added	*% of control binding*
Inhibit binding	Ca^{2+}	66
	Co^{2+}	67
	Fe^{2+}	44
	Zn^{2+}	12
Activate binding	Mg^{2+}	157
	Mn^{2+}	147
	$Mg^{2+} + Mn^{2+}$	145

TABLE IV

EFFECTS OF SULFHYDRYL REAGENTS ON BINDING

Sulfhydryl reagent	*% of control binding*
Control	100
Cleland's reagent (dithiothreitol), 6 mM	166
p-Chloromercuribenzoic acid, 1 mM	1
Iodoacetic acid, 2.5 mM	11

TABLE V

EFFECT OF COFACTORS ON BINDING

Cofactor	*% of control binding*		
Concn. (mM):	*1*	*5*	*10*
ATP	82	25	12
ADP	58	27	12
AMP	69	49	42
PP	53	68	44
NAD^+	52	—	5
NADH	107	—	88
$NADP^+$	60	—	70
NADPH	139	—	117
GTP	—	86	—
Phosphoenolpyruvate (100 mM)			95

Distribution of binding along the length of the intestine

Ample experimental evidence is available showing that in several species including rat, dog, man, and hamster the jejunum of the small intestine is able to

absorb glucose more rapidly than the lower portions. It was of interest to determine whether the distribution of binding parallels that of absorption.

It is evident from Fig. 7 that the highest specific activity for binding, as counts/min per mg of protein, was found in distal region of the intestine. Total counts bound also increased in the distal segments of the intestine. In an absorption experiment conducted under similar conditions, it was found[14] that maximal accumulation of D-glucose, D-galactose or 1,5-anhydro-D-glucitol occurred in the early or middle portion of the intestine.

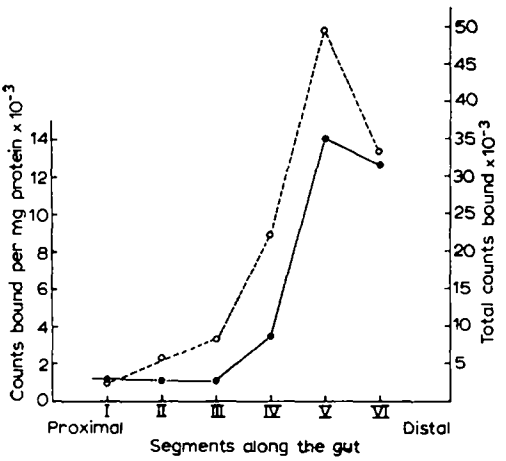


Fig. 7. Distribution of binding along the length of the small intestine. Each segment was approx. 5 cm in length. For convenience of presentation counts were reduced by 1000. O---O, total counts in the segment; ●—●, counts bound per mg protein.

Binding of glucose prior to the isolation of brush borders

Since the debinding of glucose was so relatively slow, it appeared possible to do binding experiments on intestinal sacs, wash the sac, immediately isolate the brush borders and measure the amount of radioactivity specifically bound. 2,4-Dinitrophenol was added in these experiments to reduce glucose utilization; even though it appeared likely that any substrate or metabolites reaching the cell cytoplasm would remain soluble.

When such experiments were carried out, as described in METHODS, most of the radioactivity was found to be associated with the soluble intracellular fraction as would be expected. Among the particulate fractions obtained, the fraction containing brush borders had the highest number of total counts bound as well as the highest counts per mg of protein. When the counts were compared to the activity of sucrase used as a marker for brush borders, the counts bound per mg of glucose liberated from sucrose remained about the same in the sediments until the last at which point the level of enzyme activity was so low that the data became inaccurate (see Table VI). The supernatants contained high amounts of soluble radioactivity, probably from accumulated glucose and its metabolic products.

In the presence of dinitrophenol and under the conditions mentioned above, 3-*O*-methylglucose at 1 mM concentration again did not appear to inhibit the binding

TABLE VI

Fraction	*Total counts* D-[^3H]*glucose*	*Counts bound per mg protein*	*Counts bound per mg glucose released from sucrose*
Brush border sediment	876	86	569
Supernatant from brush borders	31 462	—	—
2 100 × *g* sediment	167	37	554
8 500 × *g* sediment	384	37	738
19 200 × *g* sediment	252	38	396
Final supernatant	25 763	—	—
	Total 26 566		

of glucose to brush borders, as measured by this initially intact system, similar to the result obtained with isolated brush borders.

Binding to fractions of the brush border

When brush borders were disrupted with 1 M Tris using our usual procedures[6] no significant change in glucose binding activity per mg of protein could be observed.

Upon centrifugation of Tris-disrupted brush borders on a discontinuous glycerol gradient from 20 to 60% (w/v), five fractions can be separated. Two of these contain microvillus membranes, (Fractions C and C[1]), two are as yet unidentified (Fractions A and B) and the fifth is a sediment (Fraction D) containing in addition to heavier cell contaminants, material which resembles the core of the microvilli.

When binding experiments were conducted on all of the isolated fractions most of the observed binding was found to be associated with Fraction D. However, only 30–50% of the binding capacity of intact or Tris-disrupted brush borders applied to the gradient could be recovered. Upon further investigation, it was found that glycerol used for the gradient is a strong inhibitor of binding, and the loss of binding capacity is not regained after exhaustive washing or dialysis. It was therefore possible that the binding lost on the gradient was due to glycerol inhibition and another fraction does in fact bind as well as Fraction D.

TABLE VII

BINDING OF BRUSH BORDER SUBFRACTIONS

	Protein (mg)	*Total (counts/min bound)*	*Specific activity (counts/min bound per mg protein)*
Whole brush borders	—	—	468
Tris-disrupted brush borders	10.75	4220	416
Fractions			
(1) 450 × *g* for 10 min (undisrupted brush borders + core clusters)	2.16	2236	1032
(2) 2 100 × *g* for 10 min (cores)	1.16	1746	1085
(3) 20 000 × *g* for 30 min	2.46	318	129
(4) Final supernatant	4.00	—	—
Total	9.96	4300	

In order to resolve this, we fractionated Tris-disrupted brush borders by simple differential centrifugation, realizing that the fractions so obtained were not pure. Table VII shows the result of such an experiment. From these data it is clear that the binding capacity per mg of protein is still highest in the heavier fractions. The 20000 × *g* fraction is highest in alkaline phosphatase activity and presumably contains mostly membranes. Equilibrium dialysis experiments done on the final supernatants showed no significant binding.

By taking advantage of the slow debinding of D-[^{3}H]glucose from Tris-disrupted brush borders, an experiment was performed in which density-gradient fractions were obtained from Tris-disrupted brush borders exposed to D-[^{3}H]glucose immediately prior to centrifugation. Most of the radioactivity separated with Fraction D.

This property has been found to permit further purification of the binding material. When D fraction-containing bound D-[^{3}H]glucose is placed on a heavier glycerol gradient, reproducible bands are obtained, one of which contains D-[^{3}H]-glucose at 500 times the specific activity in the original Tris-disrupted brush borders[15].

Chromatography of glucose after binding to brush borders

The identity of the radioactive material after binding was investigated. Brush borders were incubated with L-[^{14}C]- and D-[^{3}H]glucose in the usual manner, but in large scale experiments as described in METHODS. After binding, the brush borders were briefly washed, denatured by boiling, centrifuged to remove the protein and the supernatant applied to ion exchange columns. Virtually all of the radioactivity was released to the supernatant by denaturation. After chromatography the majority of the applied radioactive material appeared in the void volume, as would be expected for the uncharged glucose molecule. Small amounts of material, comprising about 10–13% of the applied radioactivity were, however, held back on the column (see Fig. 8). This compound (Compound X) could be subsequently eluted from the column with sodium formate and must have therefore carried a negative charge. The second peak contained only the label derived from D-glucose (^{3}H) unlike the first peak which contained both ^{3}H and ^{14}C counts.

Both peaks were lyophilized and subjected to paper chromatography in methanol–ethanol–water (45:45:10, by vol.) or butanol–ethanol–water (2:1:1, by vol.) systems. The compounds were detected by radioautography and found to give single spots with an R_F corresponding to that of glucose. Since control experiments with free [^{3}H]glucose on the ion exchange column have shown no retention of radioactivity, it appears possible that the second peak which was retarded on the column contained glucose bound to a protein or a peptide fragment which released its glucose after further extraction with organic solvents. Such interpretation, although speculative, is consistent with our data obtained from Sephadex column chromatography. In these experiments glucose bound to brush borders was found to be associated with a protein fraction which eluted from the column with the void volume. We had conducted such experiments using Sephadex G-10 column in order to ascertain whether the radioactivity in isolated brush border fractions is indeed bound to macromolecules. In these experiments we have passed the glucose-bound membrane fraction through the column. Free glucose would be retained on such a column and glucose associated with macromolecules would be excluded from Sephadex and would come out in the void volume. As can be seen in Fig. 9 two peaks of radioactivity

were obtained in such an experiment. One peak is due to free glucose which debinds from the fraction passed through the column, and the other which is excluded from the column, contains protein and enzymatic activities normally associated with the membrane fraction. Only the radioactivity from D-glucose and not from L-glucose is present in the protein peak. 70% of the applied material can be recovered in both peaks.

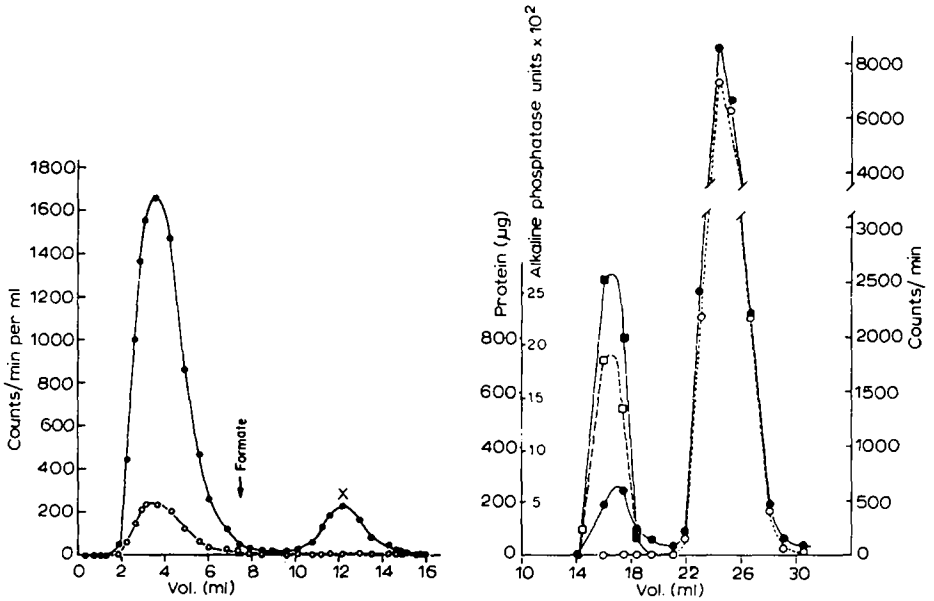


Fig. 8. Separation of bound glucose on Dowex-2 (formate) column. The column was first eluted with distilled water and after collection of 7 ml with 1 M sodium formate (pH 7.0).

Fig. 9. Distribution of enzymatic activities and of radioactive counts after chromatography of bound membrane fraction on Sephadex G-10. ●—●, D-glucose counts; ○---○, L-glucose counts; ■—■, alkaline phosphatase units; □---□, maltase. Enzyme activity expressed in I.U.

We have attempted to conduct similar experiments with Tris-disrupted brush borders and the D fraction. In such experiments we have also obtained the same two peaks, however, the recoveries were in the order of 20–30% and most of the radioactivity was found at the top of the column. It appears possible that some of the large particulate material, such as core clusters, known to be present in the D fraction, could not penetrate, and were filtered by the column.

In experiments in which we have used tritiated glucose some exchange of the label with the water was detected as a peak coming out much later on the Sephadex column.

DISCUSSION

Although considerable information is available on the behavior of the overall properties of the system for carbohydrate transport in the intestine, little is known about the nature of the membrane-associated molecules involved in this process. Since the transport process exhibits a high degree of specificity in its reaction with trans-

ported carbohydrate, a binding or recognition site can be safely presumed to exist within the transporting mechanism that is located within the brush border of the intestinal epithelial cell. The results of this study show that such a binding site, specific for the D- and not the L-glucose isomer, is present. Moreover, the binding site appears to be localized within the brush border fraction of the intestinal epithelial cell.

The available data on the overall transport process predict that we can expect relatively few such recognition sites and that the binding to such sites should be freely reversible, as implied by the steady-state kinetics of the transport process for glucose, K_m of about 1 mM. It was, therefore, surprising to see that brush borders to which glucose was bound could be briefly washed without much detectable loss of bound glucose. Moreover, the affinity for the binding was greater than that for transport with a K_m of 2 μM.

Upon closer examination of the binding specificity, it was seen that some actively transported sugars, such as 3-*O*-methylglucose, showed no inhibition, while non-actively transported sugars, such as mannose and 2-deoxyglucose, exhibited marked inhibition of the binding. No inhibition of binding by pentoses or amino acids could be detected.

It is generally assumed that the "carrier" for the transport process is localized within the membrane of the epithelial cell. We were, therefore, surprised to find most of the D-glucose-specific binding to be localized to the D fraction of the disrupted brush borders, which contains cores *plus* other heavier, contaminating material. Only 10% of the binding activity at the most, was associated with the membrane fraction. We are currently investigating possible differences between binding properties of this membrane fraction and that found in the core fraction.

The fact that maximal binding is found in the distal portion of the hamster intestine, while active transport processes in the same animal as well as in the rat and human are localized more proximally, excludes the possibility that the binding we have studied represents a simple expression of the substrate–carrier interaction that is usually associated with the active transport of carbohydrates through the intestine. Although arguments can be found to explain each of the differences between the properties of active transport and the glucose binding, it appears reasonable to assume that we are dealing with a different property of the intestine. This is supported by the combined weight of the following differences: (1) different K_m for binding; (2) no requirement of binding for Na^+; (3) different sugar specificity; (4) localization not in the membrane fraction; (5) high activity in the ileum rather than proximal jejunum; (6) lack of reversibility.

Since the binding is so high in the distal intestine, there was a distinct possibility that this binding, at least in part, was due to bacterial contamination of the brush border preparation. For this reason we have conducted a series of experiments on brush borders prepared from control rats and from germ-free rats. Brush borders prepared from germ-free animals did bind glucose. However, the binding was lower than that obtained from the controls. The reason for the lower binding is currently being investigated. It is noteworthy to point out in this connection, however, that binding of glucose to brush borders possesses different characteristics from the properties of sugar metabolism and accumulation in bacteria[16]. It is, for instance, not inhibited by a series of inhibitors such as 2,4-dinitrophenol, cyanide, azide, and penicillin.

Under the electron microscope intact microorganisms can be readily recognized. Although bacteria are sometimes, but rarely, seen in our membrane fraction, no recognizable bacteria could be identified in the fraction exhibiting high binding. We also could not detect in this fraction enzymatic activities associated with bacteria, such as hexokinase or cytochrome oxidase.

In summary, the foregoing discussion describes a strong binding of glucose to brush borders. The binding exhibits its own specificity and properties different from those of active transport. The binding also occurs in experiments using everted sacs. The interpretation of the physiological role of such binding is still elusive. It is possible that it represents a concentrative or scavenging mechanism at the distal end of the intestine, or that it is an expression of a portion of the overall process of absorption occurring after the end of the membrane-associated active transport. Definitive answers to such as yet speculative theories will have to await further experimentation.

Recently BOSMANN AND MARTIN[17] have shown monosaccharide incorporation into glycoproteins of mitochondrial membranes in an *in vitro* system. Since in our system all of the glucose is released from the protein after boiling or trichloroacetic acid precipitation, it is unlikely that glucose is covalently linked to the binding molecule.

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