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STUDIES ON THE MECHANISM OF ACTIVE INTESTINAL TRANSPORT OF GLUCOSE

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SUMMARY

D-[2-^{3}H]Glucose is actively accumulated by hamster intestinal rings and everted sacs. The isolated glucose from the tissue and the serosal fluid showed no significant change in specific activity. These data rule out an active transport mechanism involving a covalent bond with the oxygen of the hydroxyl on C-2 of glucose. At the same time an exchange reaction of the ^{3}H with water catalyzed by brush borders has been observed. This reaction is probably independent of glucose transport.

INTRODUCTION

Earlier studies on the carrier-mediated active transport in intestine with glucose and other sugar analogues have established the obligatory requirement of a pyranose ring and a hydroxyl group at C-2 position in the D-glucose configuration, in order that a sugar may be actively transported[1]. In an attempt to find out whether this hydroxyl group undergoes chemical reaction during transport, Crane and Krane[2] used hydroxymethylglucose and [2-^{18}O]glucose for transport studies. They found that hydroxymethylglucose is actively transported and that ^{18}O of the glucose[2-^{18}O] does not exchange with the medium during transport. These studies ruled out the possibility of an oxidation–reduction reaction of glucose molecule during transport and also of dehydration-type reactions. However, as pointed out by Crane and Krane[2], reactions that involved only the hydrogen of the hydroxyl group such as phosphorylation were not ruled out. Barnett *et al.*[3] studied the transport patterns of several sugars and proposed a mechanism that involved the formation of a covalent bond between the oxygen of the hydroxyl group and the carrier of the membrane.

The present paper describes experiments that were done to investigate the possibility of a covalent bond formation between glucose molecule and the sugar carrier during transport using specifically labelled sugars.

MATERIALS

D-[U-^{14}C]Glucose (230 Ci/mole), D-[2-^{3}H]glucose (620 Ci/mole), D-[3-^{3}H]-glucose (7.25 Ci/mmole) and D-[1-^{3}H]glucose (5.15 Ci/mmole) were purchased from New England Nuclear. According to the distributor D-[2-^{3}H]glucose

was prepared by incubation of fructose 6-phosphate with glucose-phosphate isomerase in the presence of ^3H_2O and subsequent dephosphorylation. This procedure should label the hydroxyl and the hydrogen on C-2. The position of ^3H was ascertained by a chemical reaction with a mixture of hexokinase, ATP and phosphoglucoisomerase. Under these conditions, 98.3% of the ^3H was released as ^3H_2O after completion of the reaction. D-[1-^{3}H]Glucose was prepared by reduction of D-glucono-δ-lactone with NaB^3H_4 and subsequent purification. D-[3-^{3}H]Glucose was prepared by catalytic reduction with $^3H_2$ gas of the 1,2- and 5,6-*O*-isopropylidene derivative of α-D-ribohexofuranose-3-ulose with subsequent hydrolysis and purification. D-[6-^{3}H]-Glucose (18 Ci/mmole) was obtained from Schwarz Mann. D-Xylose, D-lyxose, D-arabinose and L-xylose were obtained from Calbiochem; D-ribose and L-fucose were purchased from Pfanstiehl; D-galactose, D-mannose and 3-*O*-methyl-D-glucose were supplied from Nutritional Biochemicals Corporation; D-glucose was from J. T. Baker.

METHODS

Assay of glucose

Glucose was estimated using the Lloyd and Whelan[4] modification of the Tris–glucose oxidase method of Dahlquist[5]. Hexokinase was assayed according to the method of Joshi and Jagannathan[6] and phosphoglucoisomerase by the method of Slein[7]. Radioactivity was measured with a Beckman liquid scintillation counter, with scintillation fluid prepared according to Patterson and Green[8].

Time accumulation studies

For each experiment the entire intestine of one hamster was used. Rings were prepared according to the method of Crane and Mandelstam[9] and the everted sacs by the method of Wilson and Wiseman[10]. At the end of incubation the tissue was washed with buffer and was homogenized in 80% ethanol and centrifuged. The supernatant fluid was concentrated to a small volume, deionized with mixed-bed resin MB-3 (Mallinkrodt) and subjected to paper chromatography. A known aliquot of medium was processed in a similar manner.

Paper chromatography

Preparative paper chromatography of glucose was performed on Whatman No. 3 MM paper using the solvent system *n*-butanol–pyridine–water (10:3:3, by vol.) for a period of 48 h. The chromatogram was air dried, guide strips were cut and stained with silver nitrate according to the procedure of Trevelyan *et al.*[11]. A parallel strip was cut into 1 cm squares, dropped into vials containing 200 μl of water followed by 2 ml of scintillation fluid, and counted. The remainder of the chromatogram was cut based on the radioactivity and silver nitrate staining, and eluted with water. Eluates (20 ml) were taken to dryness and dissolved in 2 ml of water. Aliquots were drawn for counting and chemical estimation of glucose.

Isolation of brush borders and subfractions

Isolation of brush borders and their subfractions after Tris disruption, were prepared according to Eichholz and Crane[12].

^3H release studies with brush borders

The reaction mixture in a total volume of 0.5 ml contained 0.05 μCi of [2-^{3}H]-glucose with a final glucose concentration of 0.016 mM in Krebs–Ringer phosphate buffer (pH 6.5) and brush border protein as indicated.

After incubation at 37 °C for a period of 30–45 min 2 ml of ethanol was added to stop the reaction. The mixture was centrifuged for 2 min in a bench centrifuge, and 0.5 ml aliquots of the supernatant fraction were counted. Another series of aliquots (1 ml) were taken to dryness in a bio-dryer, dissolved in 2 ml water and 1 ml was used for counting. The difference in the counts before and after evaporation served as a measure of ^3H_2O formed in the reaction.

RESULTS

Accumulation of glucose by the intestine

Table I shows the results of the glucose accumulation into intestinal rings using 1 mM [U-^{14}C]glucose or glucose labeled with ^{3}H in the 2–OH position. The small differences seen in the T : M ratios from one experiment to the other are due to variation in animals rather than due to the different labeled sugars used. The specific activity of the isolated glucose did not change when [2-^{3}H]glucose was used. The ratio of ^{3}H to ^{14}C remained the same in the tissue and in the medium when a mixture of [2-^{3}H]glucose and [U-^{14}C]glucose was used. Similarly the specific activity of [2-^{3}H]glucose did not change significantly in the everted sac experiments (Table II).

TABLE I

ACTIVE ACCUMULATION OF GLUCOSE BY RINGS OF HAMSTER SMALL INTESTINE

The average weight of tissue used was 1.50 g in each experiment. The medium contained 50 ml of Krebs–Ringer phosphate buffer (pH 7.0) containing glucose at a final concentration of 1 mM. 1 μCi of [U-^{14}C]glucose or 5 μCi of [2-^{3}H]glucose or both were added to the flasks depending on the experiment. The incubation period was 5 min at 37 °C. The processing of tissue and paper chromatographic isolation of glucose from medium and tissue are described in Methods. (a) Based on radioactivity measurements on isolated glucose. (b) Based on chemical estimation of isolated glucose.

Sugar	*Final ratio of tissue/medium concentration*		*Specific activity (cpm/μg glucose)*	
	(*a*)	(*b*)	*Tissue*	*Medium*
[2-^{3}H]Glucose	9.7	9.1	230	235
[U-^{14}C]Glucose	7.9	7.7	206	244
Mixture of [2-^{3}H]glucose and [U-^{14}C]glucose	6.3	7.1	0.95*	0.97*

* Ratio of ^{3}H/^{14}C.

TABLE II

ACTIVE ACCUMULATION OF GLUCOSE BY EVERTED SACS OF HAMSTER SMALL INTESTINE

Final ratio of tissue/medium concentration is based on the chemical estimation of isolated glucose; 5 sacs made from one intestine were used. The medium contained 50 ml Krebs–Ringer phosphate buffer (pH 7.0) containing glucose at a final concentration of 1 mM and 5 μCi of [2-^{3}H]glucose. The incubation period was 5 min at 37 °C.

Sugar	*Final ratio of tissue/medium concentration*	*Specific activity (cpm/μg glucose)*	
		Mucosal	*Serosal*
[2-^{3}H]Glucose	8.7	233	288

Release of ^3H by isolated intestinal brush borders

In initial experiments we were attempting to demonstrate an interaction of glucose with the membrane structures during transport by measurement of the exchange of ^{3}H from [2-^{3}H]glucose with water. Such an exchange would be normally expected as the result of metabolic activity in whole tissue experiments *i.e.* from the reaction of phosphohexoisomerase on the phosphorylated [2-^{3}H]glucose leading to non-radioactive fructose 6-phosphate and ^3H_2O. Since glycolytic enzymes are soluble and washed isolated brush borders are known to be free of these enzymes we were not expecting to observe this reaction in brush border preparations. However, hamster intestinal brush borders when incubated with [2-^{3}H]glucose, in buffer, released ^{3}H as ^3H_2O. The kinetics of ^{3}H release was suggestive of an enzyme catalyzed reaction. The brush border preparations were free of both phosphohexoisomerase and hexokinase activities. The K_m for glucose was $1.1 \cdot 10^{-5}$ M (Fig. 1). The reaction was linear up to 45 min and then tapered off (Fig. 2). The rate increased with increasing amounts of brush border protein (Fig. 3). The optimal pH was 6.5 (Fig. 4). Measurement of the loss of ^{3}H to water using either intestinal rings or sacs showed an exchange

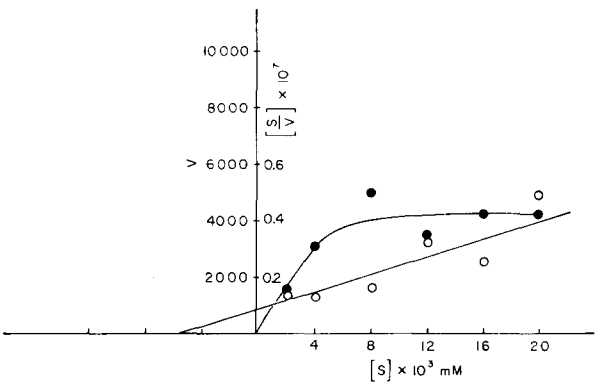

Fig. 1. K_m for glucose. Glucose solutions of concentration 0.002 mM to 0.020 mM containing 5500 to 55 000 cpm and 0.2 mg of brush border protein were used. For details of assay see Methods. -●—●-, effect of substrate concentration on velocity; ○—○, plot of S/v against S.

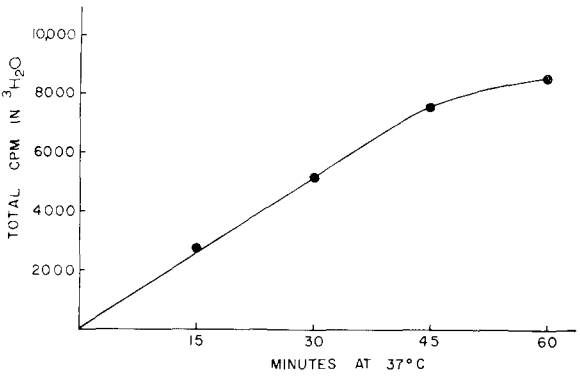

Fig. 2. Effect of time of incubation. Standard reaction mixture as described under Methods were incubated for different periods of time at 37 °C.

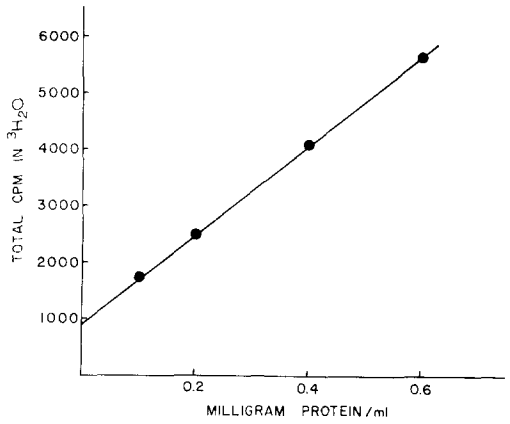

Fig. 3. Effect of protein concentration. Standard reaction mixture as described under Methods, but containing different amounts of brush border protein, were used.

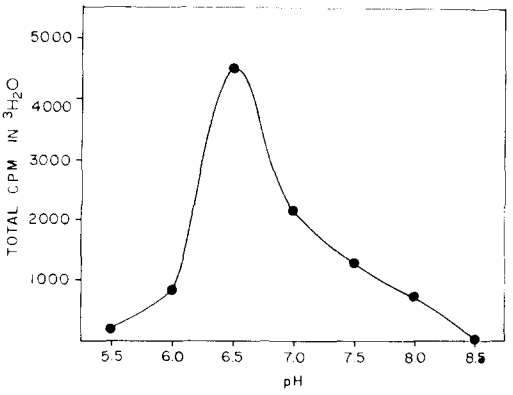

Fig. 4. pH optima for the release of ^3H from brush borders. 0.1 M sodium phosphate buffers of different pH were employed.

amounting to 20–30% of the label. The maximal rate of exchange by isolated brush border preparations was 0.1 nmole/min per mg brush border protein (Fig. 1). In comparison the rate of glucose transport in the ring experiments was calculated from Table I as 0.1 μmole/min per g wet tissue (1 g wet tissue would yield approximately 5 mg of brush border protein).

Effect of other sugars

Several sugars including mannose and 2-deoxyglucose were inhibitory. Phlorizin inhibited the release of ^3H at a concentration of 0.2 mM (see Table III). The release of ^3H from other tritiated sugar analogues was also observed (Table IV). The products

TABLE III

RELEASE OF ^3H BY HAMSTER INTESTINAL BRUSH BORDERS

The reaction mixture contained in a total volume of 0.5 ml, 0.1 μCi of [2-^{3}H]glucose, buffer, 0.4 mg of brush border protein and the inhibitor at the indicated concentration. The incubation time was 45 min. The reaction mixture was processed as given in Methods.

System	*Total cpm in ^3H_2O (percent inhibition)*
Complete	13 690
+ ATP (100 μg/ml)	13 500 (1)
+ EDTA (1 mM)	13 600 (0)
+ Phlorizin (0.2 mM)	440 (68)
+ D-Galactose (10 mM)	3 010 (78)
+ D-Mannose (10 mM)	0 (100)
+ D-Arabinose (10 mM)	5 750 (58)
+ D-Ribose (10 mM)	13 700 (0)
+ D-Xylose (10 mM)	3 020 (78)
+ D-Lyxose (10 mM)	12 800 (7)
+ L-Glucose (10 mM)	13 400 (0)
+ L-Xylose (10 mM)	12 600 (8)
+ 2-Deoxyglucose (10 mM)	0 (100)
+ 3-*O*-Methylglucose (10 mM)	685 (95)
+ L-Fucose (10 mM)	13 700 (0)

TABLE IV

RELEASE OF ^3H FROM GLUCOSE BY HAMSTER INTESTINAL BRUSH BORDERS

The reaction mixture in a total volume of 0.5 ml, contained 0.1 μCi of appropriate labeled glucose, 0.6 mg brush border protein and buffer. The incubation time was 45 min. The reaction mixture was processed as described in Methods.

Compound	*Total cpm in ^3H_2O*
[2-^{3}H]Glucose	8560
[3-^{3}H]Glucose	7500
[1-^{3}H]Glucose	4395
[6-^{3}H]Glucose	1790

of the reaction other than the ^3H_2O have not been characterized. No requirement for metal ion could be demonstrated.

Localization within brush border fractions

Brush border preparations were disrupted with 1 M Tris, and the subfractions were prepared in a glycerol gradient according to the method of Eichholz and Crane[12]. The ^3H releasing activity was localized in the "D" fraction which may contain microvillus cores as well as other large particles precipitated in the gradient (Table V).

The release of ^3H decreased to 20% on storage of the brush border preparation overnight in the refrigerator, hence fresh brush borders were used for each experiment.

TABLE V

RELEASE OF ^3H FROM [2-^3H]GLUCOSE BY HAMSTER INTESTINAL BRUSH BORDERS AND THEIR SUBFRACTIONS

The reaction mixture contained in a total volume of 0.5 ml, 0.1 μCi of [2-^3H]glucose, buffer and suitable aliquot of the fraction. The incubation time was 45 min and the reaction was stopped with 2 ml of absolute ethanol and processed as described under Methods.

Fraction	*Total cpm in ^3H_2O*
Brush borders (control)	9200
Brush borders (boiled)	35
Subfraction A	0
Subfraction B	0
Subfraction C + C′	0
Subfraction D	8400

DISCUSSION

Any description of the mechanism of active transport must explain the need for energy requirement during transport as well as its requirements for ions *etc.*, that are seen in the studies in intact systems. An endergonic chemical reaction during transport would explain the need for energy during active accumulation. Some of the earlier theories of active intestinal transport involving chemical conversion like phosphorylation dephosphorylation or mutarotase-catalyzed reactions have been shown to be untenable (for review, see ref. 1). However, there are some instances, of vectorial transport in which a chemical conversion has been shown to occur, *i.e.* the transport of glycosides in bacterial systems (for review see ref. 13). Earlier studies[1,2] of transport of various sugars led to the definition of a minimal structural requirement for intestinal active transport. The present studies on the transport of glucose labeled in C-2 position with ^3H have shown that ^3H is not released by the carrier during transport. A covalent bond involving this hydroxyl group of glucose and carrier is therefore unlikely, and chemical modification of glucose not tenable. At the same time the occurrence of an enzymatic reaction localized in brush borders, that leads to the release of ^3H from glucose has been brought to light by these studies. The experiments done to show its identity, have shown that this reaction is not catalyzed by phosphohexoisomerase or hexokinase, both of which could not be detected in our brush border preparations. The absence of hexokinase in rat intestinal

brush borders has been reported by Weiser *et al.*[14]. The disaccharidases were suspected to catalyze this reaction but this was not the case as Fraction D, and not Fraction C, catalyzed the release of ^3H. This could not result out of a kinase-type reaction as addition of ATP had no effect. Some of the properties of this system were different from the known properties of the intestinal sugar transport system. Thus the low K_m and the inhibition by 2-deoxyglucose and mannose distinguish it from the transport carrier. Were this reaction mandatory for transport, the accumulated glucose should have a lower specific activity (one half of that in the medium). The tissue accumulation and everted sac studies have shown that this is not so.

Clearly, further studies are needed to show the identity of the factor that catalyzed the release of ^3H. This reaction should be borne in mind during transport studies with [^3H]glucose as the released ^3H would complicate the measurements.

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