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# Hexose uptake in primary cultures of bovine brain microvessel endothetial cells. I. Basic characteristics and effects of D-glucose and insulin

Yoshinobu Takakura [1,*], Sandra L. Kuentzel [2], Thomas J. Raub [2], Anthony Davies [3], Stephen A. Baldwin [3] and Ronald T. Borchardt [1]

[1] *Department of Pharmaceutical Chemistry, School of Pharmacy, University of Kansas, Lawrence, KS (U.S.A.),* [2] *Drug Delivery Systems Research, Upjohn Laboratories, The Upjohn Company, Kalamazoo, MI (U.S.A.) and* [3] *Departments of Biochemistry and Chemistry and of Protein and Molecular Biology, Royal Free Hospital, School of Medicine, London (U.K.)*

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**The basic characteristics of hexose uptake and regulation of the glucose transporter (GLUT1) by D-glucose and insulin were studied in primary cultures of bovine brain microvessel endothelial cells (BMECs). A non-metabolizable glucose analog, 3-*O*-[$^3$H]methyl-D-glucose ([$^3$H]3MG), was used as a model substrate, and the uptake was studied using BMECs grown in tissue culture plates. Uptake of [$^3$H]3MG was equilibrative, temperature-dependent, and independent of sodium. The uptake also decreased gradually with culture age from 7 to 13 days. Saturation kinetics were observed for [$^3$H]3MG uptake and the apparent $K_m$ and $V_{max}$ values were determined to be 13.2 mM and 169 nmol / mg per min, respectively. Pre-incubation with high concentrations of D-glucose and 3MG accelerated [$^3$H]3MG uptake by BMECs by a counter-transport mechanism. D-Glucose, 2-deoxy-D-glucose, D-mannose, D-xylose, D-galactose and D-ribose showed significant competitive inhibition with (3H]3MG, whereas L-glucose, D-fructose, and sucrose did not affect [$^3$H]3MG uptake by BMECs. [$^3$H]3MG uptake was inhibited significantly by cytochalasin B and phloretin but not by phlorizin, 2,4-dinitrophenol, or ouabain. D-Glucose starvation of BMECs by incubation with D-glucose-free media for 24 h resulted in a significant increase (40–70%) in uptake of [$^3$H]3MG compared with control conditions (7.3 mM D-glucose). Low D-glucose treatments (2.43 and 1.83 mM) for 7 days induced a slight but significant increase (20%) in [$^3$H]3MG uptake, while long-term high glucose treatments (25 mM) showed no significant effect on [$^3$H]3MG uptake irrespective of exposure time. The increase in [$^3$H]3MG accumulation following D-glucose starvation was dependent upon starvation time (12 to 48 hr) and protein synthesis. Refeeding of D-glucose (7.3 mM) to D-glucose-starved BMECs resulted in a return of [$^3$H]3MG uptake to control levels in 48 h. The D-glucose-starvation-induced increase in [$^3$H]3MG uptake was shown to result from an increase in $V_{max}$; the $K_m$ remained constant. In addition, D-glucose-starved BMECs were shown to have an increased level of GLUT1 using an antibody against human GLUT1 and an enzyme-linked immunosorbent assay (ELISA). The increased uptake following D-glucose starvation was not significantly affected by the presence of L-glucose, was partially impaired by the presence of D-galactose, D-fructose, and D-xylose, and was completely inhibited by the presence of D-mannose and 3MG. Furthermore, preincubation of BMECs with insulin (10 $\mu$g / ml) for 20 min did not affect the uptake of [$^3$H]3MG or 2-deoxy-D-[$^3$H]glucose ([$^3$H]2DG). The present study demonstrated that hexoses can be taken up by cultured bovine BMECs by a carrier-mediated, facilitated diffusion mechanism, similar to the mechanism observed in the blood-brain barrier (BBB) and that the uptake by bovine BMECs was regulated by low levels of D-glucose but not by high levels of D-glucose or by insulin.**

* Current address: Faculty of Pharmaceutical Sciences, Kyoto University, Sakyo-ku, Kyoto 606, Japan.

Abbreviations: BBB, blood-brain barrier; BMECs, brain microvessel endothelial cells; BSA, bovine serum albumin; ELISA, enzyme-linked immunosorbent assay; GLUT, glucose transporter; HRP, horseradish peroxidase; 3MG, 3-*O*-methyl-D-glucose; 2DG, 2-deoxy-D-glucose.

Correspondence: R.T. Borchardt, Department of Pharmaceutical Chemistry, School of Pharmacy, The University of Kansas, Lawrence, KS 66045, U.S.A.

## Introduction

The BBB, which consists of brain microvessel endothelial cells (BMECs), restricts the movement of most water-soluble molecules from the blood to the brain. However, several BBB transport systems have been identified for water-soluble substrates, including hexoses, monocarboxylic acids, amino acids, amines, purines, and nucleotides [1].

Glucose is an important source of energy for the brain, and its transport across the BBB has been well studied in vivo using dogs [2–5] and rats [6,7]. Glucose uptake has also been characterized in vitro using capillary preparations isolated from rats [7–9] and cultured cells of mouse [10], canine [11], and human [12] cerebral microvessel endothelium. These studies have demonstrated that glucose transfer across the BBB involves a carrier-mediated, facilitated diffusion mechanism.

It has been suggested that transport across the BBB might be altered in some pathological conditions. For example, it was reported that glucose transport into the brain was significantly decreased in rats with diabetes induced by streptozotocin [13,14]. It was suggested that chronic hyperglycemia in the diabetes milieu may play an important role in the decrease in glucose transport across the BBB in these studies. On the other hand, McCall et al. [15] observed an increased transport of glucose across the BBB in chronic hypoglycemia. In addition, increased glucose transport from blood to brain was shown in rats under starvation conditions in which plasma glucose concentrations were low compared with normal physiological conditions [16]. These findings, although contradictory, imply that plasma glucose level is one of the most important factors responsible for the adaptive changes in glucose transport across the BBB.

It is well known that the peptide hormone insulin plays a central role in the regulation of glucose transport and metabolism in mammalian cells. Most notably, insulin enhances glucose transport in its target tissues, such as fat and muscle, within minutes [17]. In spite of a number of in vivo and in vitro studies on the effect of insulin on BBB glucose transport, conflicting results have been reported (see, for review, Refs. 18 and 19).

Recently, our laboratory developed an in vitro BBB model system consisting of primary cultures of bovine BMECs [20,21]. This in vitro BBB model system has been used to study the uptake and transendothelial transport of various solutes including amino acids [21,22], drugs [23,24], and proteins [25]. In the present study, the basic kinetic characteristics of hexose uptake into bovine BMECs were elucidated and the effects of glucose levels and insulin levels on the uptake of glucose and the expression of the brain type glucose transporter (GLUT1) were studied in an effort to clarify the mechanism for regulation of glucose transport across the BBB.

## Experimental

### *Chemicals*

D-[1-$^3$H]Glucose (19 Ci/mmol), 2-deoxy-D-[1,2-$^3$H]glucose ([$^3$H]2DG; 40 Ci/mmol), and 3-*O*-[$^3$H]-methyl-D-glucose ([$^3$H]3MG; 60 Ci/mmol) were purchased from ICN Radiochemicals (Irvine, CA, U.S.A.). L-[1-14C]glucose (47 mCi/mmol) was obtained from NEN Chemicals (Boston, MA, U.S.A.). Horseradish peroxidase (HRP) was obtained from American Qualex, La Mirada, CA. Insulin (bovine pancreas) was purchased from Sigma. All other reagents were of the highest grade commercially available.

### *Isolation and culture of BMECs*

Microvessel endothelial cells were isolated from the cerebral gray matter of bovine brains as described by Audus and Borchardt [20] and isolated BMECs were stored at −70°C. Approximately $3 \cdot 10^6$ cells were grown to confluence in 6-well culture plates pretreated with rat tail collagen and fibronectin in culture medium comprised of 1:1 minimum essential medium, Eagle's modified F-12 nutrient mix (MEM/F-12 mix), 10 mM Hepes, 13 mM sodium bicarbonate (pH 7.4), 10% plasma-derived equine serum (Hyclone, Logan, UT), 100 μg/ml heparin, 50 μg/ml gentamycin, and 2.5 μg/ml amphotericin B. Cells were refed every 2 days after seeding and monolayers were formed by 9–11 days after seeding. The histochemical, biochemical, and morphological characteristics of the cells have been reported earlier [20,21,26], and the in vitro model system has been shown to possess all the features of the BBB including tight intercellular junctions, the lack of membrane fenestrations, γ-glutamyl transpeptidase and alkaline phosphatase activities, and factor VIII antigen.

### *Uptake study of glucose analogs*

Uptake studies were carried out using confluent cell monolayers in 6-well dishes according to the method by Vinters et al. [11] with slight modifications. Cells were washed three times with 2 ml of warm glucose-free buffer (0.1% bovine serum albumin (BSA), 1 mM pyruvate, 4 mM KCl, 4.2 mM $CaCl_2$, 1.2 mM $MgCl_2$, 150 mM NaCl, 15 mM Hepes (pH 7.4)) and incubated with 1 ml of the buffer for 20 min at 37°C. After the preincubation, the buffer was removed by aspiration and 1 ml of the buffer containing D-[$^3$H]glucose, [$^3$H]2DG or [$^3$H]3MG (1 μCi/ml) and L-[$^{14}$C]glucose (0.25 μCi/ml) was added. Uptake was stopped with the addition of ice-cold PBS (5 ml) containing 0.02 mM cytochalasin B. The cell monolayers were then washed

twice with 5 ml of the PBS and were solubilized with 0.1 M NaOH (1 ml) overnight at room temperature. Aliquots were taken for determination of radioactivity and protein content. 10 ml of scintillation cocktail (Bio-Safe II; Research Products International, Mt. Prospect, Ill, U.S.A.) was added to the sample (500 $\mu$l), and the radioactivity was determined with a Beckman LS5801 scintillation counter. Specific uptake of the D-[$^3$H]glucose analog was calculated after subtraction of non-specific uptake (uptake of L-[$^{14}$C]glucose) from the total uptake of the D-[$^3$H]glucose analog. Protein was measured with a Bio-Rad protein assay kit [27] using BSA as a standard.

*Effect of metabolic and competitive inhibitors*

For the study of metabolic inhibitors, cytochalasin B, phloretin, phlorizin, 2,4-dinitrophenol (DNP), and ouabain dissolved in 95% ethanol were added to both pre-incubation and test buffer. The final concentration of ethanol was less than 1%. Control incubation buffer was also supplemented with ethanol. In the case of competitive inhibitor studies, D-glucose, L-glucose, 2DG, D-mannose, D-xylose, D-galactose, D-fructose, D-ribose, and sucrose were added to the test buffer only at a final concentration of 100 mM.

*Effects of D-glucose levels on glucose uptake in BMECs*

To study the effect of glucose levels in culture, BMECs were exposed to various concentrations of D-glucose. Typically, BMECs were grown in normal culture medium (MEM/F-12 mix) supplemented with 10% plasma-derived equine serum (final glucose concentration of 7.3 mM) for 4 days after seeding. The cells were then fed with Dulbecco's modified Eagle's medium (DMEM) without D-glucose and supplemented with 10% dialyzed equine serum (Gibco, Grand Island, NY) and D-glucose (final concentration 7.3 mM) every 2 days until the cells reached confluency. Confluent cells were incubated with D-glucose-free DMEM with dialyzed equine serum and different D-glucose-concentrations (final concentration ranging from 0 to 25 mM) for specified periods before the uptake experiments were begun. In long-term exposure experiments, BMECs were grown in DMEM for longer periods.

*Reversibility of starvation effect*

To study the reversibility of the effect of D-glucose starvation, refeeding experiments were done. Confluent BMECs were starved by incubating with D-glucose-free DMEM and dialyzed equine serum for 24 h. They were then refed with the same medium containing a normal concentration of D-glucose (7.3 mM). Control BMECs were incubated with D-glucose-containing medium. [$^3$H]3MG uptake was measured at 0, 24, and 48 h after refeeding.

*Effect of protein synthesis inhibitor*

Cycloheximide, a protein synthesis inhibitor, was used to determine whether protein synthesis was involved in the effect of D-glucose starvation. Prior to uptake experiments, confluent BMECs were cultured with or without D-glucose in the presence or absence of 0.05 $\mu$g/ml cycloheximide for 24 h.

*Anti-glucose transporter antibody*

Polyclonal antibodies were raised against a synthetic peptide corresponding to residues 477–492 from the C-terminus of human erythrocyte GLUT1 as previously described by Davies et al. [28]. The specificity of the anti-GLUT1 antibodies for bovine GLUT1 was demonstrated by ELISA using BMECs and anti-GLUT1 antibodies and also by Western blot analysis [29].

*Enzyme-linked immunosorbent assay (ELISA)*

Bovine BMECs were plated at 100000 cells/cm$^2$ in collagen- and fibronectin-coated 96-well plates. At near confluence on day 6, D-glucose starvation was begun as described above. All subsequent steps were done at room temperature. The cells were fixed for 15 min in 4% (w/v) paraformaldehyde in Ca-, Mg-free PBS, rinsed and incubated for 30 min in PBS containing 1% (w/v) BSA and 0.05% (v/v) Nonidet P-40(NP-40). Following a 1 h incubation with the appropriate dilution of rabbit anti-GLUT1 in PBS/BSA/NP-40, the cells were rinsed three times and incubated for 1 h with 50 $\mu$l of a 1:4000 dilution of goat anti-rabbit IgG conjugated to HRP. After three washes, 100 $\mu$g of the peroxidase substrate, *o*-phenylenediamine dihydrochloride, in 100 $\mu$l of 0.02 M citric acid-0.07 M sodium phosphate dibasic buffer at pH 6.3 containing 0.02% (v/v) $H_2O_2$ was added per well. Color development was stopped by the addition of 50 $\mu$l of 1 M HCl, and the optical density was read at 490 nm by using an EIA Autoreader (Bio-Tek Instruments, Inc., Winooski, VT).

*Sugar supplement experiment*

The effect of supplementing various sugars on increased [$^3$H]3MG uptake induced by D-glucose starvation in BMECs was studied. During D-glucose starvation for 24 h, several sugars were added at a concentration of 7.3 mM to medium in which BMECs were incubating. Uptake of [$^3$H]3MG was measured as described above.

*Effect of insulin*

To study insulin effects on [$^3$H]3MG uptake, BMECs were grown in normal culture medium (MEM/F-12 mix) supplemented with 10% equine serum. Confluent BMECs were incubated with serum-free culture media for 12 h before uptake experiments. Insulin dissolved in 0.01 M HCl was added to both preincubation and test buffers. Pre-incubation and test buffers for control

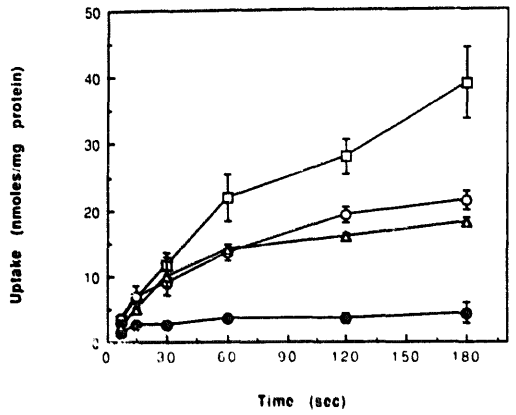


Fig. 1. Time course of radiolabeled glucose analog uptake by BMECs. Uptakes of [$^3$H]3MG (○), [$^3$H]2DG (□), D-[$^3$H]glucose (△), and L-[$^{14}$C]glucose (●) were measured at 37°C and a concentration of 5 mM. Each value except for the L-[$^{14}$C]glucose uptake values was expressed as a specific uptake corrected by a nonspecific uptake using L-[$^{14}$C]glucose. Data are mean ± S.D. (bars) values ($n$ = 3).

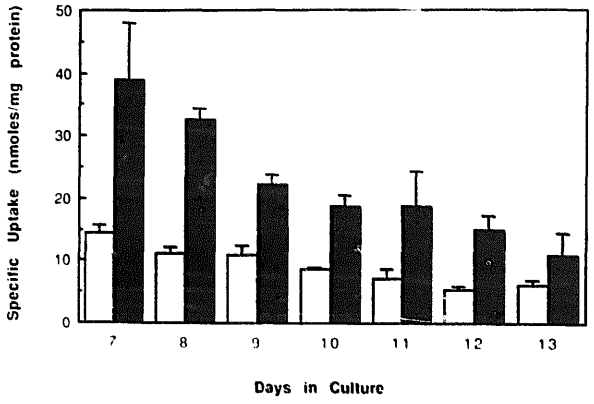


Fig. 2. Effect of culture age on [$^3$H]3MG uptake by BMECs. Uptake of [$^3$H]3MG (15 s) was measured at 5 mM (open columns) and 20 mM (filled columns) in BMECs of various culture ages. Data are mean ± S.D. (bars) values ($n$ = 3).

were supplemented with the same amount of vehicle (0.01 M HCl). The cells were preincubated with glucose-free buffer with or without insulin (10 $\mu$g/ml) for 20 min at 37°C.

The effect of insulin also was examined in 3T3-L1 adipocytes obtained from the American Type Culture Collection. Uptake experiments were carried out using confluent 3T3-L1 adipocytes differentiated from preadipocytes grown in 6-well plates in DMEM containing 10% calf serum. Differentiation was induced by the method of Frost and Lane [30].

## Results

### *Characteristics of [$^3$H]3MG uptake in BMECs*

The time courses for uptake of four glucose analogs by BMECs at 5 mM are shown Fig. 1. Rapid uptake was observed for D-type glucose analogs, while uptake of L-[$^{14}$C]glucose was significantly lower. Uptake of [$^3$H]3MG and D-[$^3$H]glucose was shown to be equilibrative and seemed to plateau after 30 s, whereas the uptake of [$^3$H]2DG continued to increase for up to 180 s.

Fig. 2 illustrates [$^3$H]3MG uptake by BMECs of various culture ages (7 to 13 days) at 5 and 20 mM. Uptake at both concentrations decreased gradually with culture age; however, an almost constant uptake was observed with confluent 9–11 day-old cultures. Uptake of5 mM [$^3$H]3MG by BMECs after a 15 s incubation was temperature-dependent with the uptake observed at 37°C (4.2 ± 0.3 nmol/mg protein) being almost 4 times greater than the uptake at 25°C (1.2 ± 0.2 nmol/mg protein). No significant specific uptake of [$^3$H]3MG was observed at 4°C (data not shown). Uptake of 5 mM [$^3$H]3MG by BMECs after a 60 s incubation was shown to be sodium-independent because the extent of uptake (15.4 ± 0.1 nmol/mg protein) when 150 mM sodium chloride was present in the medium was similar to the extent of uptake (12.2 ± 0.8 nmol mg/protein) when 150 mM choline chloride was substituted for the sodium chloride.

[$^3$H]3MG uptake increased significantly compared with control cells when BMECs were pre-incubated with an excess of unlabeled 3MG (Fig. 3) and D-glucose (data not shown). The counter-transport effect of 3MG was more dramatic than that of D-glucose. [$^3$H]3MG uptake by BMECs was concentration-dependent and saturable, as illustrated in Fig. 4. The apparent $K_m$ and $V_{max}$ were 13.2 mM and 169 nmol/mg per min, respectively.

Table I shows the effects of other sugars and metabolic inhibitors on the uptake of [$^3$H]3MG by BMECs. The uptake of [$^3$H]3MG was inhibited by D-glucose, 2DG, D-mannose, and D-xylose to a great extent (> 80%), and by D-galactose and D-ribose to a lesser extent. In contrast, L-glucose, D-fructose, and

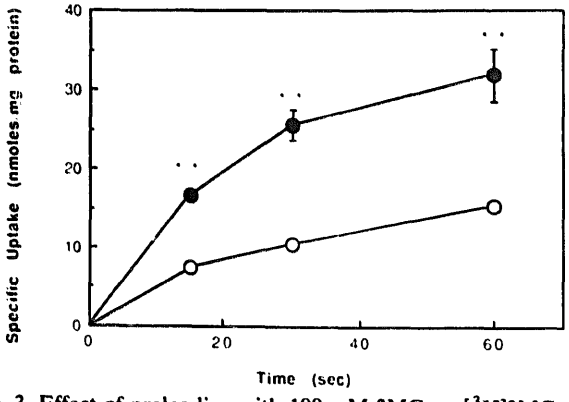


Fig. 3. Effect of preloading with 100 mM 3MG on [$^3$H]3MG uptake by BMECs. Uptake of [$^3$H]3MG was measured at 5 mM. Cells were incubated with normal buffer (control) or buffer of high substrate concentration for 20 min prior to experiments. Control (○); 100 mM 3MG pre-incubation (●). Data are mean ± S.D. (bars) values ($n$ = 3). ** $P < 0.01$.

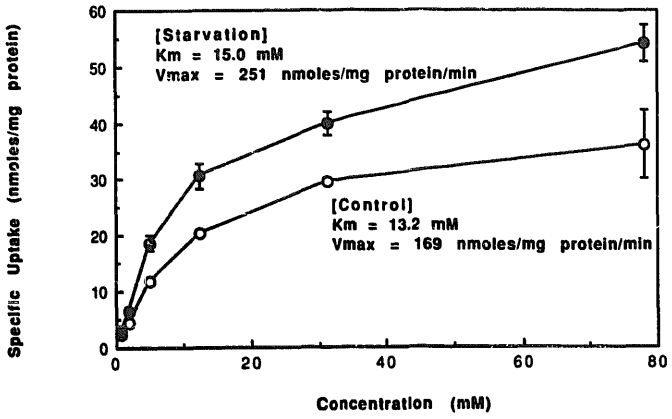


Fig. 4. Effect of substrate concentration on 3MG uptake by BMECs. Uptake of [$^3$H]3MG (15 s) was measured at various concentrations in control (○) and D-glucose-starved (●) BMECs. Kinetic parameters ($K_m$, $V_{max}$) were determined using a non-linear least-squares method. Data are expressed as mean ± S.D. (bars) values ($n$ = 3).

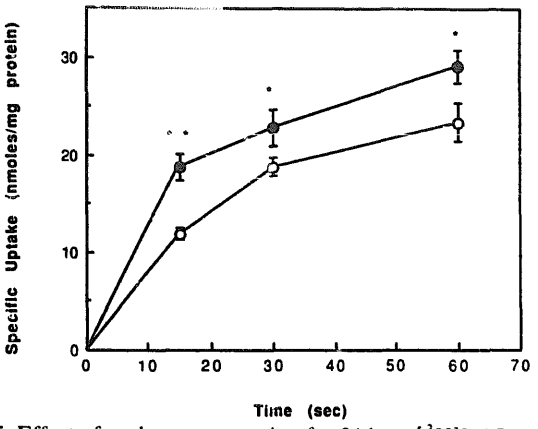


Fig. 5. Effect of D-glucose starvation for 24 h on [$^3$H]3MG uptake by BMECs. BMECs were incubated with D-glucose-free medium for 24 h before uptake experiment. Uptake of [$^3$H]3MG was measured at 37°C and a concentration of 5 mM in control (○) and D-glucose-starved (●) BMECs. Each value was expressed as a specific uptake corrected by a non-specific uptake using L-[$^{14}$C]glucose. Data are mean ± S.D. (bars) values ($n$-3). * $P < 0.05$, ** $P < 0.01$.

sucrose did not significantly affect [$^3$H]3MG uptake by BMECs. Cytochalasin B (0.02 mM) and phloretin (0.25 mM) exhibited significant inhibition of [$^3$H]3MG uptake by BMECs, whereas the uptake was not altered by phlorizin (0.25 mM), DNP (0.1 mM), or ouabain (0.1 mM).

*Effect of D-glucose on [$^3$H]3MG uptake in BMECs*

Fig. 5 shows the time courses for [$^3$H]3MG uptake in control and D-glucose-starved BMECs. [$^3$H]3MG uptake in 24 h D-glucose-starved BMECs was significantly higher (e.g., 40% at 15 s) than that in the BMECs exposed to the control condition (7.3 mM D-glucose). The equilibrium water space for [$^3$H]3MG was calculated to be 8.25 and 8.31 μl/mg protein for the control and D-glucose-starved BMECs, respectively; there was no significant difference between these values. Thus, the increase in [$^3$H]3MG uptake was not due to an increase in the equilibrium water space but to an enhanced initial rate of uptake of the hexose.

To determine the effects of intermediate levels of D-glucose on [$^3$H]3MG uptake, BMECs were treated for 4 or 7 days with 3.65 mM, 2.43 mM and 1.83 mM D-glucose. Treatment with 3.65 mM D-glucose for 4 or 7 days had no significant effect on [$^3$H]3MG uptake, while treatment with 2.43 mM and 1.83 mM resulted in a slight but significant increase (about 20%) in [$^3$H]3MG uptake (data not shown). In contrast to the effects produced by D-glucose-starvation, exposure of BMECs to high D-glucose concentration (25 mM) for up to 7 days had no effect on [$^3$H]3MG uptake (data not shown).

Fig. 6 shows the effect of different periods of D-glucose starvation on [$^3$H]3MG uptake by BMECs. The results showed that at least 12 h was required for a significant effect. In addition, the effect of D-glucose starvation increased depending on starvation time; 34, 58, and 81% increases were observed for 12, 24, and 48 h starvations, respectively. When D-glucose-starved BMECs (24 h) were refed with 7.3 mM D-glucose, the enhanced [3H]3MG uptake induced by the starvation returned gradually to control levels within 48 h after refeeding (data not shown).

To determine if protein synthesis was required for enhanced [$^3$H]3MG uptake by BMECs in response to D-glucose starvation, cells were treated with or without D-glucose in the presence or absence of cycloheximide for 24 h. Consistent with our earlier data, D-glucose deprivation for 24 h stimulated [$^3$H]3MG uptake signif-

TABLE I

*Effect of other sugars and metabolic inhibitors on 3MG uptake by BMECs*

Uptake of [$^3$H]3MG (15 s) was measured at 5 mM with or without competitive sugars or metabolic inhibitors. Data are mean ± S.D. values ($n$ = 3). n.s., not significant.

| Compounds | Concn. (mM) | Uptake (% of control) | $P$ |
|---|---|---|---|
| D-Glucose | 100 | 14.3 ± 3.6 | < 0.01 |
| L-Glucose | 100 | 95.6 ± 11.4 | n.s. |
| 2-Deoxy-D-glucose | 100 | 13.0 ± 5.6 | < 0.001 |
| D-Mannose | 100 | 12.7 ± 9.5 | < 0.01 |
| D-Xylose | 100 | 20.6 ± 5.9 | < 0.01 |
| D-Galactose | 100 | 46.0 ± 12.1 | < 0.01 |
| D-Ribose | 100 | 63.5 ± 5.5 | < 0.01 |
| D-Fructose | 100 | 78.0 ± 12.8 | n.s. |
| Sucrose | 100 | 81.2 ± 19.7 | n.s. |
| Cytochalasin B | 0.02 | 27.3 ± 4.4 | < 0.01 |
| Phloretin | 0.25 | 10.1 ± 12.2 | < 0.01 |
| Phlorizin | 0.25 | 102.4 + 12.5 | n.s. |
| DNP | 0.1 | 140.3 ± 45.9 | n.s. |
| Ouabain | 0.1 | 92.8 ± 8.0 | n.s. |

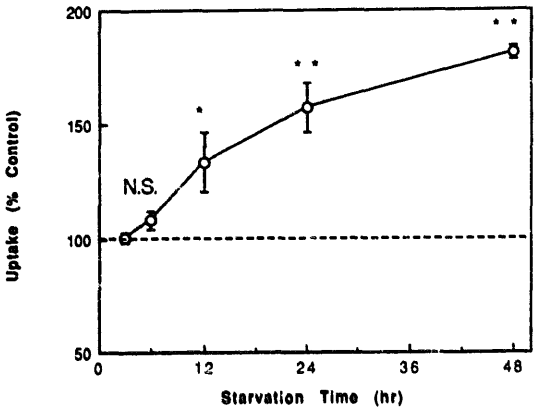


Fig. 6. Effect of D-glucose starvation on [$^3$H]3MG uptake by BMECs. BMECs were incubated with D-glucose-free medium for various time periods from 3 to 48 h. Uptake of [$^3$H]3MG (5 mM, 15 s) was measured in BMECs and data are expressed as mean ± SD (bars) values ($n = 3$) of percentage of the control. * $P < 0.05$, ** $P < 0.01$, n.s., not significant.

TABLE II

*Effect of other sugar supplement on enhanced 3MG uptake by D-glucose-starved BMECs*

All sugars were present in culture medium at 7.3 mM (initial concentration) for 24 h. All data are expressed as the mean ± S.D. of four wells. Data for each individual treatment were analyzed by Student's *t*-test. n.s., not significant.

| Sugar in culture (24 h) | 3MG uptake (nmol/mg protein) | *P* | |
|---|---|---|---|
| | | vs. no D-glucose | vs. + D-glucose |
| No D-glucose | 11.4 ± 0.5 | – | < 0.01 |
| + D-glucose | 6.7 ± 0.6 | < 0.01 | – |
| + D-mannose | 7.1 ± 0.2 | < 0.01 | n.s. |
| + D-galactose | 8.4 ± 0.5 | < 0.01 | < 0.01 |
| + D-fructose | 8.8 ± 0.7 | < 0.01 | < 0.05 |
| + 3MG | 6.0 ± 0.6 | < 0.01 | n.s. |
| + L-glucose | 11.0 ± 0.6 | n.s. | < 0.01 |
| + D-xylose | 10.4 ± 0.4 | < 0.05 | < 0.01 |

icantly (169.9 ± 7.9% as compared to 100% in BMECs treated with 7.3 mM D-glucose). This increase in [$^3$H]3MG uptake caused by 24-h D-glucose starvation could be blocked by inclusion of 0.05 μg/ml cycloheximide (116.3 ± 17.7% as compared to 169.9 ± 7.9% in BMECs treated without D-glucose and cycloheximide).

Kinetic studies on [$^3$H]3MG uptake were performed in control and D-glucose-starved BMECs (Fig. 4). The apparent $K_m$ and $V_{max}$ were calculated to be 13.2 mM and 169 nmol/mg per min for the control BMECs and 15.0 mM and 251 nmol/mg per min for the starved BMECs, respectively. An approximate 50% increase in $V_{max}$ was observed for the D-glucose-starved BMECs, while a change in $K_m$ was minimal compared with the control.

### *Effect of sugar supplement during starvation on [$^3$H]3MG-uptake in BMECs*

Table II summarizes the effects of supplementation of various sugars on D-glucose-starvation-induced increase in [$^3$H]3MG uptake in BMECs. Typically, [$^3$H]3MG uptake was significantly increased in the case of D-glucose starvation compared with the control (D-glucose treatment). No significant increase was observed when the cells were incubated with D-mannose or 3MG during D-glucose starvation. Enhanced uptake in response to starvation was not significantly affected by the presence of L-glucose, but was partially decreased by the presence of D-galactose and D-fructose, and only slightly decreased by D-xylose.

### *Expression of GLUT1 in normal and D-glucose-starved BMECs*

Total cellular expression of GLUT1 was measured by ELISA using anti-human GLUT1 antibodies and detergent-permeabilized BMECs. The specificity of the antibodies for bovine GLUT1 was demonstrated by the fact that they labeled a single band of apparent $M_r$ 54 000 on Western blots of BMEC membranes (results not shown). By ELISA, specific binding of anti-GLUT1 was significantly greater ($P < 0.001$) than control using normal rabbit antiserum at optimal dilutions 1 : 250–1 : 1000. We also found that expression of GLUT1 was dependent upon the age of the BMECs with cultures at 6–7 days expressing more than those at 10 days.

ELISA was used to measure changes in GLUT expression by BMECs in parallel to the increase in [$^3$H]3MG uptake in response to D-glucose starvation. There was no significant change in GLUT1 for 12 h when expression gradually increased, reaching a maximum of 57 ± 17% by 36 h (Fig. 7).

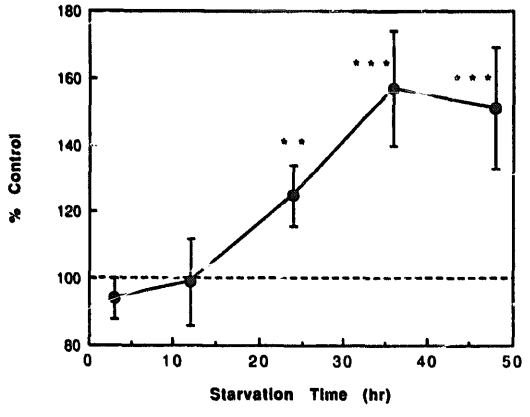


Fig. 7. Effect of D-glucose starvation on GLUT1 expression in BMECs. BMECs were exposed to D-glucose-free medium for various time periods from 3 to 48 h. The amount of total cellular GLUT1 was determined by ELISA using anti-human GLUT1 antibodies and detergent-permeabilized BMECs. Data are expressed as mean ± S.D. (bars) values ($n = 8$) as a percentage of the control. ** $P < 0.01$, *** $P < 0.001$.

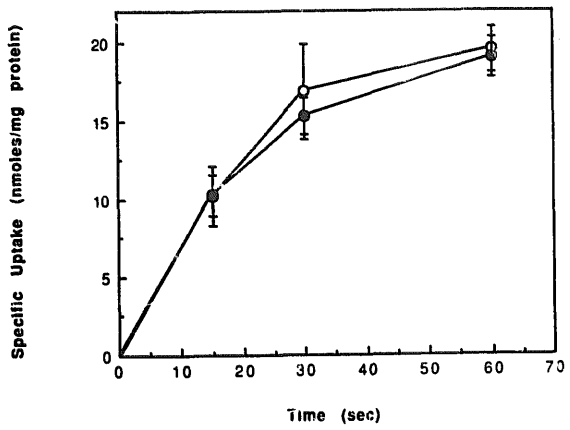


Fig. 8. Effect of insulin on [$^3$H]3MG uptake by BMECs. Prior to the experiment, BMECs were incubated with serum-free medium for 12 h. The BMECs were then pre-incubated with assay buffer with (●) or without (control; ○) insulin (10 $\mu$g/ml) for 20 min. Uptake of [$^3$H]3MG (5 mM) and [$^3$H]2DG (5 mM) was measured in BMECs and data are expressed as mean ± S.D. (bars) values ($n$ = 4).

*Effect of insulin*

The effects of insulin on uptake of [$^3$H]3MG (Fig. 8) and [$^3$H]2DG (data not shown) by BMECs were studied. Preincubation of BMECs with insulin for 20 min did not significantly affect the uptake of either glucose analog. In 3T3-L1 cells that were 30% differentiated, significant increases (150–200%) in both [$^3$H]3MG and [$^3$H]2DG uptake were observed in similar experiments (data not shown).

## Discussion

In this study, primary cultures of BMECs from bovine brains were used as an in vitro BBB model to elucidate the basic characteristics of hexose uptake and the regulation of GLUT1 by D-glucose and insulin. In preliminary studies, we evaluated four glucose analogs: D-glucose, 3MG, 2DG and L-glucose (Fig. 1). 3MG, a non-metabolizable glucose analog, and 2DG, which can be phosphorylated but not further metabolized, were chosen as glucose analogs which have affinity for the carrier of D-glucose. L-glucose was selected as a non-specific marker. Hexose uptake by bovine BMECs was shown to be highly stereospecific (D-hexoses preferential over L-hexose). Uptake of [$^3$H]3MG by the BMECs was shown to be very rapid and equilibrative. [$^3$H]2DG was taken up by the BMECs continuously since the metabolite accumulates in the cells. In the case of the natural substrate D-[$^3$H]glucose, which can be metabolized completely, the radioactivity uptake pattern was almost identical to [$^3$H]3MG uptake; however, these uptake values are probably underestimated since the radioactivity from its metabolites might be released from the cells.

From these preliminary results, 3MG was selected as a model substrate for the study of hexose uptake in the BBB model. To characterize this uptake process, uptake was carried out for 15 s, which was shown to be within the linear range (Fig. 1). BMECs grown for 9–11 days were employed for all experiments since a constant uptake was observed for these culture ages (Fig. 2). The mechanism of decreased [$^3$H]3MG uptake with culture age (Fig. 2) is unknown. However, it seems reasonable that growing cells at early ages have more nutrient uptake activity than do older confluent cells. A similar change in the expression of glucose carrier protein with culture age has been demonstrated in bovine BMECs by enzyme-linked immunosorbent assay using polyclonal antibodies against the protein (Raub, T.J., personal communication).

In BMECs cultured in normal medium (MEM/F-12 mix) supplemented with 10% plasma-derived equine serum, the equilibrium water space for [$^3$H]3MG was calculated to be 4.25 $\mu$l/mg protein. This value is in agreement with the water space of 4.2 $\mu$l/mg protein in rat brain capillaries [9] and that of 4.07 $\mu$l/mg protein in mouse cultured cerebral microvessel endothelial cells [11]. It is interesting to note that the equilibrium water space in BMECs appears to depend on culture conditions, since BMECs grown in DMEM medium with or without D-glucose and supplemented with dialyzed horse serum had equilibrium water space values for [$^3$H]3MG of 8.25 and 8.31 $\mu$l/mg protein, respectively. These differences in equilibrium water space values are probably caused by the different culturing conditions.

[$^3$H]3MG uptake by bovine BMECs was shown to be temperature-dependent and sodium-independent. These results are consistent with observations in isolated rat brain capillaries [8] and mouse cultured BMECs [11]. [$^3$H]3MG uptake was also stimulated by preloading the BMECs with an excessive concentration of D-glucose and 3MG (Fig. 3). This counter-transport phenomenon, an indication of a carrier-mediated mechanism, has been described for the BBB hexose transport in vivo [4] and in vitro [9,11]. Preloading with 3MG showed a more significant counter-transport effect on [$^3$H]3MG uptake than with D-glucose; this could be explained by the difference in metabolism of the substrates.

Saturation kinetics were observed for [$^3$H]3MG uptake, and the apparent $K_m$ and $V_{max}$ were determined to be 13.2 mM and 169 nmol/mg per min, respectively (Fig. 4). Theoretically, an accurate estimation of kinetic parameters is difficult in vitro [31]; however, the observed $K_m$ value for 3MG is comparable to that reported in rats in vivo (10 mM) [7] and in vitro (18 mM) [10]. The stereospecificity of the carrier for hexoses (Table I) was also almost identical to the results obtained in vivo [4] and in vitro [8,9,11].

The significant inhibitory effect of cytochalasin B, a non-competitive inhibitor, on [$^3$H]3MG uptake by

bovine BMECs (Table I) was similar to the published results in vivo [5] and in vitro [9,11]. Although both phlorizin and phloretin are competitive inhibitors of BBB glucose transport, phloretin (aglycon of phlorizin) is reported to be more potent than phlorizin both in vivo [4] and in vitro [9]. In the present study, 0.25 mM phloretin showed a significant inhibition of 3MG uptake by BMECs, while phlorizin was less effective at the same concentration (Table I). The lack of an effect of DNP or ouabain (Table I) on [$^3$H]3MG uptake in cultured bovine BMECs is similar to the results observed in rats in vivo [7] and in vitro [8,9]. Vinters et al. [11] reported a significant increase in [$^3$H]3MG uptake by treatment with DNP in vitro using mouse BMECs. A similar result was observed in this study; however, the DNP effect was not statistically significant.

With regard to the effect of D-glucose levels on the activity of the glucose uptake system, long-term low and high D-glucose concentrations and short-term starvation were studied. Significant adaptive changes in [$^3$H]3MG uptake by BMECs were observed in the case of D-glucose starvation (Fig. 5) or low D-glucose concentrations (data not shown). These results are similar to in vivo observations [16]. However, at high D-glucose concentrations uptake was not affected irrespective of the time of exposure (data not shown). This result is inconsistent with in vivo findings in diabetic rats [13,14]. For example, Cjedde and Crone [13] observed an approximate 30% decrease in glucose uptake by the brain of rats with chronic hyperglycemia (plasma glucose: 25.2 mM) compared with control rats (plasma glucose: 9.0 mM). McCall et al. [14] also reported that brain glucose uptake was one-third lower in rats with moderate diabetes (plasma glucose: 21.6 mM) than in control rats (plasma glucose: 8.6 mM). Despite this decrease in glucose uptake, Choi et al. [32] showed paradoxically that GLUT1 mRNA was increased in BBB capillaries from rats with experimentally induced diabetes mellitus. Although these in vivo studies concluded that increased plasma glucose was an important factor in regulating uptake of brain glucose, the present studies and the results of Choi et al. [32] suggested that other factors in vivo might be involved in decreased BBB glucose transport in diabetes mellitus.

Significant adaptive responses have been reported for various cultured animal cells when deprived of D-glucose in their growth medium [33–41]. However, to our knowledge, no information on cultured BMECs is available. When BMECs were incubated with D-glucose-free medium, [$^3$H]3MG uptake by BMECs was stimulated significantly. The increase in uptake was shown to be due to an increase in $V_{max}$ in the GLUT and not to a change in $K_m$ or water space of the cells. Furthermore, the increase in [$^3$H]3MG uptake was not observed until after 12 h of D-glucose starvation, and this response was dependent upon protein synthesis. These results indicated that D-glucose depletion resulted in an increase in the de novo synthesis of GLUT1. A similar conclusion was found using a number of different types of cultured cells [33–41]. This response was in marked contrast to increased hexose uptake by cells that had been stressed [42] or immediately following insulin treatment [42]. Under these circumstances, the rapid increase in hexose uptake was due to the translocation of an intracellular pool of GLUT to the cell surface. Matthaei et al. [43] showed that incubation of rat brain capillaries with insulin for 30 min increased intracellular GLUT but did not affect the number of cell surface GLUT. This might explain why [$^3$H]3MG uptake by BMECs was unaffected by a brief treatment with insulin. It is possible that longer incubations with insulin might have increased [$^3$H]3MG uptake in BMECs by inducing GLUT mRNA levels [43,44].

By using an ELISA which measured directly the number of total cellular GLUT1, we showed that the number of GLUT1 was increased concomitantly with the observed increase in [$^3$H]3MG uptake. Recently, Meher and Harrison [44] showed that GLUT1 mRNA levels in L6 myocytes under glucose starvation were increased 2- to 4-fold after 12 h. Our results with GLUT1 synthesis were consistent with their observations and those of Walker et al. [41].

To further characterize the effect of D-glucose starvation in BMECs, other sugar supplement experiments were carried out (Table II). The increase in [$^3$H]3MG uptake induced by D-glucose starvation was not reversed by the addition of L-glucose, which is not taken up or metabolized by the cells (Table II). However, the addition of D-mannose and 3MG to the media reduced the [$^3$H]3MG uptake activity to control levels. The enhanced [$^3$H]3MG uptake activity was partially reversed by the addition of D-galactose and D-fructose and only slightly reversed by D-xylose.

Although similar sugar supplement studies have been done in other cultured cells [33,35–38,40], consistent results have not been obtained. For instance, in chick fibroblasts, mannose and fructose reversed the effect of D-glucose depletion completely, galactose did not impair the increased uptake activity, and xylose showed an intermediate effect [34]. Germinario et al. reported that treatment with mannose and 3MG resulted in no elevation in hexose uptake, whereas the presence of D-galactose, D-fructose, and L-glucose led to normal increases in human skin fibroblasts [37,40]. In 3T3 preadipocytes, mannose reversed the starvation effect, while galactose and fructose showed no effect [38].

In general, a correlation might be expected between the ability of a sugar to reverse the enhancing effect of starvation and to inhibit the uptake of [$^3$H]3MG. However, there was no significant correlation between them

in the present study; all sugars except D-fructose and L-glucose inhibited [$^3$H]3MG uptake significantly. Although an exact mechanism is not clear at this point, it was suggested that metabolism of these sugars in the cells might affect GLUT synthesis during D-glucose starvation. However, it is especially interesting that non-metabolizable 3MG produced a significant reversal of the elevation in [$^3$H]3MG uptake, implying the existence of a mechanism independent of glucose metabolism. It is possible that 3MG might act like a metabolite of glucose, which inhibits the induction of GLUT synthesis as postulated by Germinario et al. [40].

The possible effect of insulin on BBB glucose transport is problematic [18,19]. A number of in vivo studies support the idea that insulin has no effect on glucose uptake in the BBB [2,3]. In contrast, Hertz et al. [45] showed that insulin treatment increased BBB glucose transfer in man.

Generally, in vitro experiments allow for more controllable conditions than do in vivo experiments. For example, in vivo insulin could produce direct effects on the BBB through insulin receptors on the BMECs or indirect effects through the hypoglycemia produced by the hormone. Negative insulin effects on the uptake of the glucose analogs 2DG and 3MG have been reported by several groups using isolated brain microvessels [8,9] and human and canine BMECs [12]. Our results also demonstrated that glucose uptake by cultured bovine BMECs was insensitive to a brief treatment with insulin. In contrast, Djuricic et al. [46] showed that after 20 min insulin enhanced 2DG uptake by 18-fold in isolated cerebral capillaries, and Vinters et al. [11] demonstrated that insulin stimulated 3MG uptake by cultured mouse BMECs.

Our results demonstrated that bovine BMECs were insensitive to insulin in terms of [$^3$H]3MG (Fig. 8) and [$^3$H]2DG (data not shown) uptake. The concentration of insulin and the exposure times were similar to those used by Vinters et al. [11] and Djuricic et al. [46] who have reported an insulin effect on BMECs. Significant insulin stimulation of [$^3$H]3MG and [$^3$H]2DG uptake was observed in 3T3 adipocytes, which were used as a positive control.

Facilitative transport of glucose across the plasma membrane is mediated by a family of structurally related carrier proteins with different cellular and tissue distributions [47]. Insulin-responsive tissues such as muscle and fat express a unique insulin-regulatable glucose transporter (IRGT or GLUT4); however, it is not found in the brain [48]. Recently, Slot et al. [49] showed that GLUT4 is not expressed by endothelial cells from muscle or fat. The erythrocyte-type GLUT1 is expressed mostly in brain and specifically by brain microvessels [50–54]. Our results were consistent with this.

In conclusion, the present study demonstrated that hexose uptake in cultured bovine BMECs occurs by a carrier-mediated, facilitated diffusion process, which has been shown in the BBB of other species. In addition, glucose uptake by bovine BMECs is regulated by glucose deprivation but not by high D-glucose concentrations or by insulin. The adaptive change caused by D-glucose starvation was shown to be due to increased de novo expression of GLUT1 by the cells. Thus, the in vitro model system of the BBB was shown to be useful for studying regulatory mechanisms of BBB functions at the cellular level.

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