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HEXOSE TRANSPORT IN L6 MUSCLE CELLS

KINETIC PROPERTIES AND THE NUMBER OF [^{3}H]CYTOCHALASIN B BINDING SITES

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(1) Myoblasts in culture (L6 cell line) were used as an in vitro model system, to study the kinetic and pharmacological properties of hexose transport in skeletal muscle tissue. (2) Uptake of 2-deoxy-D-[^{3}H]glucose into L6 cells grown in monolayer culture was judged rate limiting since: (a) The time course of sugar uptake extrapolated to zero, (b) a parallel inhibition of hexose uptake and phosphorylation was caused by cytochalasin B, and (c) very little backflow of the hexose was detected. (3) Uptake of 2-deoxy-D-[^{3}H]glucose by cells in monolayers was linear for at least 20 min and it was stimulated by countertransport. The K_t value was 0.83 mM. Cytochalasin B inhibited uptake non-competitively, and half maximal inhibition was achieved at 0.3 μM. Cytochalasin E (up to 5 μM) did not affect 2-deoxy-D-[^{3}H]glucose uptake. (4) L6 myoblasts, detached by trypsinization, retained the hexose transport activity. K_t in detached cells was 0.96 mM. V was 3.2 nmol/min per mg protein, and half maximal inhibition was observed with 0.25 μM cytochalasin B. (5) [^{3}H]Cytochalasin B binding to detached cells showed saturable and non-saturable components. The former could be further separated into cytochalasin E-sensitive binding (probably associated to cytoskeletal proteins) and cytochalasin E-insensitive binding, a fraction of which was inhibited by D-glucose. The D-glucose sensitive sites amount to 16.3 pmol/mg protein, and showed a K_d of 0.49 μM, which is in close agreement with the K_i of cytochalasin B inhibition of hexose uptake. These sites probably are equivalent to the hexose carrier molecules, and are present at a density of $6.8 \cdot 10^6$ sites/cell.

Introduction

Glucose is the primary energy source of skeletal muscle in the fed state. Uptake of glucose from the extracellular milieu into the cytoplasm is thought to be mediated by carrier molecules that reside on the cell membrane. Evidence for this kind of facilitated uptake in muscle stems for observations of saturability at high substrate concentrations, competition by structurally related analogs, and stereospecificity [1].

In skeletal muscle, uptake of glucose across the plasma membrane is the rate limiting step in its utilization [2]. Therefore, this stage is the target of regulatory factors such as hormones [3,4], and is also modified by muscular contraction, hyperosmolarity and anoxia [3]. Moreover, it is conceivable that, in analogy with other cell systems [5], glucose transport in skeletal muscle also varies with differentiation and growth conditions.

One possible mechanism for regulation of transport consists of modulating the number of

Abbreviations: DMSO, dimethylsulphoxide; DIDS, 4,4′-diisothiocyano-2,2′-stilbenedisulphonate.

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transporting elements on the cell membrane. Quantitation of the actual number of transporting units cannot be derived from transport measurements alone, but binding to the carrier of ligands of high affinity has been used to determine the number of transport sites in isolated cells such as erythrocytes [6–8]. The geometry of skeletal muscle precludes the homogeneous exposure of the individual fibers to externally added substrates or ligands. This has been the major reason for the paucity of knowledge of the molecular properties of hexose transport function in this tissue. An appealing alternative are myoblast cell lines which provide an adequate model system for the study of monosaccharide uptake into muscle cells. Lines of defined origin can be grown in suitable quantities, and transport measurements are not complicated by geometric factors or surgical dammage. The L6 cell line was originally established from the thigh muscle of a one-day-old rat [9]. L6 cells have been shown to possess several characteristics of skeletal muscle in vivo [10,11]. In addition, L6 cells can be studied from the myoblast stage through alignment, cell fusion and up to the stage of myotube formation.

This communication reports the use of L6 myoblasts in culture in order to characterize the kinetic and pharmacological properties of hexose uptake in muscular cells. Suspended cells were used to facilitate an even exposure of large number of cells to ligands and substrates, and the properties of hexose uptake in suspended and attached cells were compared. Finally, the suspended cell preparation was used for the quantitation of the number of hexose carrier sites, determined by binding of the inhibitor cytochalasin B.

Materials and Methods

L6 cell growth conditions. L6 cells, originally established by Yaffe [9], were provided by M. Pearson, University of Toronto. The cells were grown in 75 cm^2 flasks (Falcon, Oxnard, CA) in Eagle's minimum essential medium with 2% fetal bovine serum. The cells were incubated at 37°C, in an atmosphere of 5% CO_2. $7 \cdot 10^4$ cells were plated in 1 cm diameter plastic wells and incubated 20 to 26 h, until nearly confluent (approximately $1.25 \cdot 10^5$ cells/well). Viability, checked by Trypan blue exclusion, was > 90% under all conditions tested.

Measurement of sugar uptake in monolayer cultures. All measurements were carried out at room temperature. The medium in the wells was aspirated and the cells were washed twice with Krebs solution. For time-course determinations, 0.25 ml of 0.1 mM 2-deoxy-D-[^{3}H]glucose (8 to 40 μCi/ml) was added to each well and after incubation for the indicated times, the solution was aspirated and the cells were rapidly washed three times with ice-cold saline. For kinetic parameter determinations, 2-deoxy-D-[^{3}H]glucose at different concentrations was added to the cells for 5 min, and the wells were then rinsed three times in ice-cold saline. The uptake rate was constant for at least 5 min at all the concentrations tested. Carrier-independent transport was subtracted from all the determinations; it was estimated by adding 5 μM cytochalasin B (in DMSO) to the assay mixture. Control determinations showed that DMSO per se (up to 1% (v/v) final concentration) did not affect the transport determinations. After rinsing, the cells were digested in 0.25 ml 0.4 M NaOH, frozen thawed, and 0.20 ml aliquots were taken for liquid scintillation counting in 6 ml of Budget-Solve (Research Products International Corp.).

Uptake of 3-*O*-methyl-D-glucose was measured in the same way except that 0.1 mM of 3-*O*-methyl-D-[^{3}H]glucose was used (20 μCi/ml) and 1 mM $HgCl_2$ was added to the saline solution to inhibit efflux of 3-*O*-methyl-D-glucose during washing. This was found to be essential since this hexose can leave the cell during the washing step.

The amount of radioactive substrate trapped in the extracellular space after the protocol described above was assessed. The impermeant compounds [^{14}C]sucrose, L-[^{3}H]glucose or ^{3}H-labelled polyethyleneglycol (each at 40 μCi/ml, and either 0.1 mM or 1.0 mM) were used instead of 2-deoxy-D-[^{3}H]glucose. After a 10 min incubation period, and three cycles of rinsing, only negligible amounts of radioactivity (indistinguishable from background levels) were left in the wells. The presence of cytochalasin B during the assay did not change these results.

Due to variation in the cell number during the 20 to 26 h growth period, and to the difficulty in determining the precise number of cells in the wells, results are expressed per well rather than per

number of cells. Within each experiment, excellent reproducibility was observed.

Measurement of intracellular phosphorylated sugar. Following a hexose uptake experiment, performed as described above, cell monolayers were disrupted with 0.4 M NaOH followed by a freeze-thaw cycle. The resulting suspension was neutralized with 1 M HCl and one half of the volume was removed for total radioactivity determination. The other half was used for the assay of phosphorylated sugars as reported [12] except that 6 μl of 50 mM glucose 6-phosphate was added as carrier. This was followed by the addition of 0.2 ml of 0.15 M $Ba(OH)_2$ and 0.2 ml of 5% (w/v) $ZnSO_4$. The quantity of free 2-deoxy-D-[^{3}H]glucose was determined by counting the supernatant after centrifugation. The amount of phosphorylated 2-deoxy-D-glucose was estimated from the difference of total uptake minus non-phosphorylated hexose.

Measurement of hexose uptake in L6 cell suspensions. Three to four 75 cm^2 flasks with confluent L6 cells were digested with 0.25% Trypsin (Gibco), washed once with Eagle's minimum essential medium containing 2% fetal calf-serum, once with Krebs solution, and finally resuspended in Krebs solution at $1 \cdot 10^7$ cells/ml. At this point, cellular viability was about 90%. Uptake of 2-deoxy-D-glucose in suspension was measured by incubating 2-deoxy-D-[^{3}H]glucose solutions in Krebs with an equal volume of the cell suspension in a siliconized tube for the designated time. Cytochalasin B at a final concentration of 5 μM was used to estimate carrier-independent transport. An aliquot of the cell suspension was layered on the top of 1 ml of a mixture of dibutylphthalate/vegetable oil (10:3, v/v) and the cells were sedimented in an Eppendorff microcentrifuge for 1 min. The cellular pellets were dissolved in 250 μl of 0.4 M NaOH and counted by liquid scintillation as described above.

Binding of [^{3}H]cytochalasin B. Binding of [^{3}H]cytochalasin B to L6 cells was determined by filtration. 30 μl of a [^{3}H]cytochalasin B solution at the desired concentration were added to an equal volume of the cell suspension in the indicated medium. After 3 min, binding was stopped by the addition of 3 ml of ice-cold saline and the samples were immediately filtered through a Millipore AP prefilter and filter combination (Millipore Type EA, 1.0 μm); the filters were washed twice rapidly with saline. Both filter and prefilter were counted in Budget-Solve scintillation solution.

Experiments were performed to determine the amount of cells held by the prefilter and filter. Briefly, cells were washed in phosphate-buffered Krebs, and covalently labelled with the amino group reagent 4,4′-diisothiocyano-2,2′-[$^3H_2$]stilbenedisulphonate ([^{3}H]DIDS) (kindly provided by Dr. M. Ramjeesingh). Reaction was performed with 8 μM [^{3}H]DIDS for 30 min at room temperature. After this incubation, the cells were washed repeatedly with 5 ml of phosphate-Krebs solution containing 1 mg/ml of bovine serum albumin. When no more radioactivity eluted from the cells, they were resuspended in Tris-Krebs buffer and filtered under the same conditions used for the [^{3}H]cytochalasin B binding assay. In numerous determinations with varying cell numbers, the filter and prefilter consistently retained $>80\%$ of the cell-bound [^{3}H]DIDS radioactivity.

Other methods. Protein was determined by the method of Bradford [13] using the BioRad reagent and standard. Protein content in monolayer cultures was determined after thorough removal of medium and washing, followed by cell solubilization in 50 μl formic acid per well and collection with 200 μl H_2O. Standard curves were unaffected by the addition of this amount of formic acid.

Unless otherwise stated, the results reported are representative of at least three independent experiments performed in triplicate.

Reagents. 2-Deoxy-D-[1,2-^{3}H]glucose (30–60 Ci/mmol) and 3-*O*-[^{3}H]methyl-D-glucose (10 Ci/mmol) were purchased from New England Nuclear. [4-^{3}H]cytochalasin B (10–20 Ci/mmol) was obtained from Amersham Corporation. Unlabelled 3-*O*-methyl-D-glucose, 2-deoxy-D-glucose, L-glucose and D-glucose were purchased from Sigma Chemical Co. Cytochalasin B and cytochalasin E were obtained from Aldrich Chemical Co. All culture media and trypsin solutions were purchased from Gibco.

The composition of Krebs solution was: 122 mM NaCl, 5 mM KCl, 2.5 mM $MgSO_4$, 1 mM $CaCl_2$, 23 mM Tris (pH 7.5).

Results

A. Glucose transport in L6 myoblasts in monolayer culture

1. Kinetic parameters. Non-metabolizable analogs of D-glucose were used for the transport determinations. Fig. 1 shows the time course of uptake of 3-*O*-methyl-D-glucose into L6 cells grown in monolayers. At 0.1 mM, nearly linear uptake was observed in time when the incubations of the cells with the sugar were shorter than 1 min. Each point was calculated from triplicate determinations in the presence and absence of 5 μM cytochalasin B. In the time period analyzed uptake in the presence of cytochalasin B accounted for 5% to 25% of the total uptake. Because cytochalasin B is an inhibitor of carrier-mediated D-glucose uptake in a variety of cell types (Ref. 14, and see below) but does not inhibit diffusion of the sugar across the cell membrane, L6 myoblasts are here shown to possess a facilitated transport system (i.e. a carrier) for hexose uptake.

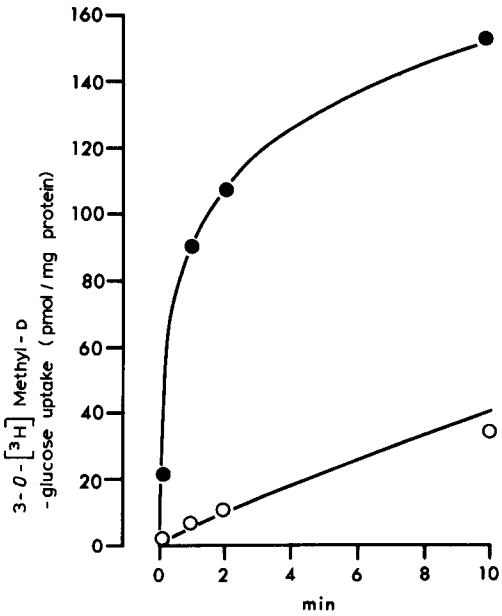

Fig. 1. Time course of 3-*O*-methyl-D-glucose uptake by L6 cells in monolayer culture. Uptake measurements were performed as described in the text (Methods): in the presence (○) or absence (●) or 5 μM cytochalasin B. Each point is the mean of a triplicate determination from which the uptake at time zero in the presence of 100 mM D-glucose has been subtracted. The results are expressed as pmol/mg protein. Each well contained 62 μg cell protein.

Uptake of non-metabolizable sugars at equilibrium allows one to calculate the value of the intracellular volume. When 0.1 mM 3-*O*-[^{3}H]methyl-D-glucose uptake was followed for up to 60 min, uptake of this hexose reached the equilibrium value of 164 pmol/mg protein in 20 min. This indicates that the equilibrium volume of 3-*O*-[^{3}H]methyl-D-glucose insde the cells is 1.64 μl/mg, or approx. 0.8 μl/10^6 cells.

The experimental protocol used involves relatively slow removal of the radioactive medium and rinsing, so it is not suitable for the accurate measurement of the rapid initial rates of 3-*O*-methyl-D-glucose uptake. 2-Deoxy-D-glucose is another non-metabolizable analog of the natural substrate D-glucose.

In metabolically active cells, this analog is taken up via the D-glucose transport system whereupon it undergoes phosphorylation by the hexokinase system [15]. In those cells the phosphorylated form of this hexose is not a substrate of the hexose-carrier, and therefore cannot leave the cell. In this fashion, a unidirectional flux of 2-deoxy-D-glucose can be measured, so long as the phosphorylating capacity exceeds the uptake rate.

Fig. 2 shows the time course of uptake of 0.1 mM 2-deoxy-D-[^{3}H]glucose by L6 cells grown in monolayer. It is observed that even after 5 min a rate of uptake of 9 pmol/min per well remains

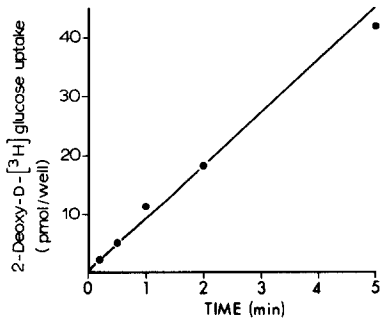

Fig. 2. Time course of uptake of 2-deoxy-D-[^{3}H]glucose by L6 cells in monolayer culture. Conditions were as in Fig. 1, except 0.1 mM 2-deoxy-D-[^{3}H]glucose (8 μCi/ml) was the assayed hexose and $HgCl_2$ was omitted, without effect, from the rinsing solution. Uptake in the presence of 5 μM cytochalasin B has been subtracted.

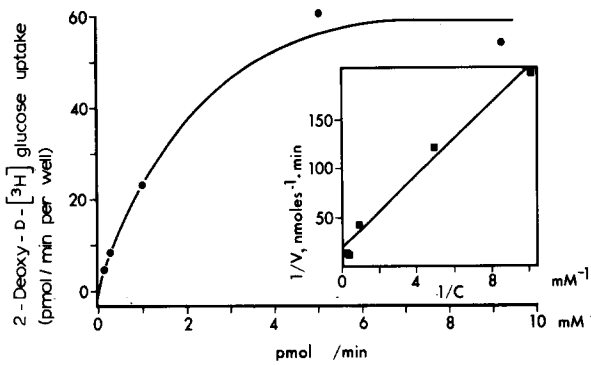

Fig. 3. Kinetics of 2-deoxy-D-[^{3}H]glucose uptake in monolayers of L6 cells. The protocol outlined in Fig. 2 was followed, using a fixed time of uptake of 2 min and varying the concentration of 2-deoxy-D-glucose in the range described in the abscissa. Uptake in the presence of 5 μM cytochalasin B has been subtracted from each point, which is the mean of three determinations. The ordinate indicates uptake per min per well. Inset: Double reciprocal plot of the uptake rate vs. concentration.

constant (in fact uptake increases linearly with time for up to 20 min). Uptake of 2-deoxy-D-[^{3}H]glucose was shown to be carrier-mediated by virtue of its susceptibility to cytochalasin B (used as described above). Nearly equal values of uptake of 2-deoxy-D-[^{3}H]glucose (0.1 mM) were detected in the presence of 5 μM cytochalasin B or 100 mM D-glucose, suggesting complete saturation of the transport system by this concentration of the natural sugar.

Fig. 3 shows the uptake of 2-deoxy-D-[^{3}H]glucose at increasing concentrations of the hexose. The measurements were made after 2 min, having determined that uptake was linear at all concentrations in that time period. A typical saturation curve was observed after subtracting the cytochalasin B-insensitive component of uptake. Analysis of the data in double reciprocal form (inset) showed a K_t of 0.83 mM and a V of 45 pmol/min per well.

In order to determine whether transport is the rate limiting step in hexose utilization, Table I analyses the effect that cytochalasin B has on total uptake of 2-deoxy-D-[^{3}H]uptake glucose and on its phosphorylation. It has been previously shown that cytochalasin B does not affect the hexokinase [15]. Therefore, in an intact cell, any effect of cytochalasin B on hexose phosphorylation should be due to a reduction in the concentration of intracellular sugar available for phosphorylation. Total uptake of 2-deoxy-D-[^{3}H]glucose was measured at two concentrations of substrate (0.1 and 1.0 mM) without or with cytochalasin B (0.1 and 0.5 μM). After 10 min, the total radioactivity taken up by the cells was split in half. One part was used to determine total uptake, and the other was used to determine the amount of phosphorylated sugar. The results indicate that at all concentrations of 2-deoxy-D-glucose and cytochalasin B, the degree of inhibition of phosphorylation paralleles quantitatively the inhibition of uptake. This is consistent with the interpretation that cytochalasin B decreases the intracellular concentration of hexose.

The experiments on Table II were designed to

TABLE I

EFFECT OF CYTOCHALASIN B ON 2-DEOXY-D-GLUCOSE UPTAKE AND PHOSPHORYLATION

[2-Deoxy-D-glucose] (mM)	[Cytochalasin B] (μM)	2-Deoxy-D-glucose uptake		Phosphorylation Inhibition (%)
		pmol/min per well [a]	Inhibition (%)	
0.1	0	5.24±0.21	0	0
	0.1	2.86±0.29	45.4	51.0
	0.5	0.76±0.03	85.5	91.0
1.0	0	20.7 ±0.7	0	0
	0.1	12.3 ±0.42	40.6	44.7
	0.5	5.62±0.6	72.8	63.3

[a] Mean±S.E. (n=3).

TABLE II

UPTAKE AND RELEASE OF 2-DEOXY-D-GLUCOSE

In the first incubation cells were incubated with 0.1 mM 2-deoxy-D-[^{3}H]glucose for 5 min. CB, cytochalasin B.

Second incubation	2-Deoxy-D-glucose uptake	
	pmol/min per well [a]	% in cells
None	3.36±0.31 (n=6)	100.0
Buffer, 4 min	3.05±0.44	90.8
Buffer, 10 min	2.69±0.30	80.0
5 μM CB, 4 min	3.28±0.33	97.8
5 μM CB, 10 min	3.01±0.05	89.6

[a] Mean±S.E. (n=3 except where indicated).

test whether a substantial intracellular pool of unphosphorylated 2-deoxy-D-[^{3}H]glucose exists inside the cell. Cells were incubated with 0.1 mM 2-deoxy-D-[^{3}H]glucose for 5 min. After this loading period, a second incubation followed in hexose-free medium. At the end of this second incubation, the amount of radioactivity in the cells was determined, and compared to that of cells that were not subjected to the second incubation. Only 10% of the accumulated cellular radioactivity was lost after 4 min, and in 10 min less than 20% was lost. Some of the leached 2-deoxy-D-[^{3}H]glucose presumably left the cells through the glucose carrier, since the exit was partly prevented by including cytochalasin B during the second incubation period.

2. Trans-stimulation of hexose uptake. For these experiments, L6 cells were loaded with 100 mM non-radioactive 3-*O*-methyl-D-glucose for 30 min, after which the extracellular medium was changed

TABLE IV

COMPETITION OF SUGARS WITH 2-DEOXY-D-GLUCOSE UPTAKE

Competing sugar (1 mM)	2-Deoxy-D-glucose uptake [a] (%)
None	100.0
D-Glucose	19.1
2-Deoxy-D-glucose	26.7
3-*O*-Methyl-D-glucose	32.0
D-Fructose	38.1
L-Sorbose	40.0
D-Xylose	40.8
L-Arabinose	54.3
D-Mannose	64.4
D-Galactose	65.3
Maltose	69.3
Sucrose	100.9
L-Glucose	118.0

[a] Measured with 0.12 mM 2-deoxy-D-[^{3}H]glucose for 2 min.

and the rates of uptake of either 2-deoxy-D-[^{3}H]glucose (0.1 mM or 0.5 mM) or 3-*O*-methyl-D-[^{3}H]glucose (0.1 mM) were determined for 5 min. Table III shows the results of this study, and compares the rates of uptake to those of cells in which 3-*O*-methyl-D-glucose was omitted from the first incubation. In all instances, uptake in 3-*O*-methyl-D-glucose-loaded cells exceeded that of control cells by a factor of 2 to 3.

3. Stereospecificity. In order to determine the stereospecificity of glucose uptake into L6 cells, the competition of different sugars with 2-deoxy-D-[^{3}H]glucose uptake was assayed. Table IV shows values of 2-deoxy-D-[^{3}H]glucose uptake in the absence and presence of a variety of sugars present at a final concentration of 1 mM. In these experi-

TABLE III

TRANS-EFFECT OF 3-*O*-METHYL-D-GLUCOSE ON HEXOSE UPTAKE

Transported species	Concn. (mM)	Hexose uptake (pmol/min per well [a])	
		Pre-incubation in buffer	In 100 mM 3-*O*-methyl-D-glucose
2-Deoxy-D-glucose	0.1	7.49±0.17	19.04±0.75
	0.5	19.15±1.67	39.80±2.80
3-*O*-Methyl-D-glucose	0.1	1.53±0.15	4.63±0.38

[a] Mean±S.E. (n=3).

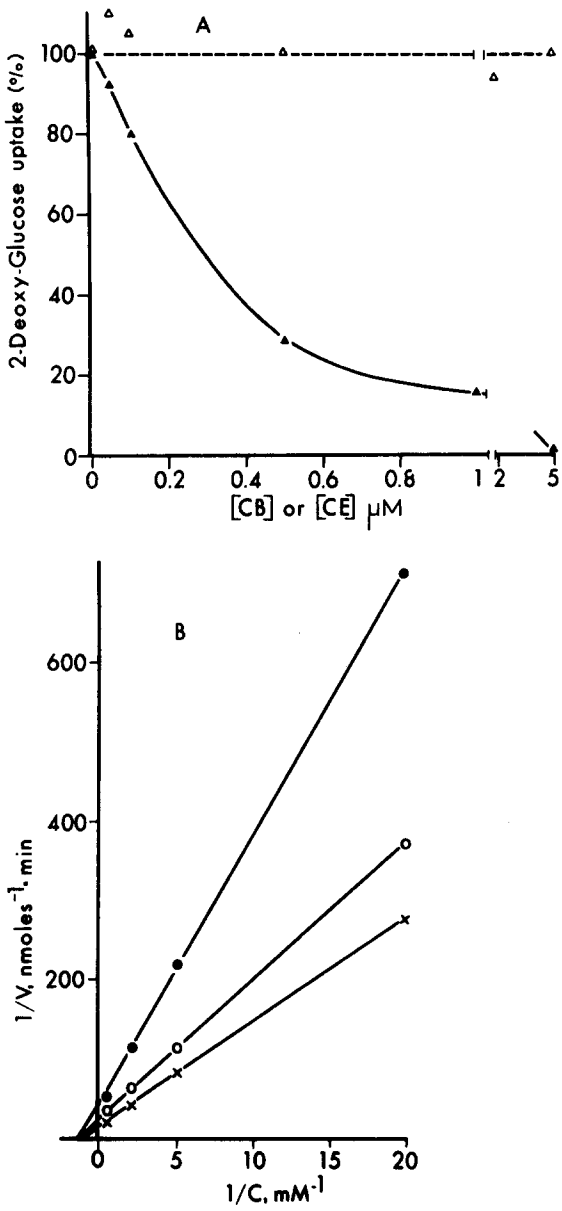

Fig. 4. Effect of cytochalasins B (CB) or E (CE) on 2-deoxy-D-[^{3}H]glucose uptake by monolayers of L6 cells. (A) Dose-response curves of cytochalasin B and cytochalasin E. Cells were pre-incubated for 5 min with the concentration of cytochalasin B (▲) or cytochalasin E (△) indicated in the abscissa. 2-Deoxy-D-[^{3}H]glycose uptake was then determined for 5 min in the presence of the drug. Points are the mean of triplicate determinations from which blanks (uptake in the presence of 100 mM D-glucose) have been subtracted. Uptake is expressed as % of the values in the absence of the drugs. (B) Effect of cytochalasin B on the kinetic parameters of 2-deoxy-D-[^{3}H]glucose uptake. Hexose concentration was varied in the range 0.05 mM to 1 mM, in the absence (×) or presence of cytochalasin B, $1 \cdot 10^{-7}$ M (○) or $5 \cdot 10^{-7}$ M (●). The triplicate determinations of each point (with blank subtracted) are plotted in double reciprocal form. Abscissa: 2-deoxy-D-glucose (mM^{-1}). Ordinate: (2-deoxy-D-glucose uptake rate)$^{-1}$.

ments we measured uptake after 2 min only in order to minimize the possible interference of the competing sugars with the hexokinase reaction following transport. Representative results of experiments performed in triplicate are presented. D-Glucose, non radioactive 2-deoxy-D-glucose and 3-*O*-methyl-D-glucose were very effective competitors of 2-deoxy-D-[^{3}H]glucose uptake. Relatively lesser interaction with the carrier was displayed by the ketohexoses D-fructose or L-sorbose. In addition, the pentoses D-xylose and L-arabinose also showed a certain degree of interference with hexose uptake. The stereospecificity of 2-deoxy-D-glucose uptake is evidenced by the lack of effect of L-glucose at this or higher concentrations (up to 100 mM, results not shown). Furthermore, the D-glucose structural isomers D-mannose and D-galactose interacted only weakly with the carrier molecule, as shown by their relatively small effect on 2-deoxy-D-[^{3}H]glucose uptake even when present in an 8-fold excess. The disaccharide sucrose was completely inefficient in inhibiting 2-deoxy-D-glucose uptake, suggesting a role for the reducing end of glucose. This was substantiated by the weak but consistent inhibition of 2-deoxy-D-glucose uptake produced by maltose (which retains a reducing glucose moiety).

4. Inhibition by cytochalasin B. This section describes the sensitivity of the glucose uptake system of L6 cells to the inhibitor cytochalasin B, its affinity for the drug, the type of the interaction and its specificity with regards to other cytochalasins.

Fig. 4A shows a dose-response curve of the inhibition of 2-deoxy-D-[^{3}H]glucose uptake by cytochalasin B. For these experiments, background uptake was measured in the presence of 100 mM D-glucose. The latter is assumed to saturate the carrier, so that the radioactivity remaining after the uptake determination solely represents entry by diffusion and extracellularly trapped 2-deoxy-D-[^{3}H]glucose. Uptake in the presence of 100 mM D-glucose represented less than 10% of the total uptake. The cells were exposed to the indicated

concentration of cytochalasin B for 5 min prior to uptake determinations. Cytochalasin B was also present during the transport assay. Half maximal inhibition of 2-deoxy-D-[^{3}H]glucose uptake was attained with 0.3 μM cytochalasin B. With 5 μM cytochalsin B, maximal inhibition was observed. In contrast to cytochalasin B, its analog cytochalasin E failed to inhibit transport in the same concentration range.

Fig. 4B is a double reciprocal plot of 2-deoxy-D-[^{3}H]glucose transport at different concentrations of cytochalasin B. The concentration of 2-deoxy-D-[^{3}H]glucose was varied and the rates of uptake were determined at 5 min in the absence or presence of cytochalasin B (0.1 or 0.5 μM). The results indicate that K_t is unaffected by the drug, whereas V decreases with increasing concentration of cytochalasin B.

B. Glucose transport in suspended L6 myoblasts

The interest in assaying the characteristics of hexose transport in suspended cells is 2-fold: (i) Changes in cell shape are known to affect transport parameters in certain cell types [16], revealing particular modes of regulation of the transport system. (ii) Large numbers of suspended cells can be accomodated in small volumes, hence reducing the requirement for incubation fluid and, most importantly, the relative volume of trapped extracellular space. This is crucial to obtain good signal to noise ratios, as is explained below for [^{3}H]cytochalasin B binding measurements.

L6 cells were resuspended as described under Methods and used for transport determinations within 15 min. Fig. 5 shows the kinetic analysis of the uptake of 2-deoxy-D-[^{3}H]glucose by these suspended cells. Fig. 5A depicts the direct relationship of uptake vs. 2-deoxy-D-glucose concentration. An Eadie-Hofstee plot of the same data is shown in Fig. 5B. The line, calculated by linear regression ($r = 0.98$) yielded values of $V = 3.21$ nmol/min per mg protein and $K_t = 0.96$ mM. An experiment was designed to compare uptake of 2-deoxy-D-glucose in monolayer and in suspension with cells from the same cellular sample. Protein was estimated in suspension, as well as in the wells after 24 h of growth. It was observed that the hexose rate of uptake in the two conditions was remarkably similar: After 5 min, the cells in monolayer culture had taken up 1.00 ± 0.01 nmol/mg protein, compared to 1.10 ± 0.12 nmol/mg taken up by the suspended cells. After 10 min, the uptake values were 2.3 ± 0.002 and 2.40 ± 0.02 nmol/mg, respectively.

The sensitivity of 2-deoxy-D-glucose uptake by suspended cells to cytochalasin B and

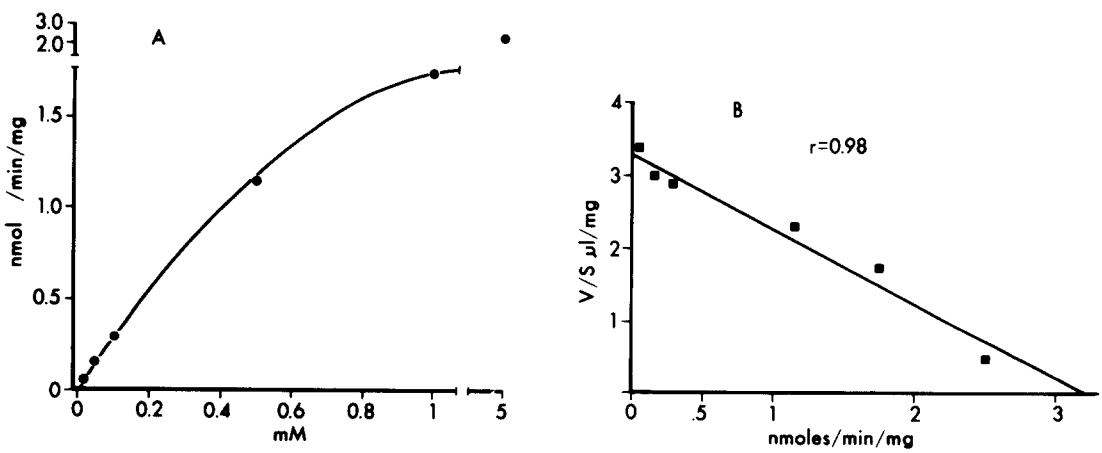

Fig. 5. Kinetic parameters of 2-deoxy-D-glucose uptake by suspended L6 cells. (A) Uptake was determined in cell suspensions as outlined under Methods. $5 \cdot 10^5$ cells in 30 μl Krebs buffer were incubated with 30 μl of deoxy-D-[^{3}H]glucose (16 μCi/ml) to yield the final concentrations expressed in the abscissa. After 5 min the cells were separated from the incubation medium through a phthalate-oil cushion by spinning for 1 min in an Eppendorff microcentrifuge. Uptake is reported in the ordinate as nmol/min per mg protein in duplicate determinations from which uptake in the presence of 5 μM cytochalasin B has been subtracted. (B) Eadie-Hofstee plot (v/S against v) of these data. The line has been fitted by linear regression analysis, and the correlation coefficient is expressed.

cytochalasin E was also tested. The results of this study were essentially similar to those obtained with attached cells. Whereas cytochalasin B inhibited 50% of the 2-deoxy-D-glucose uptake at 0.25 μM, and maximally at ≤ 5 μM, cytochalasin E was totally ineffective even at the highest concentration.

C. Binding of cytochalasin B to L6 cells

The above results have shown that cytochalasin B is an effective inhibitors of the hexose carrier activity in L6 myoblasts. The specificity at this inhibition is demonstrated by the finding that amino acid ([^{3}H]proline, α-amino[^{3}H]isobutyric acid, [^{3}H]tryptophan) uptake by L6 cells was not sensitive to cytochalasin B (not shown). Similarly, cell viability and shape were not changed by the drug. This, combined with the use of [^{3}H]cytochalasin B to identify the isolated hexose carier of red cells [17], prompted us to use [^{3}H]cytochalasin B as a specific probe for the determination of the number of hexose carrier molecules on L6 cells.

Table V summarizes results showing the specificity of [^{3}H[cytochalasin B binding to L6 cells. The cells were incubated for 3 min with varying concentrations of [^{3}H]cytochalasin B. The presence of an excess (10 μM) non-radioactive cytochalasin B is shown to decrease binding of [^{3}H]cytochalasin B to the cells indicating that at least part of the binding is saturable. Cytochalasin E (5 μM final concentration) included during the binding assay, also reduced the total binding. This is consistent with [^{3}H]cytochalasin B binding to saturable sites on the contractile elements of the cytoskeleton, which are known to bind both cytochalasin B and cytochalasin E [28]. Due to the lack of effect of cytochalasin E on 2-deoxy-D-glucose uptake, it was concluded above that cytochalasin E does not interact with the hexose carrier, and presumably does not displace [^{3}H]cytochalasin B from this site. In the experiments described hereafter, 5 μM cytochalasin E was always included during the binding assay, and specific binding was calculated by subtracting the insaturable component from the total binding.

In order to use [^{3}H]cytochalasin B to quantitate hexose carrier sites, it must be established that binding is measured at equilibrium. Fig. 6 shows the time course of binding at 0.066 and 0.33 μM [^{3}H]cytochalasin B. At either concentration, binding was at equilibrium after 2 min. In all our determinations, binding is therefore measured for 3 min.

As pointed out, when [^{3}H]cytochalasin B bind-

TABLE V

EFFECT OF CYTOCHALASINS E AND B ON BINDING OF [^{3}H]CYTOCHALASIN B

Concentration of [^{3}H]Cytochalasin B (μM)	Binding assayed in presence of		Binding of [^{3}H]cytochalasin B (cpm/mg protein)
	Cytochalasin B (10 μM)	Cytochalasin E (5 μM)	
0.05	−	−	2382
	−	+	986
0.1	−	−	4353
	−	+	1807
	+	−	328
0.3	−	−	11253
	−	+	3860
0.6	−	−	23902
	−	+	3860
1.2	−	−	52897
	−	+	26284
	+	−	9939

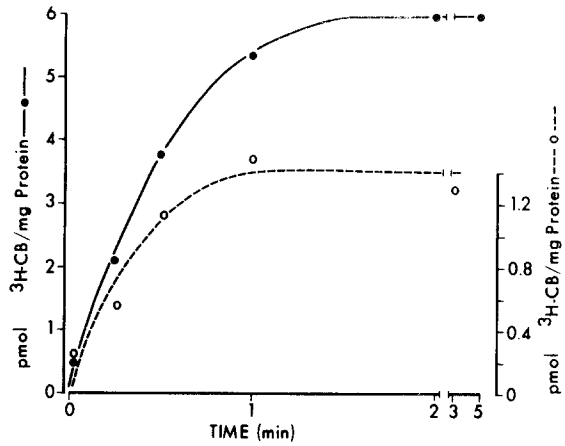

Fig. 6. Time course of [^{3}H]cytochalasin B binding to suspended L6 cells. Suspended L6 cells were incubated with [^{3}H]cytochalasin B at a final concentration of $6.6 \cdot 10^{-8}$ M (○) or $3.3 \cdot 10^{-7}$ M (●). The assay medium consisted by Krebs solution containing 5 μM cytochalasin E. The incubation was carried on for the indicated time period prior to dilution and filtration as described under Methods. The value of the non-saturable component of binding was estimated in control assays in the presence of 5 μM cytochalasin B and has been subtracted in all points.

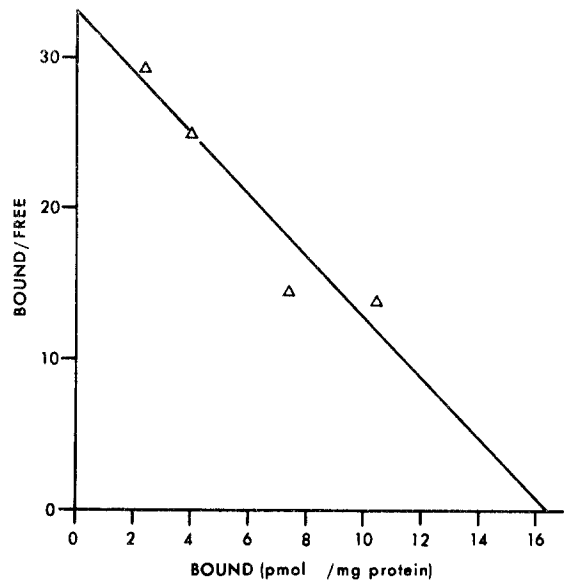

Fig. 7. Binding of [^{3}H]cytochalasin B to the hexose carrier of L6 cells. [^{3}H]Cytochalasin B binding was determined at several concentrations of cytochalasin B as outlined in Table VI. [^{3}H]Cytochalasin B binding in the presence of D-glucose has been subtracted from the value of binding in L-glucose, and is plotted in Scatchard form. At all points, the amount of [^{3}H]cytochalasin B bound was less than 5% of the total [^{3}H]cytochalasin B present. The line has been fitted by least-square analysis.

ing to whole cells is determined, several cell elements other than the hexose carrier could bind the drug. Although part of this binding can be estimated using other cytochalasins (as above), a fraction of the glucose carrier-independent binding could be cytochalasin E-insensitive. Therefore, it is desirable to have an additional criterion of specificity of binding to the hexose carrier. Table VI shows experiments designed with that aim:

TABLE VI

EFFECT OF D- AND L-GLUCOSE ON [^{3}H]CYTOCHALASIN B BINDING

[[^{3}H]Cytochalasin B] (μM)	[^{3}H]Cytochalasin B bound [a] (pmol/mg protein)					
	In the presence of				Differential binding L − D	
	L-Glucose		D-Glucose			
	Total	Saturable [b]	Total	Saturable	Total	Saturable
0.082	6.34	4.69	3.82	2.27	3.06	2.42
0.160	12.63	9.44	7.42	5.38	5.21	4.06
0.519	26.62	16.48	17.21	9.06	9.41	7.42

[a] Average of duplicate determinations, assayed in the presence of 5 μM cytochalasin E, in either 280 mM D- or L-glucose, containing 20 mM Tris-HCl (pH 7.5).

[b] Binding in the presence of 5 μM cytochalasin B subtracted from total binding.

Binding of [^{3}H]cytochalasin B to L6 cells was performed in the presence of either D- or L-glucose. The former is expected to displace the drug from the hexose carrier, with which L-glucose does not interact (see Table IV). Both total and saturable binding are reported. The data indicate that D-glucose but not L-glucose, effectively reduces the binding at all [^{3}H]cytochalasin B concentrations assayed. The last column shows the specific binding to the hexose carrier calculated by subtracting the D-glucose insensitive component from the total binding. Similar data are presented in Fig. 7 as a Scatchard plot, yielding a straight line (fitted by least squares) with a correlation coefficient of 0.94. The calculated values of this specific [^{3}H]cytochalasin B binding to L6 cells are: $K_d = 0.49$ μM and a maximum number of binding sites of 16.4 pmol/mg protein.

Discussion

Sugar uptake into skeletal muscle has so far resisted detailed characterization because of the unavailability of a muscle preparation that yields sufficient numbers of isolated cells amenable to in vitro transport studies. The approach presented in this study considers the use of myoblasts of the L6 cell line, as a plausible alternative model system for the study of hexose transport. The data obtained with L6 myoblasts can be summarized as follows: Hexose transport is mediated by a carrier, with a K_t of 0.83 to 0.93 mM, and $V = 3$ nmol/mg protein. The selectivity of hexose uptake into L6 cells (Table IV) resembles that of adult muscle (glucose > mannose > galactose > maltose) [23], suggesting that the mean features of hexose uptake of muscle are conserved in the cell line. Uptake of 2-deoxy-D-glucose was 50% inhibited by 0.3 μM cytochalasin B in L6 cells. Table II showed that not only uptake, but hexose efflux as well, was sensitive to cytochalasin B. Inhibition by cytochalasin B was structurally selective since the analog cytochalasin E did not affect 2-deoxy-D-glucose uptake. Hexose uptake was susceptible to *trans*-stimulation, increasing 2- to 3-fold when the cells were previously loaded with the non-metabolizable hexose 3-*O*-methyl-D-glucose (Table III). This property of the myoblast cell membrane was also observed in intact muscle [1], as well as in embryo heart cells [24], and recently in several cell lines [29], in sharp contrast to the findings in hepatocytes [25]. Studies on regulation of hexose transport in L6 cells under conditions that control glucose uptake in muscle, are currently underway.

A. Is hexose uptake rate limiting in its utilization by L6 cells?

Only initial rates of transport yield information pertaining the unidirectional translocation of the substrate across the membrane. 3-*O*-Methyl-D-glucose would be the ideal hexose to use in a transport study, since it is transported but not metabolised. However, due to the limited capacity of the intracellular volume (1.64 μl/mg), the cells fill up so fast that backflow becomes considerable even at short periods of time. This compromized severely the accuracy of measurement of initial flow rates (as seen in Fig. 1). The second hexose tested in this study was 2-deoxy-D-glucose, which can be phosphorylated inside the cell. The phosphorylated form of the sugar is uncapable of leaving the cell through the glucose carrier, so that back-flux is negligible. This makes it possible to measure adequately initial uptake rates, provided that the rate of sugar phosphorylation substantially exceeds that of transport. Three types of tests were performed to ensure that this is the case under our experimental conditions:

(i) The line of Fig. 2 intercepts the ordinate at a value not significantly different from zero. If transmembrane uptake of the hexose were considerably faster than its phosphorylation, an intracellular pool of the non-phosphorylated sugar would build up, only a fraction of which would be removed continuously for phosphorylation. In this case, extrapolation of hexose uptake at zero time yield a value different from zero, approaching the intracellular space at high transport rates (This latter value was found to be 15 pmol/well). Clearly, Fig. 2 shows that this is not the case for 2-deoxy-D-glucose uptake, which can therefore be regarded as slowly being transported across the membrane, and immediately phosphorylated upon entry. This result implies that transport of 2-deoxy-D-glucose was rate limiting in our uptake determinations. This is in contrast to the findings in chick embryo cardiac cells in culture, in which case the extrapolation failed to cross the origin, favoring phosphorylation as rate limiting in hexose uptake [24].

TABLE VII

HEXOSE TRANSPORT PROPERTIES IN SEVERAL CELL TYPES

Abbreviations used for hexoses: D-Glu, D-glucose; 2-d-G, 2-deoxy-D-glucose; 3-*O*-M, 3-*O*-methyl-D-glucose. The units in which V is expressed are given in the footnotes. The inhibition was non competitive (nc.) or competitive (c.). The units in which the numbers of sites are expressed are given in the appropriate footnotes. CE, cytochalasin E.

Cell type	Hexose transport			Cytochalasin B inhibition and binding					Refs.
	Hexose	K_t (mM)	V	K_i (M) ($\times 10^7$)	K_d (M) ($\times 10^7$)	No. of sites	D-Glu-sensitive	CE-sensitive	
Erythrocyte, human	D-Glu	1.6–2.8	3.6 [a]	0.7 nc.	4.9	$3.3 \cdot 10^5$ [h]			6, 7, 40
Membrane				3–7	I 4.0	200 [i]	Yes	No	6, 32
					II 1.8	75 [i]		Yes	
					III 0.3	75 [i]			
Adipocyte, rat	3-*O*-M	1.5–5	0.005–0.05 [a]						41, 42
Plasma membrane				5 n.c.	6	4 [j]	Yes	No	20, 43
Fibroblast									
Chick embryo	2-d-G	2	18–95 [b]	1.5 c.	Range	1.6 [j]	Yes		12, 44
Human	2-d-G	1.9–3.1	10–24 [b]						46, 47
Hepatocyte, rat									
Isolated	3-*O*-M	18–20	79–86 [c]						45
In culture					I 3	245 [j]	No	No	38
					II 113	1600 [j]		Yes	
Lymphocyte, human	3-*O*-M	1.7	$33 \cdot 10^6$ [c]	7 c.	L 30	$7 \cdot 10^7$ [h]		Yes	34
					M 2–4	$6.5 \cdot 10^6$ [h]	Yes	No	
					H 0.75	$4.5 \cdot 10^6$ [h]	No		

Ehrlich ascites tumor cells	D-Glu	14	$180 \cdot 10^6$ [c]	5	4–6	$14 \cdot 10^6$ [h]	Yes	No	48
Platelet, human				0.8 nc.	Range	10^4			7
L6 myoblast	2-d-G	0.8	0.45 [a] or 0.75 [b]	0.3 nc.	4.9	16.4 [j] or $6.8 \cdot 10^6$ [h]	Yes	No	This paper
Giant muscle fiber, barnacle	3-*O*-M	5	1 [d]	50					26
Sartorius muscle, frog	3-*O*-M	3–4	$(17–117) \cdot 10^3$ [c]						50
Hemidiaphragm, rat	3-*O*-M		3 [e]						51
Soleus muscle, rat	D-Glu	>40	0.014 [f]						52
Myotubes, chick embryo, cultured	D-Glu	10–20	54–84 [b]						30
Cardiac cells, chick embryo	2-d-G	2	13 [b]	Range nc.					24, 39
Heart, chick embryo	2-d-G	10–40	0.4–0.8 [g]						49

[a] nmol/min per 10^6 cells.
[b] nmol/min per mg protein.
[c] nmol/min per litre.
[d] nmol/min per cm^2.
[e] mM; % penetration per 30 min.
[f] pmol/min per g tissue.
[g] nmol/min per mg wet wt.
[h] sites/cell.
[i] pmol/mg dry wt.
[j] pmol/mg protein.

(ii) Kletzien and Perdue [12] have developed a test for determining the rate limiting step in 2-deoxy-D-[^{3}H]glucose uptake into fibroblasts. It is based on the premise that changes in the transport step will modify the subsequent phosphorylation of 2-deoxy-D-glucose only when no steady pool of this hexose exists intracellularly (i.e. only when transport is the rate limiting step). Table I showed that cytochalasin B inhibited 2-deoxy-D-glucose uptake and phosphorylation to the same extent at different concentrations of the substrate and of the inhibitor. This supports the tenet that in L6 myoblasts, transport or 2-deoxy-D-glucose, and not phosphorylation, is the rate limiting event.

(iii) Table II showed that after a 5 min load period under our usual uptake conditions, efflux of the hexose for the next subsequent 4 min amounts to less than 10% of the total uptake. This observation implies that only a small fraction of the hexose is not phosphorylated and that under our usual uptake conditions, less than 10% backflow can occur.

On the basis of the arguments discussed above, we conclude that transport of 2-deoxy-D-glucose by L6 myoblasts is rate-limiting, and that conditions are met in which initital rates of uptake are measured.

Uptake rates of 2-deoxy-D-[^{3}H]glucose in suspended L6 cells equalled those of attached cells. K_t for uptake in plated cells was 0.83 mM, which is not significantly different from $K_t = 0.96$ mM determined in suspended cells. The close agreement obtained between the rates of uptake in suspended and attached cells (see p. 272) implies that the maximal velocities were also equivalent. This suggests that neither shape nor interaction with the substratum play a major role in regulating the activity of the hexose carrier in these cells. Furthermore, the fact that the velocity of transport is not diminished by trypsinization and cell suspension suggests that hexose carrier molecules are not affected (i.e. hydrolysed or removed) by this treatment.

B. Quantitation of hexose carrier entities by [^{3}H]cytochalasin B binding

The number of transporting entities in the membrane cannot be determined solely from transport velocity measurements. Most commonly, inhibitor binding determinations have been used for this purpose. In the case of the hexose carriers of non-epithelial tissues, [^{3}H]cytochalasin B has proven to be an adequate ligand. As a prerequisite for its use as a label in L6 cells, we determined the specificity of cytochalasin B towards the sugar carrier. Control experiments showed that viability and shape were not affected and that uptake of amino acids was not inhibited by up to 5 μM cytochalasin B. On the other hand, cytochalasin B has been reported to detach myoblasts from mixed primary cultures with fibroblasts [31]. The concentration used in those studies was 5 μg/ml, or approx. 10 μM. In our hands, L6 cells barely detached with 20 μM cytochalasin B or cytochalasin E for up to 30 min. This difference in sensitivity may be due to differences in the primary culture of myoblasts as compared with the myoblast cell line. At any rate, in the 10^{-7} to 10^{-6} M range, cytochalasin B does not appear to affect L6 cells unspecifically.

Experimental difficulties involving the number of cells and the signal to noise ratio preclude the determination of [^{3}H]cytochalasin B binding to plated cells. In addition, due to the reversible nature of the binding, a fast method is required to separate extracellularly trapped [^{3}H]cytochalasin B from that bound to the cells. Filtration of suspended cells proved faster than rinsing plated cells.

This communication showed that [^{3}H]cytochalasin B binds to suspended L6 cells. Binding displayed a saturable and a non-saturable component. The former represented between 30% to 50% of the total binding, in the [^{3}H]cytochalasin B concentration range assayed. This saturable component was further dissected into cytochalasin E-sensitive and cytochalasin E-insensitive binding sites. A fraction of the saturable sites was cytochalasin E-sensitive, a characteristic of cytoskeletal elements [28] but not of the hexose carrier function (Ref. 32 and Fig. 4A). Therefore, the cytochalasin E-insensitive [^{3}H]cytochalasin B-binding sites must include the hexose carrier. The latter was finally quantitated by virtue of the displacement of [^{3}H]cytochalasin B by D-glucose (but not by L-glucose). Similar effects of D-glucose have been observed in isolated membranes of erythrocytes [32] and hepatocytes [33], as well as in lymphocytes [34] but not in membranes of adipo-

cytes [20], nor in intact normal and virus transformed mouse cells [35], rat hepatoma cells [36], polymorphonuclear leukocytes [37], cultured hepatocytes [38], nor normal human diploid fibroblasts [44]. The fact that [^{3}H]cytochalasin B binding to intact L6 myoblasts displays D-glucose sensitivity could help to determine the number of hexose carrier molecules during physiological regulation that requires cellular integrity. The D-glucose-sensitive sites of L6 myoblasts amount to 16 pmol/mg protein, and have a K_d of 0.49 μM. The close agreement of this K_d value with the K_i of inhibition of 2-deoxy-D-glucose uptake by cytochalasin B, strongly supports the identity of this group of binding sites with the hexose carrier. The determined K_d value is very similar to that found in other cell types (see Table VII). Knowing the number of binding sites per unit protein (16.4 pmol/mg) and the protein content per cell (0.69 mg/10^6 cells), we arrive at a number of $6.8 \cdot 10^6$ binding sites/cell, a value 20 times higher than that reported for red cells, and about 50 times higher than that of adipocytes. The richer content of hexose carrier molecules in L6 myoblasts should make these cells a highly suitable system for studies of the hormonal regulation of hexose transport.

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