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RATE-LIMITING STEPS OF 2-DEOXYGLUCOSE UPTAKE IN RAT ADIPOCYTES

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Summary

2-Deoxy[1-^{14}C]glucose uptake in rat adipocytes was measured as a function of time in the absence and presence of unlabelled glucose or 2-deoxyglucose. Uptake of tracer alone was linear from 2 s to 6 min. At 37 °C the rate of uptake in insulin-stimulated cells decreased markedly after a few seconds in the presence of glucose (0.5—10 mM) and after 0.5—2 min in the presence of deoxyglucose (2—10 mM). Similar data were obtained at 22 °C. With 10 mM glucose (37 °C, 30 s) approx. 80% of the intracellular radioactivity was non-phosphorylated deoxyglucose and with 10 mM deoxyglucose approx. 40% was non-phosphorylated. The results show that deoxy[^{14}C]glucose uptake after a few minutes is mainly limited by hexokinase in the presence of glucose and at least partially in the presence of deoxyglucose. The data suggest caution in using deoxyglucose uptake as a measure of transport, especially in complex kinetic studies.

In addition, the initial velocity of tracer * 3-*O*-methylglucose was found to be approx. 2-fold higher than that of tracer deoxyglucose even though both sugars inhibited the initial velocity of labelled methylglucose half-maximally at a concentration of 5 mM. These data suggest a fundamental difference between deoxyglucose and methylglucose transport.

Introduction

In recent years many investigators have sought to measure glucose transport as a function of insulin concentration in isolated adipocytes. Since conversion

Abbreviation: Hepes, *N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulphonic acid.
* A concentration much less than K_m for transport.

of labelled glucose to its final metabolic products in adipocytes cannot always be taken as a measure of glucose transport, other methods of estimating the glucose transport have been developed [1—13]. Some of these methods utilize non-metabolizable sugar analogues, such as methylglucose, L-arabinose and D-allose [1—6]. The transport proceeds by carrier-facilitated diffusion resulting in an exponential rise with time of the intracellular concentration of non-metabolizable sugars. Therefore, the rate of transport is only equal to the rate of uptake during early time periods when efflux is negligible [1—4].

Since the intracellular water space in adipocytes is so low (2—4% of the cell volume [14]) and the insulin-stimulated transport rate of methylglucose is so rapid, initial rates must be measured within 1—2 s under these conditions [1,2]. Transport of sugars with lower affinities, such as L-arabinose and D-allose, can be measured during longer time periods, but they are not suitable for measuring some kinetic constants [3,4].

In an effort to avoid these problems other investigators have utilized labelled 2-deoxyglucose, which is transported with high affinity and phosphorylated but not further metabolized, to measure transport kinetic constants of unlabelled sugars, including those of glucose [7—13]. The labelled deoxyglucose phosphate is trapped in the cell, and as long as the rate of phosphorylation by hexokinase is rapid enough to prevent any accumulation of non-phosphorylated deoxyglucose, the rate of uptake can be taken as a measure of unidirectional transport. If, on the other hand, transport is not strictly rate-limiting, the concentration of non-phosphorylated sugar in the intracellular water will start to increase from the beginning of the incubation. This increase will lead to phosphorylation rates which progressively approach the maximal capacity for phosphorylation and to increasing rates of efflux of deoxy[^{14}C]-glucose. The rate of uptake of deoxy[^{14}C]glucose (influx minus efflux) will therefore decrease progressively until, at steady state, the rate of uptake may represent only a small fraction of the unidirectional transport from the extracellular medium. It should be noted in this connection that the affinities of glucose and deoxyglucose to hexokinase are believed to be high as compared to their affinities to the transport system [15]. Therefore, a high fraction of the total phosphorylation capacity may be attained even with relatively small intracellular concentrations of non-phosphorylated sugar.

The present report was directed towards determining whether transport or phosphorylation is the rate-limiting step in deoxy[^{14}C]glucose uptake in the presence of varying concentrations of glucose and deoxyglucose. In addition, a comparison between the initial velocities of deoxy[^{14}C]glucose and [^{14}C]-methylglucose uptake (1 s incubations) is presented.

Methods

Adipocytes were obtained from epididymal fat pads of male Wistar rats, weighing 150—180 g, fed ad libitum. Hepes buffer (pH 7.4) with and without bovine serum albumin (10 mg/ml) was prepared as described previously [1]. Collagenase (type I) was from Worthington, porcine insulin was from Nordic Insulin, 2-deoxyglucose phosphate from Sigma, and phloretin from K and K Laboratories. 3-*O*-[^{14}C]Methyl-D-glucose (59—60 mCi/mmol) and 2-deoxy-

D-[1-^{14}C]glucose (54—60 mCi/mmol) were from the Radiochemical Centre, Amersham. Other chemicals were analytical grade.

Adipocyte preparation

Epididymal fat pads were added to 30-ml polystyrene cylinders containing 3 ml of 3.5% albumin buffer with 0.5 mg/ml collagenase and then minced. A smooth Teflon bar (6 × 15 mm) was added and the tissue was stirred at 37°C for 1 h. Stirring was at a rate which just kept the suspension mixed. The cell suspension was then passed through a nylon filter (PES 3000 polymen) and washed five times with 1% albumin buffer. Insulin, when present, was added to give a final concentration of 100 nM, i.e., a maximally stimulating concentration. Cells were allowed to incubate for 30 min at 37 or 24°C before beginning the experiment.

Sugar uptake I

The uptake (1—30 s) of labelled methylglucose or deoxyglucose was measured as described by Whitesell and Gliemann [1] with some modifications. Transport was stopped with 400 μl of phloretin (0.1 mM) and a 400 μl aliquot of the mixture was added to a microcentrifuge tube (550-μl) containing 100 μl of silicone oil (density 0.99 g/l, viscosity 100 cS). The microcentrifuge tube was spun within 2 min in a Beckman microcentrifuge for 30 s, cut through the oil phase and the cell pellet was added to a 3 ml vial with 2.5 ml of scintillation fluid. Zero time was determined by adding 400 μl of phloretin solution to the isotope solution before adding cells. Under these conditions the extracellular space (i.e., zero time) constituted 6% of the intracellular water space in the cell pellet. The coefficient of variation on 1 s measurements (total counts minus extracellular counts) was 5—10%. These measurements were made either at 22°C or in a room maintained at 37°C.

Sugar uptake II

The uptake of deoxy[^{14}C]glucose from 30 s to 6 min was measured in 20-ml polyethylene vials placed in a shaking water bath maintained at either 22 or 37°C. 500-μl of cell suspension (14—20%, v/v) containing 100 nM insulin were added to the vials containing 500 μl of 1% albumin buffer with 0.35 μCi of deoxy[^{14}C]glucose and, when indicated, glucose or deoxyglucose. 15 s before the end of the incubation, a 400 μl aliquot was added to a microcentrifuge tube containing 100 μl of silicone oil and spun for 15 s.

Sugar uptake III

30-s measurements of intracellular deoxyglucose and deoxyglucose phosphate * were made using method I except that the phloretin solution was at 0°C. After the addition of the 400 μl of phloretin, a 400 μl aliquot of the phloretin/cell mixture was immediately layered on to a Millipore filtering apparatus also maintained at 0°C. The filter (pore size 0.8 μm) was rinsed with 3 ml of ice-cold phloretin solution and then quickly dropped into 3 ml of boiling water. Four such filters were prepared and pooled in one extraction.

* All labelled phosphorylated sugar derivatives are included in the term deoxyglucose phosphate.

After 5 min the extract was allowed to cool, and centrifuged (60 000 × g × min). 500-μl aliquots of supernatant were either counted directly or layered on to a 0.7 × 4 cm anion exchange column (Bio Rad AG 1-8 W resin equilibrated with formate and washed with 1 mM deoxyglucose). The column was washed with five 0.5 ml aliquots of 1 mM deoxyglucose. The effluent was collected as one fraction and mixed with 9 ml of Unisolve 100 (Koch-Light laboratories) for scintillation counting.

The ability of the resin to separate deoxyglucose from deoxyglucose phosphate was tested in the following ways. Deoxy[^{14}C]glucose, when added to an extract of non-incubated cells, was recovered 98—99% from the column; and when subjected to paper chromatography (isobutyric acid/H_2O/conc. NH_4OH, 66 : 33 : 1.5 [16]) it travelled as one spot distinct from deoxyglucose phosphate. The following attempts were made to detect the presence of deoxyglucose phosphate in the effluent. 2 mmol deoxyglucose phosphate were run throught the anion exchange column, the effluent lyophilized, reconstituted to a small volume and subjected to paper chromatography. No spot was observed when tested for the presence of sugar phosphate [17], whereas a standard of 0.2 μmol deoxyglucose phosphate gave a discernable spot. These results were verified by assaying the reconstituted effluent for sugars (Ref. 18 performed by Dr. K.E. Jørgensen, University of Aarhus). No sugar could be detected, whereas an amount of less than 1% of that applied to the column was easily detectable.

Calculation of the intracellular deoxy[^{14}C]glucose concentration as a fraction of the extracellular concentrations

Cells from the same pool were incubated with [^{14}C]methylglucose for 0 s and 20 min (equilibrium) using method I, and with deoxy[^{14}C]glucose in the absence or presence of unlabelled sugar for 0 s and the appropriate time (t = 30 s) using method I plus method III. The fraction of intracellular radioactivity at time t representing non-phosphorylated deoxy[^{14}C]glucose was calculated from the method III deoxyglucose measurements as:

$$F_a = \frac{\text{cpm (time } t) - \text{cpm (time 0) passed through the column}}{\text{cpm (time } t) - \text{cpm (time 0) applied to the column}}$$

The intracellular concentration of non-phosphorylated deoxy[^{14}C]glucose as a fraction of the extracellular concentration was calculated from F_a and the deoxyglucose and methylglucose method I measurements as:

$$F_c = F_a \cdot \frac{\text{2-DG uptake (time } t) - \text{2-DG 'uptake' (time 0)}}{\text{3-O-MG (uptake (time } t) - \text{3-O-MG 'uptake' (time 0)}}$$

where 2-DG represents 2-deoxyglucose and 3-O-MG represents 3-*O*-methylglucose.

Results

In order to study the effect of glucose on deoxyglucose uptake, trace deoxy-[^{14}C]glucose uptake was measured in the presence of 0.1, 0.5, 1.0 and 10 mM glucose from 30 s to 6 min at 37°C in insulin-stimulated cells (Fig. 1). In each case the uptake with time appears linear and 10 mM glucose results in a nearly

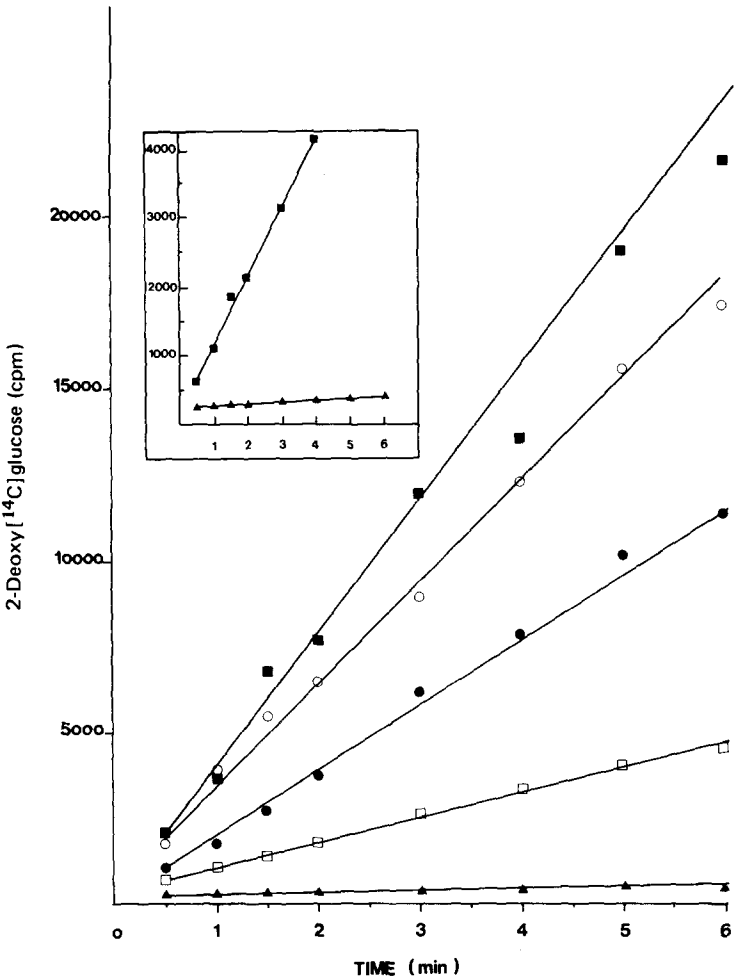

Fig. 1. *Effect of 0.0* (■), *0.1* (○), *0.5* (●), *1.0* (□) and *10.0* (▲) mM glucose on uptake of 7 μM deoxy-[^{14}C]glucose (0.5—6 min) in insulin-stimulated cells at 37°C. Measurements were made as described in Methods (sugar uptake method II) and the data expressed as cpm per sample. The insert shows results at 22°C with 0.0 (■) and 10 (▲) mM glucose.

total inhibition of deoxy[^{14}C]glucose uptake. If uptake is taken at 3 min, the inhibition constant (K_i) for glucose is calculated to be approx. 1 mM which is in good agreement with previous measurements at 24°C [12]. However, it should be noted that the lines representing incubations with glucose do not extrapolate through 0.0, and the calculated K_i values decrease with time to approx. 0.4 mM using the 6 min uptake measurements. Such behaviour would not be expected if transport were the only rate-limiting step. The insert in Fig. 1 shows essentially the same phenomenon at 22°C.

Fig. 2 shows deoxy[^{14}C]glucose uptake with time in the absence and the presence of 10 mM glucose from 1 to 30 s. The uptake of tracer alone appears linear, but in the presence of 10 mM glucose the rate is progressively reduced until a new constant value is obtained after 15 s. This final rate approaches that attained between 30 s and 6 min. Since most of the uptake occurs within the

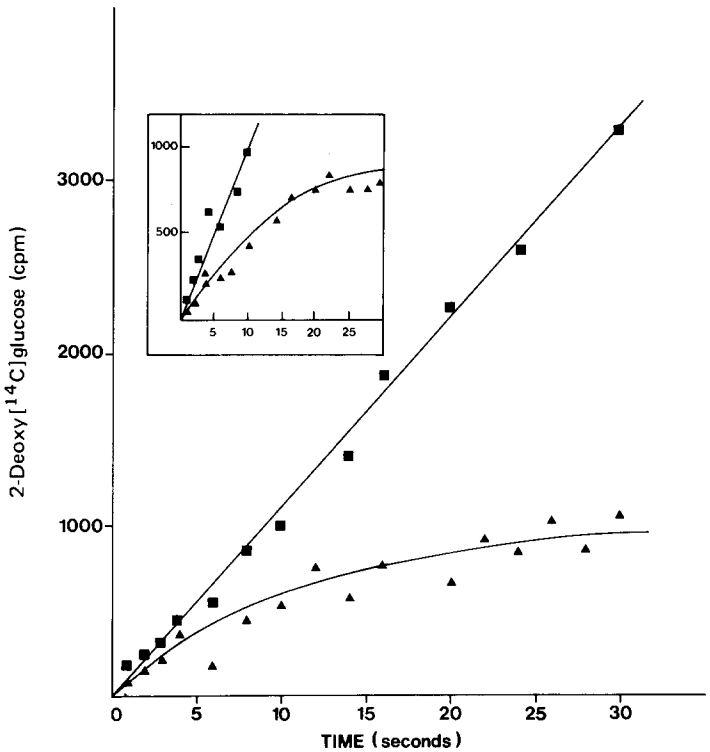

Fig. 2. Effect of 0.0 (■) and 10 (▲) mM glucose on uptake of 60 μM deoxy[^{14}C]glucose (1—30 s) in insulin-stimulated cells at 37°C. Measurements were made as described in Methods (sugar uptake method I) and the data are expressed as cpm per sample. The insert shows results at 22°C.

first 10 s even after several minutes of incubation, it is not surprising that the calculated inhibition constant is a function of time. At 1 s using quadruplicate uptake measurements in the absence and presence of 10 mM glucose we found an inhibition constant of 7 mM (data not shown). This is the same value as that obtained when [^{14}C]methylglucose was used as tracer [1]. The insert in Fig. 2 shows that essentially the same phenomenon is seen at 22°C although the change in rate appears slower than at 37°C. The data suggest that phosphorylation and not transport is the rate-limiting step in deoxy[^{14}C]glucose uptake in the presence of 10 mM glucose. To test this hypothesis we measured the intracellular concentration of deoxy[^{14}C]glucose after 30 s incubation with 60 μM deoxy[^{14}C]glucose in the presence and absence of 10 mM glucose. Table I shows that 87% of the intracellular radioactivity was non-phosphorylated deoxyglucose, indicating that transport is not rate-limiting in the presence of 10 mM glucose after incubation for a few seconds.

In order to study the effect of unlabelled deoxyglucose, deoxy[^{14}C]glucose was measured in the presence of 2 and 20 mM deoxyglucose from 30 s to 6 min (Fig. 3). Both 2 and 20 mM deoxyglucose show an apparent linear phase followed by a progressively smaller slope which eventually levels out in a manner similar to that seen for glucose. The change occurs sooner with 20 than with 2 mM deoxyglucose. A similar phenomenon is observed with 2 mM deoxy-

TABLE I

EFFECT OF GLUCOSE AND DEOXYGLUCOSE ON THE INTRACELLULAR ACCUMULATION OF DEOXY[^{14}C]GLUCOSE

Insulin-stimulated cells were incubated for 30 s at 37°C and stopped according to methods I and III. The table shows the results of a representative experiment. The filters were pooled before extraction since the individual incubations exhibit greater variations (5—10%) than individual extractions and column runs (1—2%). The indicated S.D. was calculated as the product of the percent of the extracellular deoxy[^{14}C]-glucose concentration and $\sqrt{S.D._{dg}^2/Mean_{dg}^2 + S.D._{mg}^2/Mean_{mg}^2}$ where the subscripts, dg and mg, represent deoxyglucose and methylglucose, respectively. In three other experiments the percent intracellular radioactivity as non-phosphorylated deoxy[^{14}C]glucose ranged from 57 to 87 in the presence of 10 mM glucose and from 37 to 58 in the presence of 10 mM deoxyglucose.

	% intracellular radioactivity as non-phosphorylated deoxy[^{14}C]glucose	Intracellular deoxy[^{14}C]glucose concentration as % of extracellular concentration (±S.D., n = 4)
60 μM deoxy[^{14}C]glucose	10	13 ± 1.4
60 μM deoxy[^{14}C]glucose plus 10 mM glucose	87	29 ± 3.2
60 μM deoxy[^{14}C]glucose plus 10 mM deoxyglucose	37	21 ± 6.1

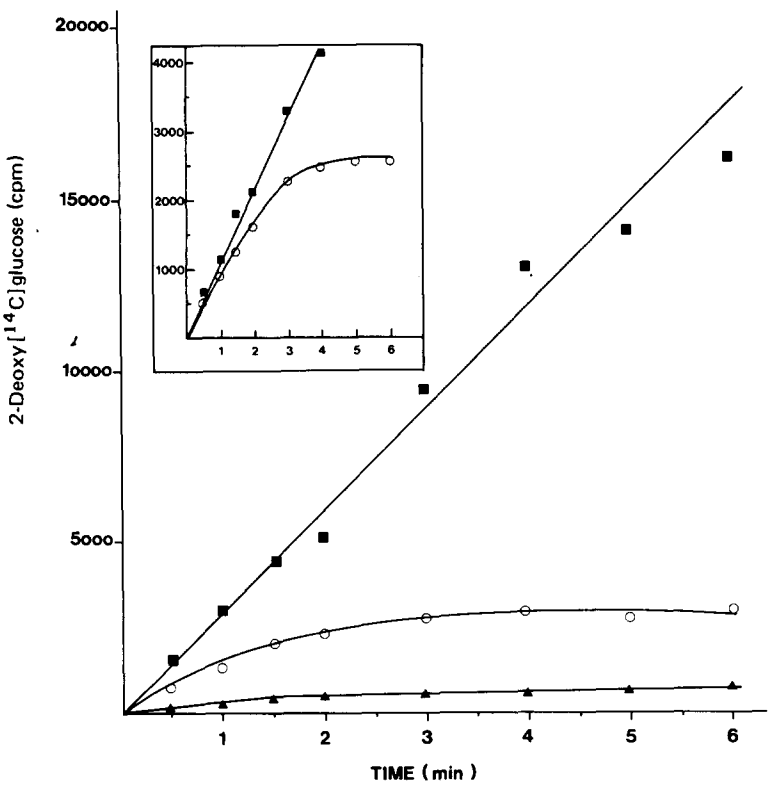

Fig. 3. Effect of 0.0 (■), 2 (○) and 20 (▲) mM deoxyglucose on uptake of 7 μM deoxy[^{14}C]glucose (0.5—6 min) in insulin-stimulated cells at 37°C. Measurements were made as described in Methods (sugar uptake method II) and the data are expressed as cpm per sample. The insert shows results at 22°C with 0.0 (■) and 2 (○) mM deoxyglucose.

TABLE II

INITIAL VELOCITY OF METHYLGLUCOSE AND DEOXYGLUCOSE AND THEIR INHIBITION CONSTANTS

Tracer methylglucose and deoxyglucose transport rates were measured utilizing 1 s incubations with insulin-stimulated cells as described in Methods (uptake method I). Uptake of the tracers was calculated as clearances defined as cpm taken up/s per cell/cpm per fl medium. Inhibition constants for methylglucose and deoxyglucose were calculated as described previously [1,3] from 1 s incubations of insulin-stimulated cells with 60 μM [^{14}C]methylgucose in the absence and presence of 5.0 mM methylglucose or deoxyglucose. The results are the mean values ± S.E. of four separate experiments.

	Methylglucose	Deoxyglucose
Clearance (fl/s per cell)	397 ± 39	173 ± 15
K_i (mM)	5.6 ± 0.54	5.02 ± 0.64

glucose at 22°C (Fig. 3, insert). These results suggest that in the presence of 2—20 mM deoxyglucose there is a progressive transfer of the rate-limiting step from transport to phosphorylation. In order to test this hypothesis we measured the intracellular deoxy[^{14}C]glucose concentration after incubation for 30 s in the presence of 10 mM deoxyglucose. Table I shows that even though the uptake in the presence of both 2 and 20 mM deoxyglucose still appears linear at this time, 37% of the intracellular radioactivity was non-phosphorylated deoxyglucose. The combined results show that at 37°C and in the presence of insulin deoxyglucose transport (2—20 mM) is not strictly rate-limiting after incubation for less than 1 min. At 22°C, phosphorylation of 2 mM deoxyglucose appears to be partially rate-limiting after approx. 2 min (Fig. 3, insert).

These experiments were performed in the presence of insulin which stimulates transport approx. 10-fold. Similar experiments were carried out in the absence of insulin and, as expected, the deviation from a linear [^{14}C]-deoxyglucose uptake appeared later. With 10 mM glucose, transport did not appear to be rate-limiting by 30 s (data not shown).

Although the data show that deoxy[^{14}C]glucose is not useful for the measurement of kinetic constants for sugars which can be phosphorylated, uptake of tracer deoxy[^{14}C]glucose alone seems to be limited only to a minor extent by phosphorylation. However, comparison of tracer [^{14}C]methylglucose and deoxy[^{14}C]glucose uptake after 1 s incubations in insulin-stimulated cells showed that methylglucose is transported approximately twice as fast as deoxyglucose (Table II). During this short incubation, uptake of both tracers is likely to reflect true unidirectional transport [1]. On the other hand, the affinity of deoxyglucose for the transport system, as judged by its ability to inhibit the initial velocity of [^{14}C]methylglucose uptake, is at least as high as that of methylglucose (Table II). This suggests a fundamental difference in the way deoxyglucose and methylglucose are handled by the transport system.

Discussion

The present study shows that in the presence of glucose, uptake of deoxy-[^{14}C]glucose is not a measure of unidirectional transport because the concentration of non-phosphorylated deoxy[^{14}C]glucose rises in the intracellular

water. Steady-state counter-transport experiments with [^{14}C]methylglucose have suggested that, in the presence of insulin, extracellular glucose (2 mM or greater) equilibrates with the intracellular water and that significant intracellular accumulation of glucose is obtained even in the absence of insulin [19]. The reason for the non-linear uptake of deoxy[^{14}C]glucose in the presence of glucose is, therefore, probably that the capacity of hexokinase to phosphorylate the tracer is progressively decreasing as the intracellular glucose concentration, and therefore the rate of phosphorylation of glucose, is increasing. It should be noted that K_m of glucose for phosphorylation is of the order of 0.1 mM [20]. It is not surprising, therefore, that even very small intracellular concentrations of glucose may inhibit phosphorylation of tracer deoxy[^{14}C]glucose. It should be noted that half-maximal inhibition of phosphorylation in insulin-treated cells was obtained with a concentration of glucose as low as 0.4 mM (Fig. 1). This concentration did not influence transport of deoxy[^{14}C]glucose in 1 s incubations since the inhibition constant of glucose was measured as 7 mM.

The data show that deoxy[^{14}C]glucose cannot be used as a tracer for the study of kinetics of transport of sugars with transport rates and affinities which approximate those of glucose. Deoxyglucose itself falls into that category since it was clearly shown to accumulate in the intracellular water. Previous investigators have failed to observe this phenomenon [12,13]. However, deoxy[^{14}C]-glucose is cleared extremely rapidly from the intracellular water when the extracellular tracer is removed, i.e., within a few seconds at 37°C and in the absence of unlabelled sugar. Therefore, all measurements of intracellular non-phosphorylated deoxy[^{14}C]glucose concentrations, including those in Table I, should be taken as minimum values. It also follows that the validity of experiments in which no appreciable accumulation of deoxy[^{14}C]glucose could be detected [12,13] depends heavily on control experiments, i.e., experiments designed to show that intracellular non-phosphorylated deoxy[^{14}C]glucose can in fact be measured. Such experiments were not presented previously.

The uptake of tracer deoxy[^{14}C]glucose appeared linear during 0—6 min (Fig. 1) and by 30 s the intracellular concentration of non-phosphorylated sugar was trivial (Table I). This suggests that deoxy[^{14}C]glucose uptake can be used as a measure of unidirectional transport in cells from normal small rats. Deoxy[^{14}C]glucose uptake has also been widely used in the study of cells from old [21] or diabetic animals [9,13] or animals fed different diets [10]. Whether or not transport is rate-limiting under these conditions will depend on the changes in the ratio V/K_m for transport relative to that for phosphorylation.

The finding that deoxy[^{14}C]glucose is transported (1 s) at a rate which is less than half of that of [^{14}C]methyglucose, although the inhibition constants of the two sugars on the initial velocity of [^{14}C]methylglucose uptake are almost the same, cannot be explained at present. This 'anomalous' behaviour of deoxyglucose adds another argument for being cautious in drawing general conclusions on transport from the study of uptake of this sugar. On the other hand, deoxyglucose may be a useful probe for studying the complex nature of the transport system and we are currently investigating this possibility.

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