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ACCUMULATION OF 2-DEOXYGLUCOSE AGAINST ITS CONCENTRATION GRADIENT IN RAT ADIPOCYTES

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Rat adipocytes were incubated at 37°C with 2-deoxy-D-[1-^{14}C]glucose ([^{14}C]2dGlc) at various concentrations and the intracellular concentrations of [^{14}C]2dGlc and deoxy[^{14}C]glucose phosphate ([^{14}C]2dGlc*P*) were measured. Using 7 μM extracellular [^{14}C]2dGlc, the intracellular [^{14}C]2dGlc concentration approached the extracelullar by 5 min in insulin-stimulated cells and by 60 min it exceeded the extracellular concentration by 50-fold. A maximum accumulation ratio of 3.5 was reached by 7 min using 1 mM and a ratio of 1.6 was reached by 1 to 3 min using 10 mM extracellular 2dGlc. The time at which the concentration of intracellular 2dGlc exceeded the extracellular was inversely related to the accumulation of 2dGlc*P*. The rate of accumulation of total radioactivity ([^{14}C]2dGlc plus [^{14}C]2dGlc*P*) decreased after 20 min using 7 μM extracellular [^{14}C]2dGlc. This change occurred later at 22°C or in the absence of insulin and sooner at higher concentrations of 2dGlc. Experiments where uptake was stopped by dilution indicated that radioactivity appearing in the medium was [^{14}C]2dGlc, but radioactivity disappearing from the cells was largerly [^{14}C]2dGlc*P*. Addition of 10 mM unlabelled 2dGlc or glucose to cells preincubated with 7 μM [^{14}C]2dGlc resulted in a more rapid loss of accumulated label from the cells, while addition of 10 mM 3-*O*-methylglucose, a non-metabolizeable sugar analogue with about the same affinity for the transport system as 2dGlc, was without effect. The results show that 2dGlc is accumulated against its concentration gradient. It is suggested that the mechanism involves first, dephosphorylation of 2dGlc*P* and second, the presence of a diffusion barrier between the site of dephosphorylation and the transport site.

Introduction

Sugars are taken up in adipocytes by carrier-facilitated diffusion and the maximal velocity of this process is increased about 10-fold by insulin [1–3]. Non-metabolizeable sugar analogues such as 3-*O*-methylglucose equilibrate rapidly with the intracellular water compartment, which constitutes only about 2% of the total adipocyte volume [1–3]. On the other hand, radioactivity is rapidly accumulated in adipocytes incubated with labelled deoxyglucose (2dGlc) because this sugar is phosphorylated by the hexokinase to deoxyglucose phosphate (2dGlc*P*). It is technically simple to measure the rate of accumulation of intracellular radioactivity (believed to be 2dGlc*P*) and this method has been widely utilized as a measure of hexose transport in adipocytes (for review, see Ref. 4). The rationale for this approach rests on the following hypotheses: 2dGlc is taken up by carrier-facilitated diffusion and is then immediately phosphorylated by the hexokinase; 2dGlc*P* is not metabolized any further and is irreversibly trapped in the cells [5].

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Abbreviations: 2dGlc, 2-deoxyglucose; 2dGlc*P*, 2-deoxyglucose phosphate; [^{14}C]2dGlc, 2-deoxy-D-[1-^{14}C]glucose; [^{14}C]2dGlc*P*, 2-deoxy[^{14}C]glucose phosphate.

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The rate of accumulation of 2dGlc*P* is only a measure of 2dGlc transport from the extracellular fluid when two criteria are fulfilled: (1) phosphorulation is not rate-limiting for the uptake and (2) the accumulated 2dGlc*P* does not escape the cell. We have recently shown that phosphorylation does become rate-limiting for 2dGlc uptake into insulin-stimulated rat adipocytes by about 30 s in the presence of glucose (about 1 mM or more) and at least partially rate-limiting in the presence of 2dGlc [4]. The present study was designed to determine whether intracellularly trapped 2dGlc*P* could be released from the cell. In the course of the study it was unexpectedly found that free 2dGlc could be accumulated in concentrations greatly exceeding those in the extracellular buffer. Such a phenomenon has not been reported previously for any sugar in tissues with insulin-sensitive carrier-facilitated hexose transport systems.

Methods

Adipocytes were prepared from epididymal fat pads from male Wistar rats, weighing 150–180 g, fed ad libitum as described previously [4]. Hepes buffer (pH 7.4) with and without bovine serum albumin (10 mg/ml) was prepared as described previously [2]. Collagenase (type I) was from Worthington, porcine insulin from Nordic Insulin, 2,4-dinitrophenol from BDH laboratories, Chemicals Division, and phloretin from K&K laboratories. 3-*O*-[^{14}C]methyl-D-glucose (59–60 mCi/mmol) and 2-deoxy-D-[1-^{14}C]glucose (52–60 mCi/mmol) were from the Radiochemical Centre, Amersham. Other chemicals were analytical grade.

Insulin, when present, was added to the cells to give a final concentration of 100 nM, i.e., a maximally stimulating concentration. Cells were then allowed to incubate for 15 min at 37°C before the beginning of the experiment.

All experiments were carried out in a cold room (3°C). Cells (2.5% v/v) were incubated with stirring in 1% albumin-Hepes buffer (pH 7.4), 7 μM 2-deoxy-D-[1-^{14}C]glucose and 0 to 10 mM unlabelled 2dGlc in a waterbath set at 37 or 22°C as indicated. The cell concentrations were low enough to ensure that the extracellular concentration of 2dGlc did not decrease more than 10% during the experiment. 30 s before the incubation was to be terminated, four 400-μl aliquots of the cell suspension were rapidly removed, placed in microfuge tubes containing 100 μl silicone oil (3°C) and spun for 15 s. There was sufficient airflow through the microfuge to ensure that the temperature in the tubes did not rise during the centrifugation. The final temperature in the silicone oil was not more than 10°C. Two of the tubes were immediately cut through the oil and the cell layers dropped into a glass test-tube containing 1.4 ml boiling water. The remaining two tubes were cut through the oil and the total content of radioactivity in the cell layers (2dGlc plus 2dGlc*P*) was determined by scintillation counting. After 5 min of boiling, the cell extract was added to 1.5 ml conical plastic tubes, the volume adjusted back to 1.4 ml and spun in a microfuge. 600 μl of the centrifuged cell extract was placed in a counting vial with 2.5 ml water plus 10 ml Unisolve 100 (Koch Light Laboratories) and the content of radioactivity determined by scintillation counting. 600 μl of the cell extract was layered on an anion-exchange column (0.7 × 4 cm, BioRad AG1-8X washed with 1 mM 2dGlc). The column was washed with 0.5-ml aliquots of 1 mM 2dGlc. The effluent was collected as one fraction, mixed with 10 ml Unisolve 100 and the radioactivity (i.e. 2dGlc) determined by scintillation counting. The ability of the resin to separate 2dGlc from 2dGlc*P* has been reported previously [4]. Furthermore, the material passing through the column moved as 2dGlc by paper chromatography using methods described previously [4]. Extracellular 2dGlc in the cell pellet was determined by incubating [^{14}C]2dGlc for 1 min with cells preincubated with 80 μM cytochalasin B [6] following the procedure outlined above. This value was substracted from the total radioactivity put on the column as well as from the radioactivity in the column effluent, and the fraction of intracellular radioactivity representing 2dGlc was determined. The measurements of radioactivity in the unextracted cell pellets were corrected for extracellular 2dGlc and then converted to total intracellular concentrations (2dGlc plus 2dGlc*P*) using intracellular water space values obtained as described previously [2]. The concentrations of intracellular 2dGlc and 2dGlc*P* were then calculated from the total intracellular concentrations and the fractions of intracellular radioactivity determined to be 2dGlc.

The figures show typical results and each experiment was repeated at least twice.

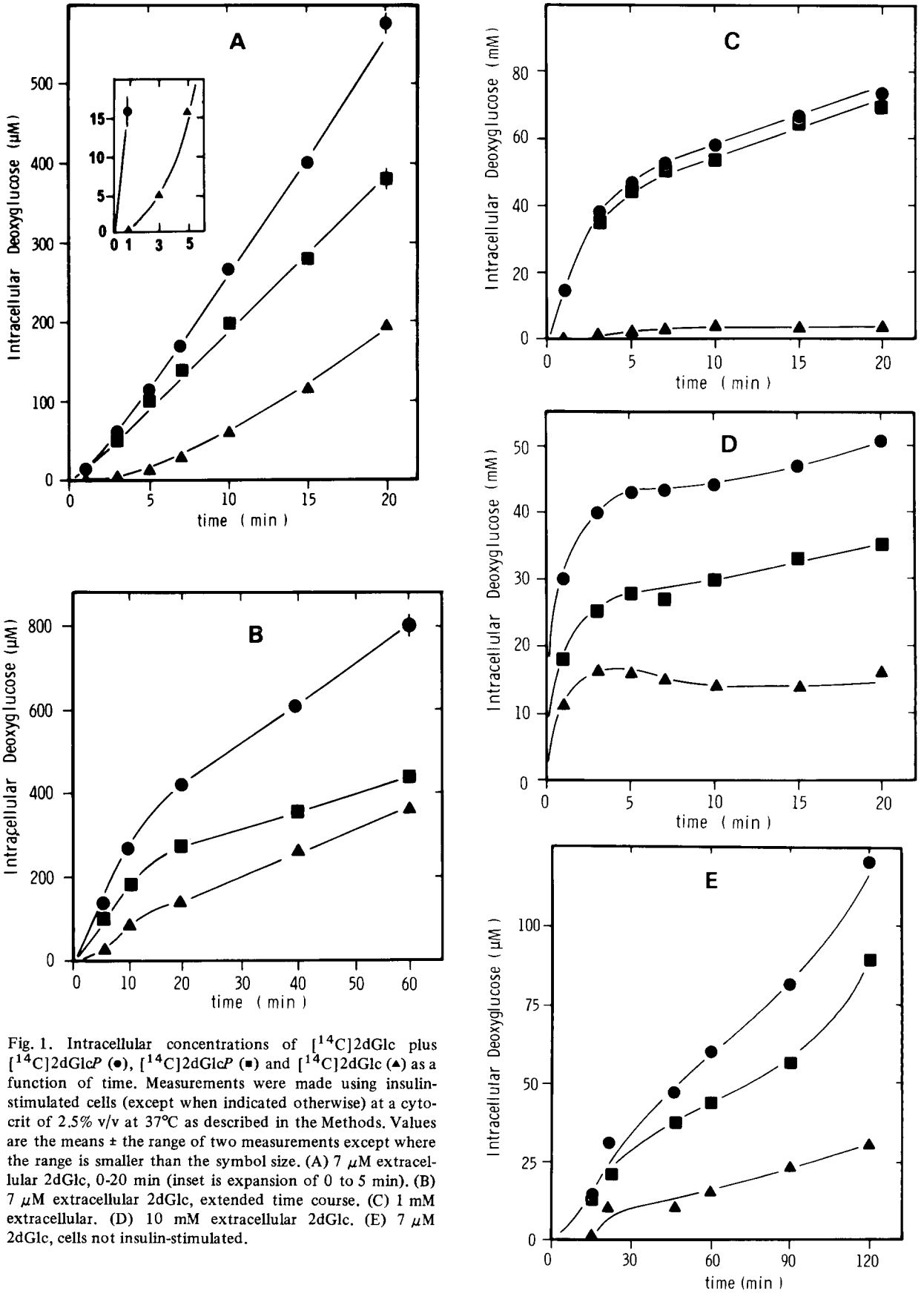

Fig. 1. Intracellular concentrations of [^{14}C]2dGlc plus [^{14}C]2dGlc*P* (●), [^{14}C]2dGlc*P* (■) and [^{14}C]2dGlc (▲) as a function of time. Measurements were made using insulin-stimulated cells (except when indicated otherwise) at a cytocrit of 2.5% v/v at 37°C as described in the Methods. Values are the means ± the range of two measurements except where the range is smaller than the symbol size. (A) 7 μM extracellular 2dGlc, 0-20 min (inset is expansion of 0 to 5 min). (B) 7 μM extracellular 2dGlc, extended time course. (C) 1 mM extracellular. (D) 10 mM extracellular 2dGlc. (E) 7 μM 2dGlc, cells not insulin-stimulated.

Results

Fig. 1A shows the intracellular concentration of 2dGlc and 2dGlc*P* as a function of time from 1 to 20 min when cells were incubated with 7 μM [^{14}C]2dGlc. The accumulation of radioactivity (2dGlc plus 2dGlc*P*) appears linear, which is consistent with previous studies showing a linear uptake for at least 6 min when cells were incubated with 60 μM [^{14}C]-2dGlc [4]. Fig. 1A (inset) also shows that by 1 min the intracellular concentration of 2dGlc was close to zero whereas there was a considerable accumulation of 2dGlc*P*. Identical results were obtained when cells were preincubated for 10 min with 7 μM unlabelled 2dGlc (data not shown). By 3 min the ratio of the intracellular to the extracellular 2dGlc concentration (accumulation ratio) had reached 0.75 and by 5 min it was 2.5 (Fig. 1A). By 30 min this ratio had increased to 30 and by 60 min to 50 (Fig. 1B) and may not have reached a maximum. Thus, there is a dramatic intracellular accumulation of 2dGlc against its concentration gradient after a period of a few min.

When the extracellular concentration of 2dGlc was raised to 100 μM, the accumulation ratio reached a maximum of 14 by 7 min (data not shown), at 1 mM a maximum ratio of 3.5 was reached by 5 min (Fig. 1C) and at 10 mM a maximum ratio of 1.6 was reached between 1 and 3 (Fig. 1D). 45 μM [^{14}C]-2dGlc equilibrated rapidly with the intracellular water ($t_{1/2}$ approx. 4 s) and the accumulation ratio did not exceed 1 after 10 min of incubation when cells were preincubated with 1 mM dinitrophenol to deplete the cells of ATP (data not shown) [7]. It is apparent from the results that accumulation of 2dGlc is energy-dependent.

The time at which the intracellular concentration of 2dGlc exceeded the extracellular concentration was inversely related to the extracellular concentration. At an extracellular 2dGlc concentration of 7 μM this time was between 3 and 5 min (Fig. 1A) at 1 mM it was between 1 and 3 min (Fig. 1C) and at 10 mM less than 1 min (Fig. 1D). When the incubation was at 22°C this time was between 5 and 7 min with 7 μM 2dGlc and less than 3 min with 1 mM or more (data not shown). When cells were not insulin-stimulated (37°C) it was between 10 and 20 min (Fig. 1E).

Fig. 1B shows that the rates of accumulation of both 2dGlc and 2dGlc*P* decreased after 20 min to new approximately constant levels. At 100 μM extracellular 2dGlc, this change in rate occurred by about 10 min (data not shown), at 1 mM by about 3 min (Fig. 1C) and at 10 mM in less than 1 min (Fig. 1D). When the incubation was at 22°C and with 7 μM extracellular 2dGlc the change in rate occurred at about 40 min (data not shown). There was no change in rate evident up to 120 min when the cells were not insulin-stimulated (37°C) (Fig. 1E). The decreased rate of accumulation might be attributed either to an exit of 2dGlc and/or 2dGlc*P* from the cell or to a decreased rate of inward transport and/or phosphorylation. To distinguish between these two possibilities, cells were incubated with 7 μM unlabelled 2dGlc for 60 min followed by addition of 7 μM [^{14}C]2dGlc uptake curve was the same as that seen when [^{14}C]-2dGlc was present during the first 60 min of incubation (data not shown). These data suggest that the decreased rate of accumulation is attributable to an exit of 2dGlc and/or 2dGlc*P* from the cells and not to a decreased rate of transport and/or phosphorylation.

Fig. 2 shows intracellular concentrations of 2dGlc and 2dGlc*P* in cells incubated with 7 μM [^{14}C]2dGlc where uptake was effectively stopped by removal of [^{14}C]2dGlc from the medium after about 10 min (see Fig. 2, legend). The intracellular concentration of 2dGlc continued to rise for about 9 min and then began to fall. The intracellular concentration of 2dGlc*P*, on the other hand, decreased from about the time that transport into the cell was effectively stopped. At least 80% of the radioactivity appearing in the medium during the 20 min incubation after washing was 2dGlc (see Fig. 2, legend). The incubation medium did not dephosphorylate 2dGlc*P* (data not shown) and most, if not all, of the 2dGlc*P* disappearing from the inside of the cell must therefore be dephosphorylated within the cell and eventually transported out of the cell. This slow loss of 2dGlc from the cell may explain the decreased accumulation rates shown above.

Another effective method of stopping 2dGlc accumulation is to block hexokinase. Since 10 mM 2dGlc inhibits the rate of phosphorylation of 7 μM [^{14}C]-2dGlc by more than 99% [4], one would expect to see a loss of accumulated 2dGlc*P* similar to that seen in Fig. 2. Fig. 3 shows the intracellular concentrations of 2dGlc and 2dGlc*P* in cells incubated with 7 μM [^{14}C]2dGlc where phosphorylation was inhibited by

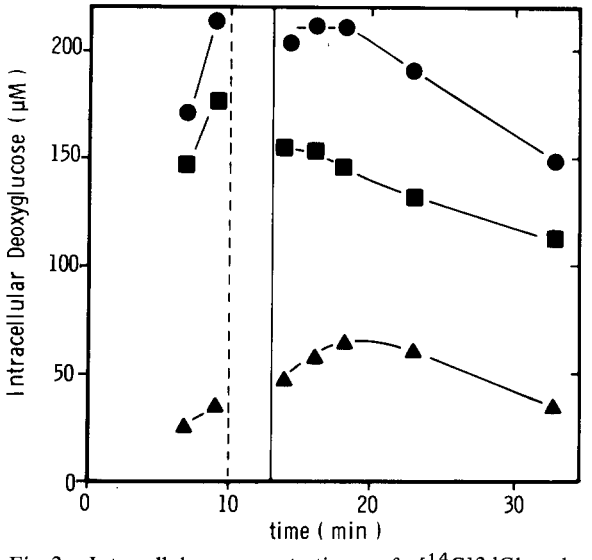

Fig. 2. Intracellular concentrations of [^{14}C]2dGlc plus [^{14}C]2dGlc*P* (•), [^{14}C]2dGlc*P* (▪) and [^{14}C]2dGlc (▲) as a function of time before and after 45-fold dilution. Measurements were made using insulin-stimulated cells at a cytocrit of 2.5% v/v at 37°C in the presence of 7 μM extracellular 2dGlc (before dilution) as described in the Methods. At 9 min cells were allowed to float and the medium was removed at 12.5 min followed by the addition of fresh medium at 13 min. Values are the means of two measurements with ranges smaller than symbol size. The increase in 2dGlc accumulation between 9 and 13 min was no more than 5 to 10% of that during the preceeding 9 min of incubation. This reduced accumulation rate can be explained by the limitation of isotope in the resulting extracellular space. Aliquots of the medium were analysed and no 2dGlc*P* could be detected. However, since the extracellular radioactivity only increased by 12% after dilution, and the separation and counting procedures are only accurate within 2.5% for each sample, only 80% of the radioactivity appearing in the medium is 2dGlc with certainty, even though no 2dGlc*P* was detected. This result was obtained several times and the 80% should therefore be regarded as a minimum figure.

addition of 10 mM unlabelled 2dGlc after 10 min of incubation. Under these conditions, intracellular radioactivity decreased more rapidly than seen after washing (Fig. 2). Addition of 10 mM glucose, which also inhibits phosphorylation of 2dGlc [4], caused a similar loss. In contrast, addition of 10 mM 3-*O*-methylglucose, which does not inhibit phosphorylation [1], did not cause efflux and in fact uptake continued at a slower rate consistent with its inhibition of 2dGlc transport (data not shown).

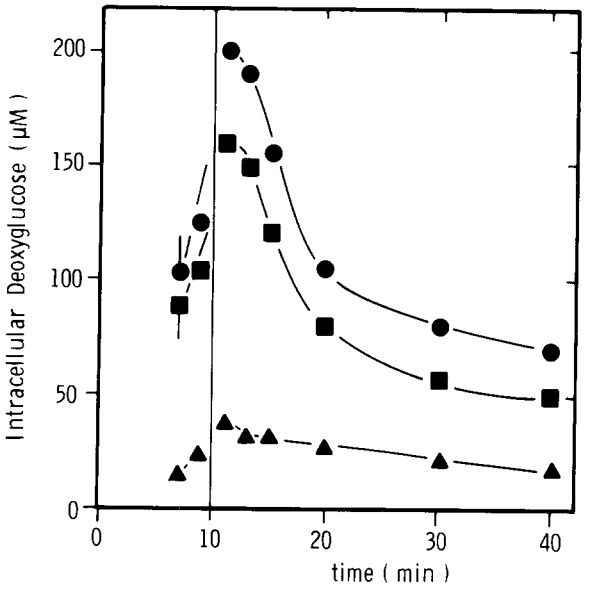

Fig. 3. Intracellular concentrations of [^{14}C]2dGlc plus [^{14}C]2dGlc*P* (•), [^{14}C]2dGlc*P* (▪) and [^{14}C]2dGlc (▲) as a function of time before and after addition of 10 mM 2dGlc. Measurements were made using insulin-stimulated cells at a cytocrit of 2.5% v/v at 37°C as described in the Methods. Values are the means ± the range of two measurements except where this is smaller than the symbol size.

Discussion

The most striking result of the present study is the marked intracellular accumulation of 2dGlc against its concentration gradient. It might be proposed that 2dGlc is first accumulated by an active transport system followed by phosphorylation. However, the finding that the intracellular [^{14}C]2dGlc concentration was essentially zero, whereas [^{14}C]2dGlc*P* was accumulated appreciably after incubation with 7 μM [^{14}C]2dGlc for 1 min (Fig. 1A, inset), argues against this hypothesis. Furthermore, the time course of accumulation of [^{14}C]2dGlc and [^{14}C]2dGlc*P* did not change when the cells were preincubated with 7 μM unlabelled 2dGlc, indicating that moderate intracellular concentrations of 2dGlc*P* did not inhibit phosphorylation of newly transported [^{14}C]2dGlc. Finally, active hexose transport has never been described in insulin-sensitive systems. We therefore regard this possibility as very unlikely.

Another possibility is that 2dGlc might be bound to an intracellular macromolecule. We have found no

evidence for such binding using gel filtration on Sephadex G25 (unpublished observation), but the possibility cannot be excluded.

The experiments showed that intracellular 2dGlc*P* was dephosphorylated (Fig. 2, legend). We therefore propose that the accumulation of 2dGlc is caused by the following sequence. First, phosphorylation to 2dGlc*P* followed by dephosphorylation in a cellular compartment where the newly formed 2dGlc cannot readily diffuse to the carrier system. It is necessary to postulate such a diffusion barrier; if it were not present, intracellular 2dGlc would equilibrate rapidly with the extracellular fluid ($t_{1/2}$ approx. 4 s [8]) and no appreciable accumulation would occur.

This hypothesis can explain the experimental observations: at trace concentration (i.e., 7 μM, which is assumed to be much lower than K_m for hexokinase) 2dGlc is initially transported from the extracellular medium, through the transport system (which is presumed to embrance at least two carriers for 2dGlc entry [8]) to the inward-facing side of the membrane. The premise rests on the finding that trace 2dGlc quickly equilibrates with the intracellular water (half time of approx. 4 s) when the rate of accumulation of 2dGlc*P* is essentially zero due to preincubation of the cells with 10 mM glucose [4,8]. Once arrived intracellularly, trace 2dGlc is immediately phosphorylated (in the absence of glucose) so that the intracellular 2dGlc concentration is close to zero for the first few min (Fig. 1A, inset). Some of the accumulated 2dGlc*P* is then dephosphorylated. This hypothesis is based on the finding that, when uptake and phosphorylation are stopped by dilution, there is a decrease in the intracellular 2dGlc*P* concentration without an accompanying appearance of 2dGlc*P* in the medium (Fig. 2, legend). When uptake proceeds, the rate of formation of intracellular 2dGlc increases as a function of time because the concentration of substrate for dephosphorylation (2dGlc*P*) increases, leading to an accumulation of 2dGlc.

The model also has to account for the slow efflux of trace [^{14}C]2dGlc (Fig. 2) and its acceleration by the addition of 10 mM 2dGlc (Fig. 3) but not 10 mM 3-*O*-methylglucose. It is proposed that trace [^{14}C]-2dGlc*P* in the absence of 10 mM 2dGlc is dephosphorylated at a rather rapid rate followed by rephosphorylation of a large fraction. It is necessary to postulate that the diffusion barrier mentioned above separates the site of dephosphorylation and that of rephosphorylation (hexokinase). If this was not the case, newly formed trace [^{14}C]2dGlc would be rapidly rephosphorylated and no accumulation of 2dGlc would occur. The addition of 10 mM 2dGlc (a concentration much higher than K_m for hexokinase [9,10] would quickly occupy a large part of the hexokinase, block rephosphorylation of [^{14}C]2dGlc and therefore increase its exit from the cell.

The present results are in contrast to those of Kobayashi and Olefsky [11,12], who reported an intracellular 2dGlc concentration of less than 0.01 mM after incubation of insulin-stimulated cells for 3 min at 22°C with 1 mM extracellular 2dGlc and 0.48 mM after incubation with 10 mM. Chandramouli and Carter [13] reported intracellular 2dGlc concentrations of 3.7 mM and 13.9 mM following 1 h incubations at 37°C with extracellular concentrations of 1 mM and 10 mM, respectively. These values are quite similar to those obtained in the present study. However, Chandramouli and Carter stated that the intracellular 2dGlc concentrations were not significantly different from those in the medium, due to the errors inherent in their methods. While the present manuscript was being prepared, Wieringa et al. [14] stated in a preliminary report that accumulated [^{14}C]2dGlc*P* can be dephosphorylated and released from adipocytes and, in addition, that a high concentration of unlabelled 2dGlc or glucose in the extracellular medium would accelerate the decrease in intracellular radioactivity. These authors also reported that accumulation of intracellular radioactivity is an oscillating process with variations in intracellular radioactivity of about 40%. This is in contrast to our previous [4] and present results. Wieringa et al. did not report accumulation of intracellular 2dGlc.

Previous studies on rat diaphragm [15] and caudofemoralis muscle [16] have failed to demonstrate the presence of 2dGlc in the intracellular water even after stimulation with insulin. On the other hand, recent studied on the perfused rat lung have shown the appearance of appreciable intracellular 2dGlc concentrations, although the accumulation ratio never exceeded unity [17]. A phosphatase capable of breaking down 2dGlc*P* to 2dGlc was demonstrated in the lung tissue and dephosphorylation of 2dGlc*P* may therefore have contributed to the generation of the intracellular 2dGlc.

In hamster kidney cortex slices a 2dGlc accumulation ratio of 14 was found in the presence of 0.01 mM extracellular 2dGlc [18]. However, in this system accumulation of 2dGlc clearly preceeded that of 2dGlc*P* and it was concluded that phosphorylation of 2dGlc occurs passively after active membrane transport of 2dGlc.

Unlike the perfused rat lung and the hamster kidney cortex, yeast cells have been reported to accumulate 2dGlc against a concentration gradient as a result of dephosphorylation of accumulated 2dGlc*P*. However, in this system, direct coupling of transport and phosphorylation (i.e., phosphorylation was necessary for transport) was proposed to explain why accumulated 2dGlc was not transported out of the cell down its concentration gradient [19]. In contrast, we have previously shown that 2dGlc transport proceeds independently of phosphorylation in the rat adipocyte [8].

Thus, the mechanism of accumulation of intracellular 2dGlc proposed in the present study appears to be unique for the adipocyte. However, 2dGlc is not a natural sugar and it remains to be seen whether dephosphorylation of glucose6-phosphate may also occur. With glucose as a substrate, glucose6-phosphate concentrations do not increase nearly as much as do the 2dGlc*P* concentrations observed in the present study, since glucose6-phosphate is further metabolized [20]. However, we have recently demonstrated that transmembrane transport of hexose occurs through two barriers in series and that the permeability of the second barrier can be regulated by a glucose metabolite, perhaps glucose6-phosphate [8]. It seems possible that dephosphorylation may play a role in regulating glucose6-phosphate concentrations.

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