Biochimica et Biophysica Acta, 772 (1984) 244–250
Elsevier

BBA 72091

ACCELERATED NET EFFLUX OF 3-*O*-[^{14}C]METHYLGLUCOSE IN ISOLATED FAT CELLS

JØRGEN VINTEN

Department of Medical Physiology B, Panum Institute University of Copenhagen, Blegdamsvej 3 C, DK 2200, Copenhagen (Denmark)

(Received July 19th, 1983)
(Revised manuscript received December 21st, 1983)

Key words: Carbohydrate transport; Methylglucose; Insulin; (Rat adipocyte)

(1) A flow-tube apparatus suited for measurement of rapid efflux of sugars from adipocytes is described. (2) Due to heterogeneity of fat cell populations, a conventional analysis of the time-course of net efflux of 3-*O*-methylglucose based on the integrated rate equation can produce gross errors in estimates of kinetic parameters. (3) The half-saturation constant and maximum transport capacity for 3-*O*-methylglucose transport were found to be about 3-fold higher for net efflux than for equilibrium exchange flux, both in insulin-stimulated and non-stimulated adipocytes. This suggests asymmetric kinetic parameters for 3-*O*-methylglucose transport.

Introduction

Transport of 3-*O*-methylglucose in isolated fat cells has been studied in order to explore the properties of the glucose transporter [1–4] and to assess whether changes of the membrane permeability to glucose contributes to the changes in glucose uptake brought about by various agents or physiological conditions [5,6]. For measurement of transport, the method of Vinten et al. [1] and later the method of Whitesell and Gliemann [3] or various modifications hereof [4] have been used. From measurements using these modifications it has been concluded that the kinetic parameters for 3-*O*-methylglucose transport are symmetrical and that the kinetic parameters for equilibrium exchange flux and net fluxes are the same [4]. This implies that at any given concentration of 3-*O*-methylglucose, the initial rate of net efflux should be identical with the initial rate of equilibrium exchange flux. A preliminary attempt to confirm this by use of the method of Vinten et al. [1] was not successful, and this precipitated the present study.

Methods

Experimental procedure

Chemicals and cell preparation. Labelled sugars were obtained from New England Nuclear, collagenase was from Worthington (Type 1), and phloretin from K&K laboratories. Porcine insulin was from Novo, and (when present) was used in a final concentration of 1 $\mu mol \cdot l^{-1}$. The composition of the isotonic, albumin-containing, buffered salt solution used for washing and incubations was given earlier [1]. Other chemicals were obtained from Sigma. Isolated fat cells were prepared from the epididymal fat pads of male Wistar rats weighing 110–130 g using the method of Foley et al. [7]. During incubation with collagenase, 3-[^{3}H]glucose (0.7 μCi/ml, 12.0 Ci/mmol) was present in order to label the intracellular lipids. After washing of the fat cells, ^{3}H activity concentration in intracellular lipids was determined. This made it possible to correct subsequent measurements for variations in sample size.

Flow-tube measurements. When efflux of 3-*O*-[^{14}C]methylglucose was rapid, the rate was mea-

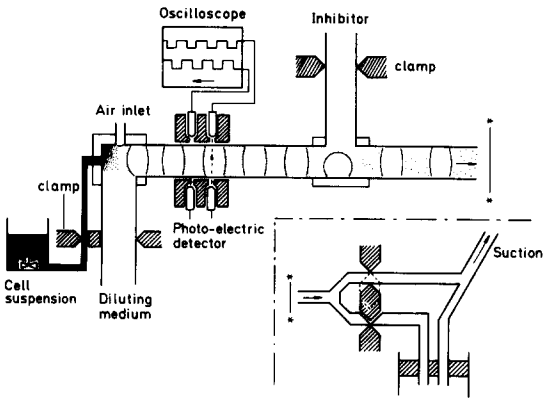

Fig. 1. Sketch of the flow-tube apparatus used for measurement of rapid 3-*O*-[^{14}C]methylglucose exit. Cell suspension and diluting medium are led to the primary mixing chamber by polyethylene tubes (i.d. 0.51 mm and 2.0 mm, respectively) replaced by silicone rubber tubes at magnetic clamps. Polyethylene reaction tube (i.d. 2.0 mm) connects the primary mixing chamber with the secondary mixing chamber, in which efflux is stopped by addition of phloretin. Suction is applied to the outlet and the system is operated by magnetic clamps. Effluent is collected by switching a clamp on outlet (shown in the insert) after a period of time sufficient to flush tubes thoroughly. The system together with the diluting medium and cell suspension was kept at 37 °C by immersion in a water bath, and air to the inlet was taken near the water surface. The air segments served as a means of obtaining rapid mixing. This could alternatively be achieved by using a high linear flow rate but the resulting turbulent flow tended to disrupt the fat cells.

sured by means of a flow-tube apparatus made of polyethylene tubes connected with silicone-rubber tubes and machined pieces of plexiglas, shown in Fig. 1. Fat cells in a stirred suspension (25–30% by vol.) were allowed to equilibrate with 3-*O*-[^{14}C]methylglucose (5 μCi/ml) at the desired initial intracellular concentration. Hereafter, flow, driven by a subatmospheric pressure applied to the effluent collecting vial (insert), was initiated by releasing the magnetic clamps. The flow of fat cell suspension fused with a flow of albumin-free buffer ('Diluting medium' in Fig. 1) in a mixing chamber whereby the fat cell suspension was diluted about 50-times. In equilibrium exchange experiments the buffer contained unlabelled 3-*O*-methylglucose in a concentration identical to that used for the initial equilibration, while it contained none in net efflux experiments. The latter situation is often named 'zero-trans efflux' in the literature [4,8,9]. At this point, the flow of diluted suspension was divided into segments by bubbles of air from an air inlet. Two light beams passing transversely to the travelling direction of the segments were recorded by means of a storage oscilloscope. From the recording, the transit time of the segments in the reaction tube could be calculated. As the segments left the reaction tube they were diluted 1.6-fold in a second mixing chamber with a buffered, isotonic salt solution containing phloretin (0.4 mmol·l^{-1}, 'inhibitor' in Fig. 1). This stopped efflux of 3-*O*-[^{14}C]methylglucose. The cells in 7-ml portions of the effluent were recovered by centrifugation (1200 × *g*, 40 s) through silicone oil ($d = 0.96$ g/ml), and their ^{3}H and ^{14}C activities were determined by scintillation counting. Different transit times in the reaction tube were obtained by changing its length and/or the suction applied to the effluent collecting vial. The amount of 3-*O*-[^{14}C]methylglucose in the cells at the start of washout was determined by moving the inlet for the phloretin solution to the primary mixing chamber. For determination of the amount present after an infinite time, diluted cell suspension collected from the primary mixing chamber was incubated for 5 min whereafter phloretin solution was added manually.

The performance of the mixing chambers was checked by observing the colour-shift of a pH-indicator (Bromocresol purple) added to the cell

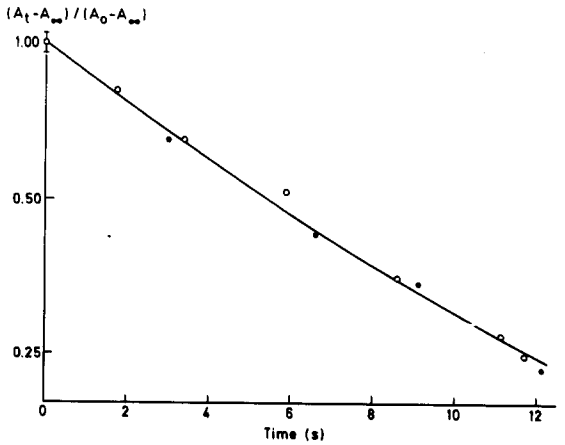

Fig. 2. Net efflux of 3-*O*-[^{14}C]methylglucose (initial intracellular concentration 25 mmol·l^{-1}) in insulin-stimulated fat cells. Filled symbols measured by the method of Ref. 1, open symbols measured by the flow tube. The curve represents Eqn 2 with parameters $K^n = 15$ mmol·l^{-1}, $J_m^n = 5.1$ mmol·l^{-1}·s^{-1}, $r = 0.55$.

suspension in test experiments. In these tests, pH and buffer capacities of the solutions were adjusted so that a colour shift required a near complete mixing. As an alternative control of the apparatus, the rate of 3-*O*-[^{14}C]methylglucose efflux from human erythrocytes was measured at 38 °C with the present apparatus (this required as the only modification that a silicone oil with $d = 1.04$ was used for recovery of cells by centrifugation) and with the flow tube described by Brahm [10]. The two methods were found to agree within 10% and the rate coefficient for efflux of 3-*O*-[^{14}C]methylglucose in human erythrocytes (100 μmol $\cdot$ l^{-1}) was 2.2 s^{-1} (Vinten, J. and Brahm, J., unpublished data). When efflux of 3-*O*-[^{14}C]methylglucose was slow, a previously described method was used [1]. In some experiments (e.g., Fig. 2) efflux was measured by both methods. All experiments were performed at 37 °C.

Calculations. The time-course of net efflux of 3-*O*-[^{14}C]methylglucose was compared to the predictions of two models:

$$t = \left[\left(1 + K^{n}_{1/2}/C^{osm}_{E}\right)\left(C_0 - C_t^{app}\right) + \left(C^{osm}_{I}/C^{osm}_{E}\right)K^{n}_{1/2}\log_e\left(C_o/C_t^{app}\right)\right]/J^{n}_{m} \quad (a)$$

$$\Leftrightarrow t = f\left(C_t^{app}\right)$$

$$\Leftrightarrow C_t^{app} = f^{-1}(t) \quad (1)$$

and

$$C_t^{app} = \int_0^{\infty} g\left(J^{n}_{m}, \overline{J^{n}_{m}}, r\right) f^{-1}(t)\,\mathrm{d}J^{n}_{m} \quad (2)$$

where t is the time after start of efflux, C_0 the intracellular concentration of 3-*O*-[^{14}C]methylglucose at start of efflux, C_t^{app} is the amount of intracellular 3-*O*-methylglucose at time t divided by the initial intracellular volume, C^{osm}_{E} the osmolar concentration of non-permeating solutes in the diluting medium, C^{osm}_{I} the corresponding concentration in the intracellular water. $K^{n}_{1/2}$ and J^{n}_{m} are, respectively, the half saturating concentration (mmol $\cdot$ l^{-1}) and maximum flux (mmol $\cdot$ s^{-1} $\cdot$ (liter of intracellular water)$^{-1}$) for net efflux. Eqn. a is equivalent to Eqn. 2 of Ref. 11. Function f^{-1} is the inverse of f, and g is the log-normal probability density function of J^{n}_{m} with arithmetic mean equal to $\overline{J^{n}_{m}}$ and (relative) dispersion equal to r. Thus, Eqn. 1 describes the saturable net efflux in a regular two compartment system or a system composed of identical cells in an infinite dilution medium, whereas Eqn. 2 describes the efflux from a sample of cells with a log-normal distribution of cellular maximum fluxes. Note that the predictions of the models are indistinguishable for small values of t (i.e., in the initial part of the time-course).

Equilibrium exchange efflux data were treated as described earlier [1] with the exception that the gamma distribution function was replaced by the log-normal distribution which has been used for similar purposes by others [12,13]. Since this produced no perceptible change in the fit, the advantage of using a commonly known distribution was considered more important than the ease of computation offered by the gamma distribution. A relative dispersion of 0.55 was used throughout. The function f^{-1} and the definite integrals were calculated numerically on a digital computer using standard methods. The symbol A_x which appears in the figures is the ratio ^{14}C-activity/^{3}H-activity in a sample of cells at time x. A_t is thus proportional to C_t^{app}.

Results

Kinetic parameters for net and equilibrium exchange efflux

The concentration dependencies of the initial rate of net and equilibrium exchange efflux of 3-*O*-[^{14}C]methylglucose in insulin-stimulated fat cells are shown in a Hanes-type plot in Fig. 3. It appears that J^{n}_{m} and $K^{n}_{1/2}$ are substantially larger than the corresponding parameters for equilibrium exchange efflux. Parameter estimates obtained by linear regressions are given in Table I. Since this analyses is based on initial rates, the estimates are independent of whether cell populations are assumed to be heterogeneous or not.

In Fig. 4 the net and equilibrium exchange efflux of 3-*O*-methylglucose (initial concentration 50 mmol $\cdot$ l^{-1}) in cells not stimulated by insulin are shown. The rate of both types of efflux is about 13-times lower than the corresponding rates measured in insulin-stimulated cells, and in a series of six similar experiments this factor varied in the range from 8.3 to 13.6. In these experiments the

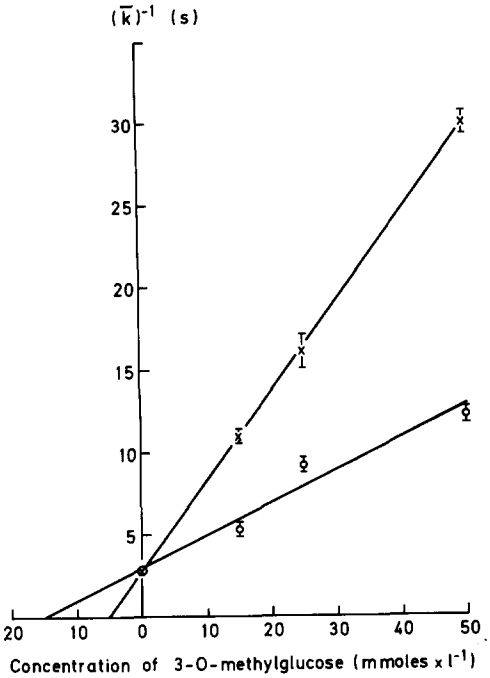

Fig. 3. Concentration dependencies of net and equilibrium exchange efflux in insulin-stimulated fat cells. Initial rates ($\bar{k}$) of 3-*O*-[^{14}C]methylglucose exit were determined in plots like those shown in Figs. 2 and 5. Each point is the mean of the reciprocals of four individual determinations of $\bar{k}$, except the point at a near zero concentration (⊗) which is the mean of eight determinations. Bars denote S.E., crosses (×) represent equilibrium exchange conditions, open circles (○) net efflux, lines represent linear regressions. Parameter estimates are given in Table I.

presence of extracellular 3-*O*-methylglucose (50 mmol · l^{-1}) reduced the initial rate of 3-*O*-[^{14}C]methylglucose efflux to (43 ± 8)% (mean ± S.E.) of that obtained when 3-*O*-methylglucose was absent from the diluting medium. Fig. 4 also shows the efflux of 3-*O*-[^{14}C]methylglucose initially present in a concentration of 50 μmol · l^{-1} (tracer efflux). At this concentration, which is practically zero compared to $K^{n}_{1/2}$ and $K^{ee}_{1/2}$, no difference was found between initial rate of net and equilibrium exchange efflux. Furthermore, the ratios between initial rates of tracer efflux and effluxes at 50 mmol · l^{-1} were the same as the corresponding ratio in insulin-stimulated cells. This indicates that insulin is without effect on both $K^{ee}_{1/2}$ and $K^{n}_{1/2}$.

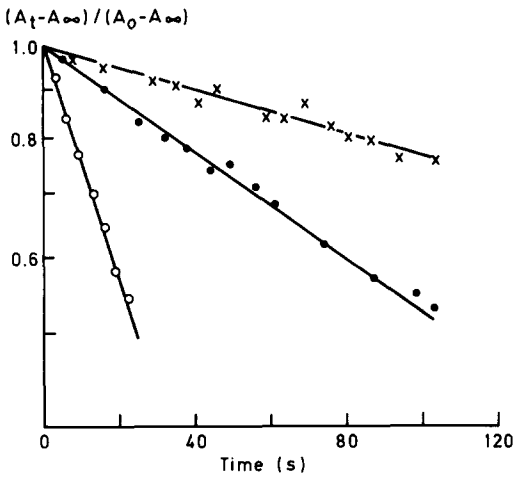

Fig. 4. Efflux of 3-*O*-[^{14}C]methylglucose in fat cells not stimulated by insulin. Concentrations of 3-*O*-[^{14}C]methylglucose: open circles (○) 50 μmol·l^{-1}, closed circles (●) 50 mmol·l^{-1} net exit, crosses (×) 50 mmol·l^{-1}, equilibrium exchange.

Progress curve for efflux of 3-O-[^{14}C]methylglucose

Fig. 5 shows a comparison of the time-course of equilibrium exchange (50 mmol · l^{-1}, crosses) and net efflux (50 mmol · l^{-1}, open circles) in insulin-stimulated cells. The fraction of releasable activity still present in cells at time t is plotted on a logarithmic ordinate as function of time. In addition to the experimental results five calculated curves (A–E) are shown. Curves A to D all simulate net efflux and the values used for parameters J^{n}_{m} and $K^{n}_{1/2}$ are those given in Table I for net efflux conditions. Curve A represents efflux from a homogeneous cell population (Eqn. 1) and curves B and C represent efflux from heterogeneous cell populations (coefficient of variation for J^{n}_{m} between cells 0.55). For curves B and C osmolarities of 350 and 300 mosmol · l^{-1} of the diluting medium were used, respectively. These curves are thus illustrating the effect of 'osmotic balancing', assuming that fat cells behave like osmometers with infinite water permeability. Curve D is a straight line extending the common initial slope of curves A to C. Curve E is a simulation of equilibrium exchange efflux in heterogeneous cells, J^{ee}_{m} and $K^{ee}_{1/2}$ from Table I, coefficient of variation 0.55. The modest deviation from a straight line is due to the fact that only the early part of the progress curve is shown. In the experiment shown the net

TABLE I

KINETIC PARAMETERS FOR 3-*O*-METHYL-D-GLUCOSE EFFLUX IN INSULIN-STIMULATED FAT CELLS

	J_m^n (mmol·l^{-1}·s^{-1})	$K_{1/2}^n$ (mmol·l^{-1})	J_m^{ee} (mmol·l^{-1}·s^{-1})	$K_{1/2}^{ee}$ (mmol·l^{-1})
Present results	5.06 ± 0.30	14.8 ± 2.2	1.82 ± 0.04	5.15 ± 0.64
Ref. 4	1.19 ± 0.07	2.66 ± 0.26	0.84 ± 0.002	4.45 ± 0.26
Ref. 21		5.65 ± 2.05		
Ref. 1			1.7	5
Ref. 2			1.7	6

efflux was initiated by diluting the extracellular phase by a medium with osmolarity identical to that of the loading medium, mannitol replacing 3-*O*-methylglucose. In other experiments the diluting medium contained only sucrose or NaCl or KCl in osmotic concentrations equal to that of the loading medium. These changes produced no detectable effects on the time-course of net efflux in insulin-stimulated cells (initial concentration of 3-*O*-[^{14}C]methylglucose was 50 mmol·l^{-1}, data not shown). To test the effect of 'osmotic balancing' efflux of 3-*O*-[^{14}C]methylglucose from cells loaded to a concentration of 57 mmol·l^{-1} and diluted in a medium with osmolarity 300 mosmol·l^{-1} was compared to efflux from cells equilibrated with 50 mmol·l^{-1} and diluted in a medium with 350 mosmol·l^{-1}. This difference in conditions pro-

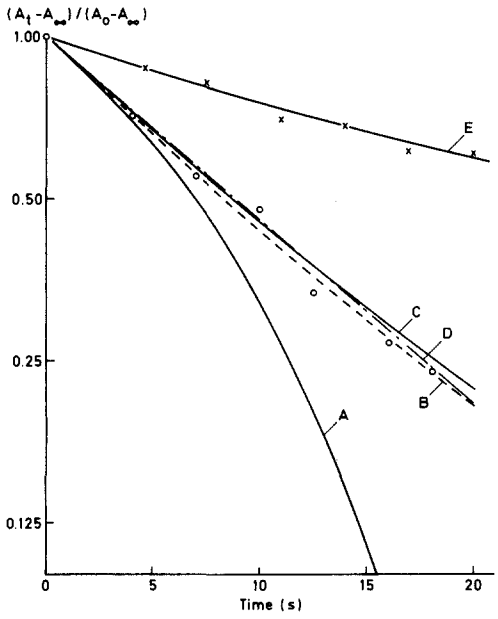

Fig. 5. Net (open circles, ○) and equilibrium exchange (crosses, ×) efflux of 3-*O*-[^{14}C]methylglucose (50 mmol·l^{-1}) in insulin-stimulated fat cells. The curves represent: A, saturable net efflux in an ideal two-compartment system Eqn. 1 (⇔ Eqn. 2 with $r = 0$); B, saturable net efflux in a parallel multicompartment system, (Eqn. 2, $r = 0.55$), 50 mmol·l^{-1} of mannitol present in the diluting medium; C, as B, but no mannitol and with 57 mmol·l^{-1} of 3-*O*-[^{14}C]methylglucose intracellularily prior to dilution; D, monoexponential process with initial rate identical to A, B and C; E, equilibrium exchange efflux in a multicompartment system with $r = 0.55$. The appropriate parameter estimates from the experiment shown in Fig. 3 were also used for computation of curves.

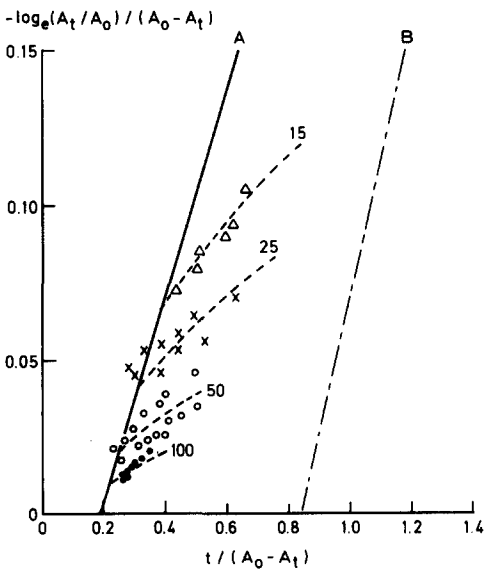

Fig. 6. Linearized plot of net efflux progress data in insulin-stimulated cells. Lines represent Eqn. 1 with parameters of the present paper (A) and with those of Ref. 4 (B). Broken curves are the predictions of Eqn. 2 with parameters from the present paper, the numbers indicate the initial concentrations of 3-*O*-[^{14}C]methylglucose. Data obtained at these concentrations are also shown.

duced no detectable difference between the first 75% of the time-courses (data not shown). This was compatible with the predictions of of Eqn. 2 (see Fig. 5).

Estimation of kinetic parameters in a linearized plot of the integrated rate equation

Fig. 6 shows a plot commonly used [4,11] for linearization of integrated rate equations similar to Eqn. 2. In the plot a line satisfying this equation intersects the abscissa and ordinate at points close to $1/J_m^n$ and $-1/K_{1/2}^n$, respectively. All measurements from experiments on systems conforming to Eqn. 1 and differing only with respect to initial concentrations should give points situated along the same straight line. It appears from Fig. 6 that this can only apply to points representing very early parts of the time courses of experiments with different initial concentrations. Furthermore, all points representing measurements at the different initial concentrations fit the respective (broken) curves derived from Eqn. 2 quite well.

Discussion

Shape of the progress curve for 3-O-[^{14}C]methylglucose exit

The presently observed time courses of 3-*O*-[^{14}C]methylglucose net exit from samples of more than 10^4 fat cells are clearly incompatible with the predictions of a two compartment model based on simple saturation kinetics. It has earlier been established that a multicompartment model is necessary to account for the lack of log-linearity of the time-course of 3-*O*-[^{14}C]methylglucose exit in the equilibrium exchange situation and that heterogeneity of the cells with respect to the ratio between maximum cellular transport capacity (mmol · s^{-1} per cell) and cellular distribution space for 4-*O*-[^{14}C]methylglucose (l per cell) was responsible for this [1,14]. It was to be expected that a similar model for net efflux would fit the observations with the same value for the relative dispersion as that found to fit with equilibrium exchange experiments, when in both experimental situations transport is mediated by the same integral membrane protein (the glucose transporter [15,16–20]) and originates from the same distribution space.

Since the predictions of Eqn. 2 produces an acceptable fit to the net efflux data, the estimate of $K_{1/2}^n$ from initial rate measurements can still be regarded as representing a property of the glucose transporter, and more complicated models (e.g., based on a concentration dependent substrate affinity) can be left out of consideration.

It is evident that the integrated rate equation plot of Fig. 6 is of limited value for estimation of kinetic parameters from progress curves in heterogeneous cell populations, since all but the initial measurements are biased due to over-representation of cells with a low transport/distribution-space ratio.

A recent report on 3-*O*-[^{14}C]methylglucose transport in adipocytes describes no deviations from the predictions of Eqn. 1 [4]. The reason for this is not clear, but it could be suggested that the narrower range of initial concentrations used by the authors has made a deviation from two compartment kinetics less conspicuous. Also, it cannot be ruled out that the cell pools used by the quoted authors were less heterogeneous than those of the present paper.

Accelerated net exit

The present results clearly show that $K_{1/2}^n$ and J_m^n for 3-*O*-methylglucose efflux are larger than the corresponding parameters for equilibrium exchange. It is likely that this is due to asymmetry of the kinetic parameters for net fluxes, since it has been claimed that the initial rate of 3-*O*-[^{14}C]methylglucose net influx is the same as that found in equilibrium exchange flux [3,4] and since a marked asymmetry of the inhibitory properties of several non-transported glucose analogues has been demonstrated [21]. On the other hand it has been reported that the kinetic parameters for net efflux and equilibrium exchange are the same [4,21]. In these reports, however, different values for $K_{1/2}^n$ are given, and the experimental methods differ (cf. Table I). Furthermore, calculations are based on the integrated rate equation, and they are thus dependent on the implicit assumption that fat cells are homogeneous with respect to the ratio between transport and distribution space for 3-*O*-methylglucose.

The present results show that the (relative) effect of extracellular 3-*O*-methylglucose on efflux

of 3-*O*-[^{14}C]methylglucose was the same whether cells were stimulated by insulin or not. This implies that insulin produced no qualitative changes in 3-*O*-methylglucose transport, and is thus in accordance with the hypothesis that insulin acts by recruiting transporters to the cell membrane from an intracellular store [15,17–20].

The fact that changes in transmembrane ion gradients (and likely also in membrane potential) was without impact on the accelerated net exit suggests that this property is inherent in the glucose transporter of fat cells. It has been reported that the binding site for cytochalasin B on the glucose transporter of human erythrocytes faces the cell interior [22,23]. If this is the case also in fat cells, the difference between the glucose concentration reported to half-inhibit cytochalasin B binding to the glucose transporter from fat cells (about 35 mmol · l^{-1}, [17]) and the glucose concentration reported to half-inhibit entry of non-metabolizable sugars (about 8 mmol · l^{-1}, [3,24]) could indicate an asymmetry of the kinetic parameters for glucose transport similar to that found in the present studies on 3-*O*-[^{14}C]methylglucose transport.

Acknowledgements

The financial support from the Novo Foundation as well as the skilled technical assistance of Mrs. Ulla Blankensteiner and Mrs. Jonna Harpøth is gratefully acknowledged.

References

1 Vinten, J., Gliemann, J. and Østerlind, K. (1976) J. Biol. Chem. 251, 794–800
2 Vinten, J. (1978) Biochim. Biophys. Acta 511, 259–273
3 Whitesell, R.R. and Gliemann, J. (1979) J. Biol. Chem. 254, 5276–5283
4 Taylor, L.P. and Holman, G.D. (1981) Biochim. Biophys. Acta 642, 325–335
5 Vinten, J. and Galbo, H. (1983) Am. J. Physiol. 244, E129–E134
6 Foley, J.E., Cushman, S.W. and Salans, L.B. (1980) Am. J. Physiol. 238, E180–185
7 Foley, J.E., Laursen, A.L., Sonne, O. and Gliemann, J. (1980) Diabetologia 19, 234–241
8 Hankin, B.L. and Stein, W.D. (1972) Biochim. Biophys. Acta 288, 127–136
9 Foster, D.M. and Jaquez, J.A. (1976) Biochim. Biophys. Acta 436, 210–221
10 Brahm, J. (1977) J. Gen. Physiol. 70, 283–306
11 Karlish, S.J.D., Lieb, W.R., Ram, D. and Stein, W.D. (1972) Biochim. Biophys. Acta 255, 126–132
12 Creese, R., Neil, M.W. and Stephenson, G. (1956) Trans. Faraday Soc. 52, 1022–1032
13 Sten-Knudsen, O. (1953) Acta Physiol. Scand. 104, 105–110
14 Gliemann, J. and Vinten, J. (1974) J. Physiol. 236, 499–516
15 Suzuki, K. and Kono, T. (1980) Proc. Natl. Acad. Sci. U.S.A. 77, 2542–2545
16 Carter-Su, C., Pessin, J.E., Mora, R., Gitomer, W. and Czech, M.P. (1982) J. Biol. Chem. 257, 5419–5425
17 Cushman, S.W. and Wardzala, L.J. (1980) J. Biol. Chem. 255, 4758–4762
18 Karnieli, E., Zarnowski, M.J., Hissin, P.J., Simpson, I.A., Salans, L.B. and Cushman, S.W. (1981) J. Biol. Chem. 256, 4772–4777
19 Kono, T., Robinson, F.W., Blevins, T.L. and Ezaki, O. (1982) J. Biol. Chem. 257, 10942–10947
20 Lienhard, G.E., Kim, H.H., Ransome, K.J. and Gorga, J.C. (1982) Biochem. Biophys. Res. Commun. 105, 1150–1156
21 Holman, G.D. and Rees, W.D. (1982) Biochim. Biophys. Acta 685, 78–86
22 Devés, R. and Krupka, R.M. (1978) Biochim. Biophys. Acta 510, 339–348
23 Baldwin, J.M., Lienhard, G.E. and Baldwin, S.A. (1980) Biochim. Biophys. Acta 599, 699–714
24 Foley, J.E., Cushman, S.W. and Salans, L.B. (1978) Am. J. Physiol. 234, 112–119