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D-Glucose Transport into Suspended Human Fibroblasts

Rapid Measurement of Uptake by Silicone Oil Filtration Centrifugation, and Comparison of Different Cell Detachment Procedures

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Summary: The uptake of ^{14}C-labeled D-glucose into the cellular space of human diploid fibroblasts (Flow 2000) – grown to confluency and detached with trypsin-EDTA – was studied using silicone-oil-layer-filtering centrifugation. This method is rapid enough to enable the determination of initial transport rates, which are not complicated by subsequent metabolism of the hexose taken up into the cells. D-Glucose uptake shows saturation kinetics with a K_m of 1.8mM and maximal transport capacity of 4–8 nmol/(10^6 cells × min) at 20 °C. This saturable transport system is responsible for at least 80% of the total glucose taken up into the cells in the concentration range tested (0.1–10mM D-glucose in incubation medium). The glucose carrier is stereospecific, is independent of sodium and potassium ions, and is inhibited by cytochalasin B. Its temperature dependence reveals an activation energy of 31 kJ/mol (7.5 kcal/mol; $Q_{10} \approx 1.5$). As detachment of the cells from the culture flasks is necessary for applying silicone-layer-filtering centrifugation, various detachment procedures were tested. In the enzymatic procedure cells were treated with either trypsin or pronase. In the chelating method, $Ca^{2\oplus}$ and $Mg^{2\oplus}$ ions were chelated by EDTA and $K^{\oplus}$ ions with sodium tetraphenylborate. For mechanical detachment, cells were grown initially on plastic foil. After each of these detachment procedures the transport of D-glucose was the same. It is therefore concluded that this method of rapid measurement of D-glucose uptake in suspended human fibroblasts may serve as an alternative to the uptake measurement with glucose analogues in attached cells when studying the hexose transport system in human diploid fibroblasts.

Enzymes:
Trypsin (EC 3.4.21.4);
Pronase P (*Streptomyces griseus* neutral proteinase, EC 3.4.24.4).

Abbreviations:
EDTA = Ethylenediamine tetraacetic acid;
Hepes = 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid;
Glc = D-glucose;
2-dGlc = 2-deoxy-D-glucose;
3-MeGlc = 3-*O*-methyl-D-glucose;
NHI = National Institute of Health.

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D-Glucosetransport in suspendierten menschlichen Fibroblasten. Messung mittels Silikonöl-Filtrationszentrifugation und Vergleich verschiedener Verfahren zur Ablösung der Zellen

Zusammenfassung: Mittels der Silikonöl-Filtrationszentrifugation wurde die Aufnahme ^{14}C-markierter D-Glucose in diploide, menschliche Fibroblasten (Flow 2000) untersucht, nach Ablösen der konfluenten Kulturen durch Trypsin-EDTA-Lösung. Diese Methode erlaubt die Bestimmung initialer Aufnahmegeschwindigkeiten für D-Glucose. Aufgrund der kurzen Meßzeiten kann eine klare Differenzierung zwischen Aufnahmevorgang und nachfolgender intrazellulärer Metabolisierung der D-Glucose vorgenommen werden. Die D-Glucoseaufnahme zeigt Sättigungsverhalten mit einem K_m-Wert von 1.8mM und einer maximalen Transportkapazität von 4–8 nmol/(10^6 Zellen × min) bei 20 °C. Im untersuchten Konzentrationsbereich (0.1–10mM D-Glucose im Inkubationsmedium) erfolgt die D-Glucoseaufnahme in die Zellen zu mindestens 80% über dieses sättigbare Transportsystem. Der Glucosecarrier zeigt Stereospezifität, arbeitet unabhängig von Natrium- und Kaliumionen und wird durch Cytochalasin B gehemmt. Aufgrund der Temperaturabhängigkeit errechnet sich eine Aktivierungsenergie von 31 kJ/mol (7.5 kcal/mol; $Q_{10} \sim 1.5$). Da zur Anwendung der Silikonöl-Filtrationszentrifugation das Ablösen der Zellen von der Züchtungsfläche erforderlich ist, wurde der Einfluß verschiedener Zellablösungsverfahren (enzymatisch, Komplexieren von Kationen, mechanisch) auf die Aufnahme von D-Glucose untersucht: Das Ablösen der Zellen mit Trypsinlösung oder mittels kurzzeitiger Inkubation in Pronaselösung, das Komplexieren von Calcium- und Magnesiumionen mit EDTA und von Kaliumionen mit Tetraphenylbor-Natrium, sowie das mechanische Ablösen auf Plastikfolien gezüchteter Zellen erbrachten vergleichbare Ergebnisse. Die Messung der D-Glucoseaufnahme in suspendierten menschlichen Fibroblasten mittels Silikonöl-Filtrationszentrifugation erlaubt die Verwendung des physiologischen Substrates des Glucose-Transportsystems. Sie stellt somit bei Untersuchungen des Hexose-Transportes in diploiden menschlichen Fibroblasten eine Alternative zu den Transportmessungen mit Glucoseanalogen in substratfixierten Zellen dar.

Key-words: Transport kinetics, glucose carrier, trypsinization, human diploid fibroblasts.

The measurement of glucose transport by human diploid fibroblasts in vitro is complicated by its rapid uptake and quick metabolism within the cells, with subsequent release of lactate[1]. The kinetics of this uptake process, therefore, cannot easily be ascertained, since current techniques used for such measurements in monolayer cell cultures are not rapid enough. Many investigations on hexose transport in fibroblast strains and lines have for this reason been carried out using glucose analogues such as 2-deoxyglucose which is only phosphorylated, with uptake kinetics linear over a longer time range than D-glucose, and 3-*O*-methylglucose which is not metabolized at all. It should, however, be noted that 3-*O*-methylglucose may not be taken up into the cells exclusively by the glucose transport system[2].

To bypass these difficulties in characterizing the glucose transport system we have measured the uptake of D-glucose into suspended human fibroblasts using the silicone-layer-centrifugation method[3]. This method, which has been successfully applied for measuring hexose uptake into rat liver cells[4,5], allows kinetic measurements within seconds and is, therefore, rapid enough for establishing the kinetics of glucose uptake. A major problem with this approach is the necessity of having the fibroblasts in suspension. It was essential to establish that procedures used for detachment of the cells did not per se alter the functional properties of the glucose carrier. We therefore compared the influence of certain detaching procedures on glucose uptake kinetics of human diploid fibroblasts in vitro. These procedures included enzymatic digestion, chelating agents and mechanical detachment.

Material and Methods

Culture conditions

Commercially available diploid fibroblastoid cells obtained from human foetal lung (Flow 2000) were maintained in Eagle's Minimum Essential Medium with Eagle's salts (Gibco Labs.) and 12% foetal calf serum. Two charges of foetal calf serum (Microbiological Ass. Inc. and Flow Labs. – screened for mycoplasma) were used without noticeable differences with respect to culture growth and transport measurements. The cultures were incubated at 37 °C in an aerated atmosphere with 5% CO_2. Penicillin (100 units/m*l*) and streptomycin (100 μg/m*l*) and 2.2 g $NaHCO_3$/*l* were added to the medium. Culture medium was procured in powder form and was freshly constituted before use. Non-essential amino acids were supplemented and the pH was adjusted to 7.4. The cultures were grown in 174-cm^2 plastic culture flasks (Nunc). Subcultivation was performed by detaching the cells with 0.2% trypsin in a solution of salts (see below) containing 0.1mM EDTA and 5.6mM D-glucose. If not stated otherwise, cultures which were in phase II of their lifespan[6] were used for the measurements after they had attained confluency. Periodic tests for mycoplasma contamination using the method of double labelling with [^{3}H]uracil and [^{14}C]uridine[7] gave negative results. Cell counts were performed under phase-contrast in three independently filled Neubauer chambers.

Detachment procedures

For *enzymatic digestion* either 0.2% trypsin (Serva, 1:250; Cat.No. 37290) or 0.05% pronase (Serva, Cat.No. 33634) were used. These enzymes were dissolved in a salt solution containing 137mM NaCl, 5.4mM KCl, 4.8mM $NaHCO_3$, 0.1mM EDTA and 0.001% phenol red as pH indicator; addition of 0.1mM EDTA to the trypsin solution produced a better dispersion of cells than trypsin alone.

Detachment with chelating agents was performed either with 0.1mM and 1.0mM EDTA, respectively, in the salt solution given above, or with 5mM sodium tetraphenylborate[8] in a salt solution in which KCl was replaced by an equivalent amount of NaCl. Solutions were buffered with 20mM Hepes. The final pH was adjusted to 7.4 at 20 °C with 1N NaOH. Cultures were washed three times with the salt solution before detaching agents were applied. The time interval between the addition of the detaching agents and the start of glucose measurements is given in Table 4. In the case of 0.1mM EDTA[9] and sodium tetraphenylborate only partial detachment was achieved even after prolonged incubation.

For *mechanical detachment* cells were grown on plastic foil[10]. Subconfluent cultures were carefully washed with the salt solution described above and detached by gently stretching the foil. This yielded small clumps of cells. It was not possible to obtain cell counts in this case because of the danger of cell destruction during the process of dispersion through vigorous pipetting.

Measurement of sugar uptake

Silicone-layer-filtering-centrifugation

Filtering-centrifugation[3,11] was carried out with a Beckman Microfuge using 0.4 m*l* polypropylene tubes (Beckman Instruments München). The tube was filled with 20 μl 1M $HClO_4$ at the tip. This was followed by 70 μl silicone oil (AR 20:100 = 1:3, Wacker Chemie, München), on top of which 250 μl of the fibroblast suspension (5×10^5 to 1.5×10^5 cells/m*l*) were added. If not stated otherwise, all incubations were carried out at 20 °C in the 0.2% trypsin solution. Incubation was started by addition of the radioactive labelled substance in a volume of 10 μl and was terminated by centrifugation of the fibroblasts through the silicone oil into the perchloric acid. Within one second after the beginning of centrifugation, more than 85% of the cells were found already separated from the medium as shown by labelling of cell space by tritiated water.

For *measurements of radioactivity*, 20 μl of the supernatant salt solution was taken and counted in Instagel in a Packard liquid scintillation counter with correction for quench. The content of the plastic tube was then frozen; the tip of the tube containing the perchloric acid-fraction with a thin adhering film of silicone oil was cut off with a blade and suspended in 300 μl water. After vigorous shaking and centrifugation of the protein precipitate, 200 μl of the supernatant was taken for counting. After dissolving the protein precipitate in 100 μl 1N NaOH at 60 °C for 60 min and neutralization with 1N HCl, radioassay showed that during the time range we used for the determination of glucose uptake rates (10–45 sec), less than 1% of the [^{14}C]D-glucose found within the cells was incorporated into the perchloric acid insoluble fraction. For this reason, radioassay was carried out in most experiments simply by cutting off the tip and putting it straight into the scintillation cocktail. This procedure gave identical results to the above mentioned method. In similar experiments not shown here the perchloric acid in the centrifugation tube was replaced by 1N NaOH. The differences in quenching of NaOH and perchloric acid samples were corrected with quench curves obtained either with NaOH or perchloric acid as quencher. No significant difference in the cell associated radioactivity could be found between cells centrifuged into perchloric acid and cells centrifuged into sodium hydroxide. This indicates that the amount of [^{14}C]CO_2 and [^{14}C]$HCO_3^{\ominus}$ inside the cells, generated by glucose metabolism within the time range of our measurements, is negligible. The conditions of incuba-

tion were chosen so that less than 1% of the added radioactive substance appeared in the sediment after centrifugation.

Determination of cell space

Determination of the cell space of human diploid fibroblasts by filtering-centrifugation was carried out in two different ways:

a) Incubation of the cells with [^3H]water and inulin [^{14}C]carboxylic acid

The volume of [^{3}H]water found in the sediment after centrifugation expresses the intracellular space of the fibroblasts and its film of adherent medium. The latter can be measured with inulin [^{14}C]carboxylic acid (abbreviated as inulin), which does not penetrate the cells. The amount of [^{14}C] inulin found in the sediment remains constant with increasing incubation time (1 to 15 min). Only the inulin fraction, soluble in 50% ethanol was taken for the determination of the inulin space, because this fraction gave more reproducible results than the water soluble inulin. Non-radioactive inulin in excess did not alter the amount of [^{14}C]inulin in the sediment, indicating that inulin was not unspecifically bound to the plasma membrane. Subtraction of this inulin space from the total water space yields the inulin-impermeable water space, which is defined as "cell space" hereafter. This cell space is equal to 79 ± 6% (SD, n = 150) of water space. Water- and inulin spaces ($\mu l/10^6$ cells) respectively are calculated according to the following formula:

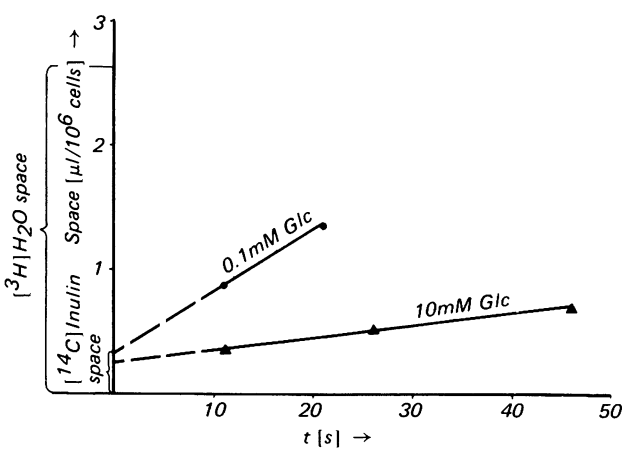
$$\text{Space} = \frac{\text{R.act.}_S \times V_M}{\text{R.act.}_M \times \text{cells}};$$

R.act.$_S$ = radioactivity in sediment fraction (in disintegrations/min = dpm);
R.act.$_M$ = radioactivity in incubation volume (in disintegrations/min = dpm);
V_M = volume of incubation medium (in μl);
cells = number of cells in incubation volume (in millions)

b) Determination of the cell space via glucose uptake kinetics

Incubation of the fibroblasts with [^{14}C]D-glucose and subsequent centrifugation yields [^{14}C]D-glucose in the sediment which consists of the [^{14}C]glucose taken up by the cells plus the [^{14}C]glucose remaining in the adherent medium. Since the latter is independent of incubation time, the amount of ^{14}C radioactivity obtained in the sediment after zero time incubation allows calculation of the volume of the adherent medium (see formula above), indicated as zero time glucose space. For practical reasons zero time yields of ^{14}C radioactivity were extrapolated from the yields obtained after different incubation times. This extrapolation can be correctly done provided that glucose uptake measurements are performed within the linear range and that there is no unspecific absorption of glucose to the plasma membrane. Fig. 1 shows a typical experiment, in which cell space was determined using two different concentrations of [^{14}C]glucose and [^{14}C]inulin, respectively. The good agreement of the inulin space and the zero time glucose space confirms that both preconditions for obtaining a correct value of the latter space were actually fulfilled. The measurement of initial rates of glucose uptake is limited by the accuracy of the determination of cell-adherent medium in the sediment

Fig. 1. Linear uptake of D-glucose into suspended human fibroblasts.



Abscissa: incubation time of cells with [^{14}C]-glucose.

Ordinate: virtual fraction of the incubation solution calculated from [^{14}C]activity in the sediment, representing a certain volume of incubation solution depleted of D-glucose by uptake into the cells plus incubation solution adherent to the cells.

Extrapolation to zero-time yields the latter volume. A good agreement of the spaces accessible by inulin at 3-min incubation time and glucose at zero-time incubation can be seen. Each point represents the mean from triplicate measurements. Calculated cell space: via [^{14}C]inulin; 2.28 $\mu l/10^6$ cells, via 0.1mM [^{14}C]D-glucose: 2.29 $\mu l/10^6$ cells, via 10mM [^{14}C]D-glucose: 2.36 $\mu l/10^6$ cells.

and the time range at which the linearity of uptake is preserved. To confirm the validity of uptake measurements control experiments as shown in Fig. 1 were included in all experiments to be discussed subsequently.

Uptake of radioactively labelled substances into cell space

The cells were incubated with [^{3}H]water and the ^{14}C-labelled substance whose uptake was to be measured. The cells were then centrifuged through the silicone oil and the concentration C_c of the [^{14}C]substance in the cell space (in nmol/μl) was calculated using the following formula:

$$C_c = \frac{(^{14}\text{C R.act.}_S - \alpha \times {}^{14}\text{C R.act.}_M) \times {}^{3}\text{H R.act.}_M \times C_M}{({}^{3}\text{H R.act.}_S - \alpha \times {}^{3}\text{H R.act.}_M) \times {}^{14}\text{C R.act.}_M}$$

R.act.$_{S,M}$ = radioactivity of [^{14}C]substance and [^{3}H]-water respectively either in sediment fraction S or incubation volume M (in disintegrations/min = dpm);

C_M = concentration of [^{14}C]substance in incubation volume (in mmol/l).

α = ratio of R.act.$_S$: R.act.$_M$ for [^{14}C]inulin.

The value α represents the medium fraction adherent to the cells, which had been carried through the silicone oil.

Multiplication of C_c with the cell space (in $\mu l/10^6$ cells) yields the amount of the ^{14}C-labelled substance (in nmol) taken up by 10^6 cells. For further explanation see[11].

Measurement of protein and phosphorylated glucose

Protein was measured by the method of Lowry et al.[12]. The determination of the phosphorylated D-glucose was carried out with the anion-exchanger AG 1X8 acetate (400 mesh, Biorad Lab., München) as described by Baur and Heldt[5].

Radionuclides

All radioactive substances were purchased from Amersham-Buchler, Braunschweig, and used with the spec. act. indicated: [^{3}H]water (0.5 to 2×10^{-4} Ci/mol); inulin [^{14}C]carboxylic acid (mean molecular weight 5000, 7.5 Ci/mol, 0.2 to 0.7mM); [^{14}C]D-glucose (uniformly labelled, 0.15 to 50 Ci/mol); [^{14}C]L-glucose (1.3 Ci/mol); [^{14}C]D-glucose 6-phosphate (1.3 Ci/mol); [^{14}C]D-fructose (1.3 Ci/mol); [^{14}C]D-fructose 6-phosphate (1.3 Ci/mol); [^{14}C]sucrose (1.3 Ci/mol). The purity of the radioactivly labelled hexose phosphates was tested by thin-layer chromatography prior to use.

Non-radioactive sugars

Non-radioactive D-glucose, L-glucose, D-glucose 6-phosphate, D-fructose, D-fructose 6-phosphate, sucrose and cytochalasin B were obtained from Sigma Chemie GmbH, München. All other substances were of analytical grade and were purchased from Merck, Darmstadt, or Serva, Heidelberg.

Results and Discussion

Kinetics of hexose uptake

The uptake of D-glucose at 20 °C into detached human diploid fibroblasts in suspension is a fast process, as is demonstrated in Fig. 2. At a concentration of 20mM D-glucose in the medium, the uptake into cell space is linear only within the first minute of incubation; at 1mM glucose concentration, linearity is obtained only within the first 20 seconds. In uptake measurements over a longer time range (1–10 min), not shown here, it was

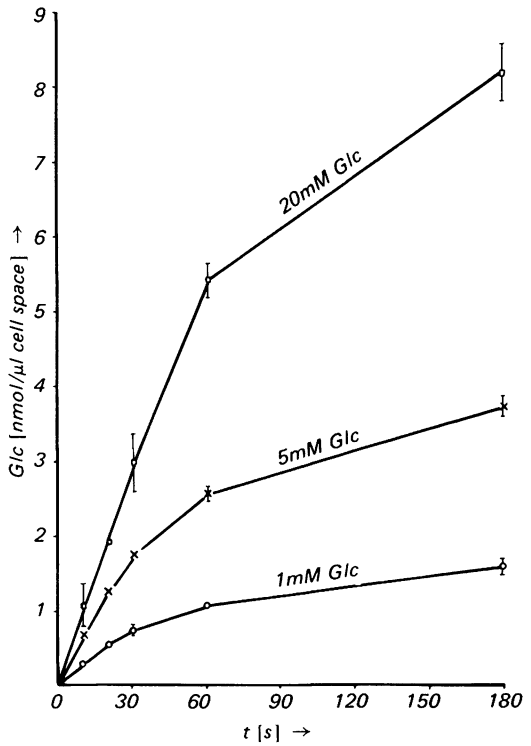


Fig. 2. Time course of D-glucose uptake into suspended human fibroblasts at a glucose concentration of either 1, 5 or 20mM in the incubation solution.

Cell space 2.8 $\mu l/10^6$ cells; temp. 20 °C. Mean values from triplicate measurements are given with standard deviations (SD), the standard deviations lying within the symbols used, if arrows are not indicated.

seen that in the case of 1mM [^{14}C]D-glucose in the incubation medium, the amount of radioactivity found within the cells reached equilibrium after about 3 min. The equilibrium could be a mere reflection of the rapid excretion of [^{14}C]lactate resulting from metabolized glucose[13,1]. All three curves in Fig. 2, however, yield an uptake value of zero when extrapolated to zero time, which is consistent with the assumption that the measurements indicate initial transport rates. From measurements like these, initial rates of uptake can be evaluated, which are demonstrated for various sugars and sugar phosphates in Table 1. The strong stereospecificity of the uptake process (D- versus L-glucose) as well as the relative impermeability of the cell membrane for hexose phosphates (D-glucose phosphate and D-fructose phosphate) is clearly seen. The transport rates for D-fructose and sucrose are more than one order of magnitude lower than the one for D-glucose.

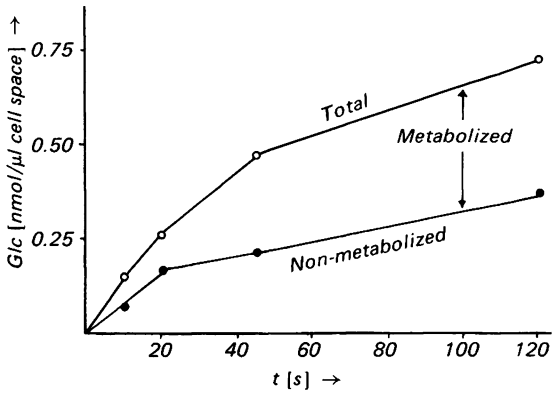


Fig. 3. Uptake of D-glucose into suspended human fibroblasts and determination of the glucose fraction metabolized after different incubation times with [^{14}C]D-glucose.

D-Glucose concentration in the incubation solution: 0.5mM; cell space 2.7 $\mu l/10^6$ cells; temp. 20 °C. For further experimental details see text.

Metabolic influence on uptake measurements

The glucose taken up into the cells was rapidly metabolized as shown in Fig. 3. In this experiment the cellular incorporated radioactivity was allowed to pass through the anion-exchanger AG1X8 acetate. Free glucose was eluted with H_2O, whereas the metabolized fractions were retained in the column and, thereafter, eluted with 2M HCl. After correction for extracellular radioactivity (inulin space), the concentrations of free and total (free + metabolized) glucose inside the cells were plotted as a function of incubation time. Even after 10 s a considerable amount of glucose was already metabolized. Though the concentration of total glucose inside the cells exceeded that of glucose in the incubation medium after 55 s, the level of free glucose reached only 75% after 120 s. With regard to this rapid metabolism, it has to be remembered that part of the glucose would have been converted to lactate and excreted into the incubation medium – a process which has been detected as early as one minute after incubating rat hepatoma cells[13,14] and human fibroblasts[1]. Such being the case, under our experimental conditions, the calculated transport rates for D-glucose would be underestimated. To check this possibility, the fibroblasts were incubated with iodoacetic acid, and glucose uptake was measured thereafter. This inhibitor blocks glyceraldehyde-3-phosphate dehydrogenase[15] and consequently lactate formation, without impair-

Table 1. Initial rates of sugar uptake into cell space of suspended human fibroblasts.

Sugar concentration in incubation medium: 1mM; cell space: 1.6 μl; temp. 20 °C. Measurement of uptake for D-glucose was carried out at t = 11 s and t = 21 s, for other sugars at t = 11 s and t = 61 s. Each measurement was done three times. Calculation of uptake rates was carried out as described in methods. Substrate spaces ($\mu l/10^6$ cells, mean ± SD): L-glucose 0.49 ± 0.02 (11 s), 0.52 ± 0.04 (61 s); D-fructose 0.50 ± 0.03 (11 s), 0.55 ± 0.02 (61 s); D-glucose 6-phosphate 0.49 ± 0.01 (11 s), 0.55 ± 0.03 (61 s); D-fructose 6-phosphate 0.52 ± 0.01 (11 s), 0.55 ± 0.06 (61 s); sucrose 0.48 ± 0.04 (11 s), 0.55 ± 0.08 (61 s); inulin space 0.48 $\mu l/10^6$ cells. For further explanation see methods.

Sugar	Sugar uptake [nmol/(10^6 cells x 20 s)]
D-Glucose	0.45
L-Glucose	≤ 0.01
D-Fructose	≤ 0.02
D-Glucose 6-phosphate	≤ 0.02
D-Fructose 6-phosphate	≤ 0.01
Sucrose	≤ 0.03

ment of hexose transport[1]. This method was preferred to the direct determination of lactate in the incubation medium for technical reasons. When measuring D-glucose uptake after 20 s (the time normally used) no influence of iodoacetic acid on D-glucose uptake [calculated in nmol/(μl cell space × min)] was observed (Table 2). At 60 s the control value decreased to 65 %, as would be expected from the kinetics of glucose uptake shown in Fig. 2. An increase of 25 % was achieved by addition of iodoacetic acid prior to measurement. From these experiments one may conclude that excreted radioactive lactate was negligible within the first 20 seconds and the recorded values approach true initial rates.

Table 2. Influence of iodoacetic acid on D-glucose uptake into suspended human fibroblasts.

Cells were incubated for 2 min with iodoacetic acid prior to uptake measurement with 1mM D-glucose; temp. 20 °C. Cell spaces: 2.9 $\mu l/10^6$ cells (control); 2.8 $\mu l/10^6$ cells (iodoacetic acid).

	Measuring time [s]	Uptake rates [nmol/(μl cell space × min)]
Control	20	0.81 ± 0.09 (n = 3)
	60	0.52 ± 0.09 (n = 3)
Iodoacetic acid (1mM)	20	0.77 ± 0.07 (n = 3)
	60	0.64 ± 0.12 (n = 3)

Concentration dependence of D-*glucose uptake*

The initial rates of D-glucose uptake at various concentrations in the medium reveal a hyperbolic saturation curve. S (concentration in the medium) plotted against S/v [v = uptake rate in nmol/(μl cell space × min)] yields a linear function which enables the determination of Michaelis constant (K_m) and the maximal transport capacity (V) of the carrier system, as demonstrated in Fig. 4. The factor S/v has been plotted as a function of S, so that the participation of a non-saturable component in the uptake rate may easily be detected by a corresponding drop in linearity at increasing concentrations:

$$v_{tot} = \frac{V \times S}{K_m + S} + K_{NS} \times S \rightarrow$$

$$\frac{S}{v_{tot}} = \frac{K_m + S}{V + K_{NS} \times K_m + K_{NS} \times S}$$

when v_{tot} = total uptake rate and K_{NS} = rate constant of non-saturable uptake. Thus, the results in Fig. 4 show that D-glucose uptake is mediated exclusively by a saturable carrier process up to concentrations which equal at least three times the K_m value. At higher concentrations a slight drop in linearity was observed in part of these experiments (see Fig. 4, 10mM), which could reflect glucose uptake by simple diffusion[16]. This non-saturable component, however, never exceeded 20% at 10mM D-glucose, the highest used in our experiments.

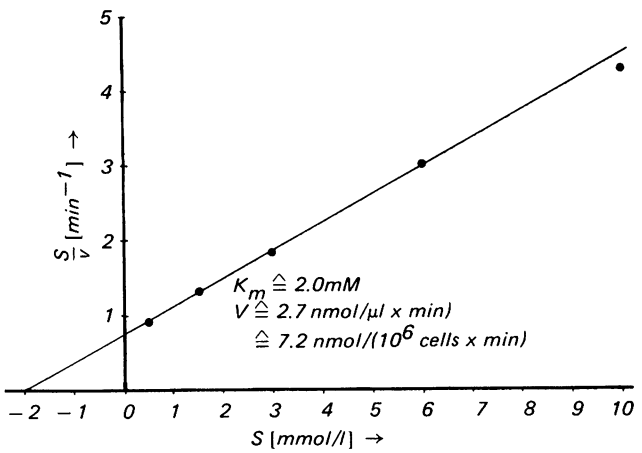


Fig. 4. Concentration dependence of D-glucose uptake into suspended human diploid fibroblasts.

Cell space 2.7 $\mu l/10^6$ cells; temp. 20 °C. For regression analysis, the values for S = 0.5, 1.5, 3.0 and 6.0mM were used. Each point represents the mean from triplicate measurements. S: concentration of D-glucose in the incubation solution in mmol/l; v: uptake rate in nmol/(μl cell space × min); S/v in mmol × l^{-1}/nmol × μl^{-1} × min^{-1} = min^{-1}.

Table 3. Comparison of glucose uptake systems in various animal cells in culture.
(2-dGlc: 2-deoxy-D-glucose; 3-MeGlc: 3-*O*-methyl-D-glucose; Glc = D-glucose) K_i: indirect determination by inhibition of glucose uptake by 2-deoxyglucose and vice versa resp.

Cell type	Culture conditions during uptake measurement	Sugar tested	Temp. [°C]	K_m [mM]	V [nmol/ 10^6 cells x min]	V [nmol/ mg prot. x min]	Activat. energy [kcal/mol]	Ref.
Human diploid fibroblasts (Flow 2000)	Cells grown to confluency, suspended in trypsin-EDTA	Glc	20	1.8 ± 0.4 (SD)	6.4 ± 2.2 (SD)	12.8 ± 4.4 (SD)	7.6 (4–30 °C)	See Fig. 4, 5, 6 Table 4 and text
	Values estimated from data of Fig. 5		37		7 – 14	14 – 28		See Fig. 4, 5, 6 Table 4 and text
Human diploid fibroblasts (HSWP)	Confluent attached cells	Glc 2-dGlc	37 37	~2 (K_i) ~2 (K_i)				[1]
Mouse embryo tertiary fibroblasts (BALB)	Plasma membrane vesicles from confluent cultures	Glc	Room temp.	2.0		1.3		[17]
Mouse embryo fibroblasts (NIH Swiss)	Attached cells, exponential growth phase	2-dGlc	37	2.1 – 2.3		18.2 – 19.2		[29]
Mouse embryo cells (STU)	a) Attached cells $(2.9 - 16) \cdot 10^4$ cells/ cm^2; b) After dispersal by treatment with trypsin-EDTA	2-dGlc	37	1.1 – 2.0 1.1 – 2.0	1.9 – 4.0 1.9 – 4.0			[18]
Mouse 3T3 cells	Confluent cell layer	2-dGlc Glc	37	1.7 1.8 (K_i)				[2]
Chick embryo fibroblasts	Confluent cell layer	3-MeGlc	22	1.1		0.6		[20]

Cell type	Culture conditions during uptake measurement	Sugar tested	Temp. [°C]	K_m [mM]	V [nmol/ 10^6 cells x min]	V [nmol/ mg prot. x min]	Activat. energy [kcal/mol]	Ref.
Chick embryo cells (body wall)	Attached cells, growing cultures	2-dGlc 3-MeGlc	39 Room temp.	0.51 ± 0.28 3.5 ± 0.54		1.8 ± 0.28 0.82 ± 0.10		[19]
Chick embryo cells (body wall)	Attached cells, growing cultures	2-dGlc					10.6	[23]
Chick embryo cells	Attached cells, growing cultures	2-dGlc	–	2.7		1.6		[16]
Novikoff rat hepatoma cells	Suspension culture, exponential growth phase	2-dGlc Glc	37	1.3 – 2.5 1 – 2	1 – 5 4 – 8		15.6 (22–37 °C)	[13]
Novikoff rat hepatoma cells	Suspension culture, exponential growth phase	3-MeGlc	23	2.4 ± 0.8	20*			[21]
Primary rat liver cells	Suspended cells after liver treatment with collagenase	Glc	20	30		110	22 (4–18 °C) 7 (18–37 °C)	[5]
Human glioma cells	Attached cells $(2.8 - 25) \cdot 10^4$ cells/ cm^2	2-dGlc	37	4.5 – 6.2		45 – 100		[30]
Mouse neuroblastoma cells (C1300/ Nb41A3)	Attached cells $(2-12) \times 10^4$ cells/cm^2 12×10^4 cells/cm^2 2×10^4 cells/cm^2 12×10^4 cells/cm^2	2-dGlc Glc	37	0.5 – 4.9 3.2 (K_i)		1.9 – 18.3	 4.6 8.9	[22]

* Calculated from the value given in the legend of fig. 4 (V = 0.26 ± 0.03 nmoles/μl cell water x sec., and the average value for intracellular water space (1.3 $\mu l/10^6$ cells) described in methods.

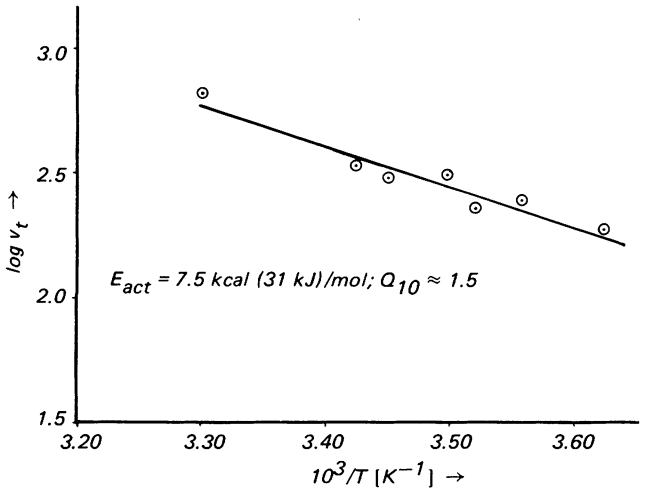


Fig. 5. Temperature dependence of the uptake of D-glucose into suspended human fibroblasts.

Concentration of D-glucose 1mM; cell space 1.4 $\mu l/10^6$ cells. The cell suspension was obtained from growing cultures (subconfluent monolayers). Initial rates of glucose transport v_t were measured as nmol D-glucose transported per 10^6 cells and 15 s. With regard to activation energies calculated from these measurements it should be noted that uptake measurements could not be performed at saturating glucose concentration for technical reasons.

Temperature dependency

The temperature dependence of D-glucose uptake, because of its rapidity, was tested only between 3 °C and 30 °C. This has been plotted in Fig. 5 as the logarithm of the initial rates versus the reciprocal of temperatures. From the Arrhenius plot an activation energy of 7.5 kcal (31 kJ)/mol ($Q_{10} \sim 1.5$) was obtained.

Comparison of uptake parameter

The kinetics for D-glucose uptake shown in Fig. 4 are representative of this strain of fibroblasts in phase II[6], as measured in suspension after trypsinization of confluent cultures. From 21 confluent cultures the following mean values were obtained at 20 °C: K_m = 1.8 ± 0.4 (SD) mM; V = 2.4 ± 0.6 (SD) nmol/(μl cell space × min) and 6.4 ± 2.2 (SD) nmol/(10^6 cells × min), respectively. Taking protein content as 0.52 ± 0.14 (SD) mg/10^6 cells (n = 24 cultures), an uptake rate of 8–16 nmol/(mg protein × min) was estimated. The dependence of these parameter on in vitro age of the cultures is discussed elsewhere*. The characteristics of this glucose transport system is compared in Table 3, with those obtained by other authors using fibroblast cultures and various other animal cells in culture, under different experimental conditions. For the purpose of comparison, the V values of our measurements done at 20 °C have been extrapolated to 37 °C; though it should be emphasized that the linearity of temperature dependence was checked only up to 30 °C (Fig. 5). There is good agreement of the K_m value of the glucose transport system described here with the one obtained with membrane vesicles of mouse embryo tertiary fibroblasts[17] and the estimated values for D-glucose uptake in HSWP human diploid fibroblasts[1] and mouse 3T3 cells[2]. The V values for D-glucose uptake into the strain of cells reported here are higher than those reported in mouse embryo cells[18] and chick embryo fibroblasts[19,16] for 2-deoxyglucose, and in chick embryo fibroblasts[20] for 3-*O*-methylglucose, but comparable with the maximal transport capacity for D-glucose uptake in Novikoff rat hepatoma cells[13]. Direct comparison of these data may be complicated by differences in culture conditions and growth phases of the cell cultures used. It may be noted, however, that Wohlhueter et al.[21] measured a relatively high V value for 3-*O*-methylglucose transport in rat hepatoma cells using a similar rapid kinetic technique to that employed by us. Considering the high V values obtained by us, it has to be taken into account, that in our experimental procedure the cells were starved from glucose for 15–60 min prior to uptake measurement. This could induce a higher transport capacity. From the results described by Salter and Cook[1],

* Cremer, T., Werdan, K., Stevenson, A.F.G. & Lehner, K., unpublished.

however, one may conclude that this time interval is not sufficient to induce considerable increase in glucose transport capacity.

The activation energy for D-glucose transport into Flow 2000 fibroblasts (Fig. 5) is comparable with the one described by Walum and Edström[22] for mouse neuroblastoma cells (Table 3) and by Baur and Heldt[5] for suspended rat liver cells (in the range 18–37 °C), but lower than the one reported for chick embryo cells[23] and rat hepatoma cells[13].

Influence of sodium and potassium ions

D-Glucose transport is independent of sodium ions: D-glucose uptake rates were 0.32 ± 0.02 nmol/(μl cell space × 20 s) in the presence of sodium ions (137mM), and 0.39 ± 0.02 without sodium ions, where NaCl has been replaced by equivalent concentrations of choline chloride (temp. 20 °C, preincubation period in $Na^{\oplus}$ and choline medium resp.: 30 min, $n = 3$; [D-glucose] = 1mM). The non-dependence on potassium ions can be deduced from the experiment of Table 4 (III), where sodium tetraphenylborate has been used to detach the cells by complexing potassium ions. No significant differences in K_m and V values with respect to other detachment procedures were found.

Cytochalasin B inhibition of the glucose carrier

D-Glucose uptake is specifically inhibited by cytochalasin B (control: 1.13 ± 0.10 nmol/(μl cell space × min); 2μM cytochalasin B: 0.14 ± 0.02; temp. 20 °C, $n = 3$; preincubation period with cytochalasin B: 5 min; [D-glucose] = 1mM). The specificity of this inhibitor for the sugar transport system in chick embryo fibroblasts[16] is retained in trypsinized, suspended human fibroblasts. This strong inhibition is an affirmation of the results shown in Fig. 4, that D-glucose uptake is mainly due to a carrier process, since this inhibitor specifically blocks the carrier-mediated D-glucose uptake[16]. It may be noted, however, that Graff et al.[31] presented evidence that the non-mediated hexose uptake can also be inhibited by cytochalasin B. The concentration of cytochalasin B for half-maximal inhibition of hexose non-mediated diffusion (L-glucose, Novikoff rat hepatoma cells) was, however, 10μM, five times the concentration we used in our experiments.

Table 4. D-Glucose uptake into suspended human fibroblasts.
Comparison of various detaching agents. Temp. 20 °C; incubation time: time between start of cell detachment and beginning of glucose uptake measurements. For experimental details see legends to Fig. 4 and 6. The cells grown on plastic foil detached as small clusters, which made exact cell counting impossible (see methods). Therefore the determination of the cell space (as μl/10^6 cells) and the calculation of the maximal transport capacity in nmol/(10^6 cells × min) were not feasible. No carrier-independent D-glucose uptake up to 6mM glucose in the medium was detectable in any of the described experiments.

Experimental Series Nr.	Incubation procedure	Incubation time [min]	Cell space [μl/10^6 cells]	K_m [mM]	D-Glucose transport (V) [nmol/(μl cell space × min)]	[nmol/(10^6 cells × min)]
I	Trypsin (0.2%) + EDTA (0.1 mM)	30	2.4	2.0	2.6	6.4
	Pronase (0.05%)	15	2.5	2.2	2.0	4.9
	EDTA (0.1 mM)	45	2.4	1.9	2.0	4.8
II	Trypsin (0.2%) + EDTA (0.1 mM)	30	2.5	1.6	2.2	5.4
	Trypsin (0.2%) + EDTA (0.1 mM)	30	2.3 } 2.6 ± 0.3	2.0 } 1.6 ± 0.3	3.0 } 2.4 ± 0.5	7.0 } 6.2 ± 0.6
	Trypsin (0.2%) + EDTA (0.1 mM)	30	2.9	1.3	2.1	6.2
	EDTA (1 mM)	30	3.1	1.9	2.7	8.5
	Mechanical detachment of cells grown on plastic foil	10	–	1.5	2.9	–
III	Sodium tetraphenylborate (5 mM)	75	2.7	1.9	1.8	4.7
	Pronase (0.05%)	15	3.0	2.1	1.9	5.6

Comparison of detaching agents on D-*glucose uptake*

The results presented in the previous paragraphs demonstrate the presence of a glucose carrier in the cell membrane of trypsinized human fibroblasts. The question arises as to whether the trypsinization procedure per se leads to an alteration of the characteristics of this transport system. To test this possibility, two different experimental approaches have been used:

1) To detect a possible proteolytic digestion of the glucose carrier by trypsin, the parameters of glucose transport were determined after different incubation times in 0.2% trypsin and 0.1mM EDTA. Fig. 6 shows one of several experiments, in which glucose transport of the same culture was measured after 30 and 90 min resp. of incubation. The cell space ($\mu l/10^6$ cells) remained constant during this period, indicating that there was no time-dependent swelling or shrinking of the cells. The cells did not become permeable to inulin, more than 90% of them excluded trypan blue, and subcultures grew satisfactorily after 90 min trypsin incubation. The close agreement of K_m and V values found after both incubation times indicates the resistance of the glucose carrier to trypsin digestion. There is no indication that incubation times with trypsin shorter than 30 min would have led to higher transport rates (data not shown), but the possibility cannot be excluded that even the shortest time of 10 to 15 min necessary to obtain a suitable single cell suspension might be sufficient to destroy a trypsin-sensitive fraction of glucose carriers.

2) To investigate this question, uptake kinetics obtained after incubation with trypsin and EDTA were compared with those obtained after detachment of cells with EDTA alone (Fig. 6 and Table 4) and other detachment procedures (Table 4). In the presence of 0.1mM EDTA part of the cells became detached from the surface of the culture vessel only after 45 min incubation time. These cells, however, gave rise to similar uptake kinetics, the difference lying within the normal range when measurements of different cultures are considered. The non-carrier-mediated D-glucose uptake at a concentration of 10mM D-glucose in the incubation solution, which can be calculated

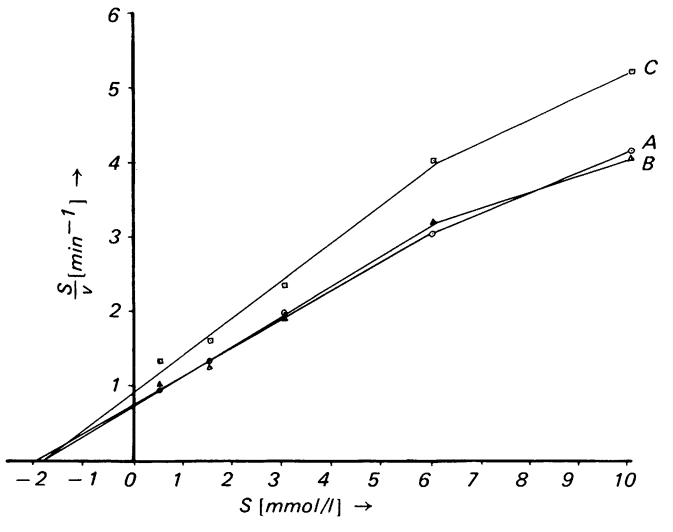


Fig. 6. Concentration dependence of D-glucose uptake into suspended human fibroblasts: influence of different incubation times with 0.2% trypsin, 0.1mM EDTA and comparison with cell detachment by 0.1mM EDTA only. *Curve A:* 0.2% trypsin + 0.1mM EDTA, incubation time 30 min, K_m = 2.0mM, V = 2.6 nmol/(μl x min); *curve B:* 0.2% trypsin + 0.1mM EDTA, incubation time 90 min, K_m = 1.9mM, V = 2.5 nmol/(μl x min); *curve C:* 0.1mM EDTA (without trypsin), incubation time 45 min, K_m = 1.9mM, V = 2.0 nmol/(μl x min). 30 min after the beginning of the detachment procedure with trypsin and EDTA, glucose uptake was measured using half of the cell suspension, the other half was incubated for further 60 min in the trypsin/EDTA solution before measurements were repeated. These results are representative for those obtained in four similar experiments. Cells from another culture were detached by treatment for 45 min with a solution which contained 0.1mM EDTA, while trypsin was omitted. Each point represents the mean from triplicate measurements. Performance of all measurements in each of the curves in Fig. 3 and 6 lasted for approximately 20 min. Temp. 20 °C; for regression analysis, the values for S = 0.5, 1.5, 3 and 6mM have been taken into account. For the units of S and S/v see legend to Fig. 4.

from the graphs of Fig. 6, amounts to 12% for EDTA (45 min), 10% for trypsin plus EDTA (30 min) and 16% for trypsin plus EDTA (90 min), indicating that the non-saturable component of D-glucose uptake also remained unaltered by trypsin treatment. Experiments as described in Fig. 6 were also carried out with pronase. Though measurements done immediately after detachment of the cells with pronase ($\leqslant$ 15 min) normally yielded K_m and V values comparable to those obtained after trypsin detachment (Table 4), prolonged incubation of the cells with pronase (60 to 90 min) gave results that were less reproducible than those with trypsin treatment. The experimental data (not shown) obtained from six cultures, however, allow no final conclusion as to whether K_m, V or both parameters are indeed altered by prolonged pronase treatment. Besides detachment of the fibroblasts by cleaving peptide bonds and chelation of divalent cations by EDTA, suspended cells were obtained by chelation of potassium ions by sodium tetraphenylborate and mechanical detachment of cells grown on plastic foil. All detachment procedures used yielded comparable K_m and V values (Table 4), none of which lay beyond the standard deviation of the mean values obtained from 21 trypsin detachment experiments, as mentioned above.

Our results, therefore, confirm the observations of various other authors that – despite of the fact that trypsin causes numerous effects on the plasma membrane[24,25] – the glucose transport system of various cells seems to be relatively resistant to tryptic digestion[9,14,26,27]. This is consistent with the view that membrane proteins involved in transport processes are not readily accessible to external hydrolytic enzymes.

Conclusion

The advantage of the advocated method of measuring initial transport rates of the glucose carrier of human diploid fibroblasts lies in its rapidity, which allows the use of its physiological substrate D-glucose instead of analogues. This advantage is taken at the cost of converting the cells from the normal attached to a suspended state. This conversion could possibly cause a reduction of exposed plasma membrane owing to the change of form from a flat spread cell to a spherical one[28] and lead to a redistribution of carrier molecules in such a way as to make them less accessible to their substrate.

A meaningful comparison of the size of the plasma membrane of human diploid fibroblasts in their attached versus their suspended state would, however, require an investigation of the exact changes of number and form of differentiated surface features, like microvilli, filopodia etc., associated with different detachment procedures.

Bissell et al.[9] described a diminished uptake of 2-deoxyglucose into suspended primary and transformed chick cells compared to the uptake of the attached cells; differences between the hexose uptake of the normal and the virus-transformed cells in the attached state were, however, retained in the suspended state. Plagemann[18] studying the transport parameters of 2-deoxyglucose in mouse embryo cells, found no differences in K_m and V values between trypsin-EDTA detached cells and cells grown in monolayers; even the growth density dependece of the maximum transport capacity for 2-deoxyglucose was retained in the suspended cells (Table 3).

Measuring the uptake of D-glucose into suspended cells may serve as alternative to measuring the uptake of glucose analogues into attached cells, when studying the glucose transport system of human diploid fibroblasts. Results concerning changes of glucose transport associated with X irradiation* and in vitro senescence of human diploid fibroblasts** will be presented elsewhere.

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