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AGE-DEPENDENT CHANGES IN 3-O-METHYLGLUCOSE TRANSPORT IN CHICK EMBRYO FIBROBLASTS

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Summary :

Transport accross the cell membrane of 3-O-methylglucose, a non-phosphorylatable glucose analogue, was measured in primary cultures of fibroblasts from 8-, 12- and 16-day chick embryos. Transport of this hexose was found to be 3.5 times and 2 times faster in fibroblasts from 16-day embryos than in fibroblasts from 8- and 12-day embryos, respectively. Compared with 8- and 12-day embryos, the rate of efflux in fibroblasts from 16-day embryos was found to be increased. 3-O-methylglucose transport in these cells did not result in an accumulation of the hexose against a concentration gradient. It was concluded that in fibroblasts from older embryos a facilitated diffusion system for hexose transport was stimulated. Embryo differentiation could be associated with a change in the plasma membrane by increasing either the number or the mobility of the glucose carriers, since the Vmax of the transport system for 3-O-methylglucose increased in fibroblasts from older embryos, while the affinity or Km of the system remained unchanged.

INTRODUCTION

In the tissues of higher organisms, active glucose transport against a concentration gradient has been demonstrated both in the small intestine and in kidney epithelia (1-3), whereas in most other cells, glucose is transported by facilitated diffusion (carrier-mediated transport (4-7)). This implies that the glucose molecule is in the first place bound to a mobile carrier protein which allows the substrate to permeate the cell membrane. An important feature of this model is that the transport is neither energy-dependent nor uni-directional. Thus, if substrates are used which are not metabolized within the cells, it is possible to measure the influx and efflux of the substrates. For such experiments, 3-O-methylglucose has been widely used (8-10). Transport of this sugar has therefore been examined and its rate has been found to be increased in transformed cells (11) and related to the changes of the cell surface properties which are responsible for part of the expression of malignant behaviour (12-13).

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Embryo cells are of particular interest since they exhibit differential cell surface properties related to the stage of embryo development (14-17) and also undergo changes in hexose transport. In an attempt to investigate this hypothesis, we studied the characteristics of 3-O-methylglucose transport in fibroblasts from chick embryos at various stages of development.

MATERIAL AND METHODS.

Cells. The fibroblasts were obtained from 8-, 12- and 16-day chick embryos (Société Jourdain, France) by the method of Rein and Rubin (18), modified as previously described (Aubery and Bourrillon, 19).

Cell cultures. Primary monolayer cultures were made in 16 mm diameter wells in 0.5 ml of Eagle's minimum medium (Société Egic, France), supplemented with 1 % glutamine, 1 % antibiotics (penicillin, streptomycin) and 10 % foetal calf serum. The initial seeding concentration was 10^6 cells/ml (0.5×10^6 cells/well). Cultures were grown in humidified air containing 5 % CO_2 at 37 °C. A sample of cells was counted in a haemocytometer. Each measurement refers only to viable cells and represents the mean of six samples. The experiments were done on subconfluent cultures ($5\ 10^5$ cells/well), which in fibroblasts from 8-, 12- and 16-day embryos occurred after 48, 72 and 96 h, respectively. Confluency was then reached 24, 48 and 96 hours later respectively in fibroblasts from 8-, 12- and 16-day embryos showing a differential proliferative capacity in relation to the age of embryos (19).

Measurement of 3-O-methylglucose (3-O-MeG) uptake. Medium was aspirated and the cells were washed three times with 3 ml (9 ml total) of Dulbecco's phosphate buffered saline (PBS) pH 7.4 for 1 min. each. Cells were incubated at 20 °C in the presence of the labelled sugar ([^{3}H] -3-O-methyl-D-glucose ; specific activity, 6.5-7.7 Ci/mmol., Radiochemical Centre Amersham) at a final concentration of 100 µM in 400 µl of phosphate-buffered saline, pH 7.4 (PBS). At appropriate times, medium was aspirated and cells were washed three times with 3 ml of ice-cold PBS (washing procedure took less than 15 sec.) containing 1 mM phloretin in 1 % ethanol to prevent the release of labelled sugar (20). Cells were then dissolved in 500 µl of 0.1 M NaOH and radioactivity counted in a liquid scintillation spectrometer (Intertechnique SL 30) in 10 ml of scintillation fluid (ACS, Amersham).

Measurement of 3-O-methylglucose efflux. The efflux of labelled sugar from pre-loaded cells was determined by incubating cells in PBS containing [^{3}H] -3-O-methylglucose (1 µCi/ml), for 30 min. at 20 °C. The fluid was then aspirated and the cells washed rapidly once with 2 ml PBS and then incubated for different periods of time in 400 µl PBS minus the labelled sugar. This latter incubation was run either in the absence or presence of unlabelled sugar at a final concentration of 100 mM. The radioactivity in the cells was determined for each incubation period.

Measurement of the Km and Vmax of 3-O-methylglucose transport. All substrate concentrations were determined at 30 sec. in fibroblasts from 8-day embryos and at 15 sec. in fibroblasts from 12- and 16-day embryos. At least three cultures were used for each determination. The non-specific uptake through diffusion was determined in the presence of 100 mM 3-O-methylglucose. The large excess of 3-O-methylglucose was used to prevent specific carrier mediated uptake without affecting non-specific uptake through diffusion (20, 21).

RESULTS.

I - Total 3-O-methylglucose uptake. Cultures of fibroblasts from 8-, 12 and 16-day chick embryos were incubated with 3-O-methylglucose (100 µM, final concentration) for varying periods of time and the amount of isotope taken up by the cells was measured as described under "METHODS".

In fibroblasts from 8-day embryos, the increase of the total uptake of 3-O-MeG was linear with time for at least 30 sec. and only for 15 sec. in fibroblasts from 12- and 16-day embryos. At 15 sec., fibroblasts from 16-day embryos transported 3-O-MeG at a rate 3.5 times faster than those in fibroblasts from 8-day embryos and only 2 times faster than those in fibroblasts from 12-day embryos (Fig. 1). At equilibrium, which was reached within 15 min., the transport of 3-O-MeG reached a similar level in fibroblasts from 8-, 12- and 16-day embryos. In order to determine whether transport of 3-O-MeG was an "uphill" process in these cells, we determined the intracellular concentration of the accumulated sugar. Measuring cell size in a Coulter Particle Counter with a size-distribution analyser (M. CORNIC, personnal communication), we found a mean cell volume of 1100 μ^3/cell corresponding to 1.1 µl per 10^6 cells in fibroblasts from all stages of development. The data in Fig. 1 show that at equilibrium fibroblasts incubated with 100 µM 3-O-MeG had accumulated an amount of approximately 0.1 nmoles of hexose per 10^6 cells corresponding to an internal concentration of 95 µM in fibroblasts from all stages. This value was identical to the extracellular

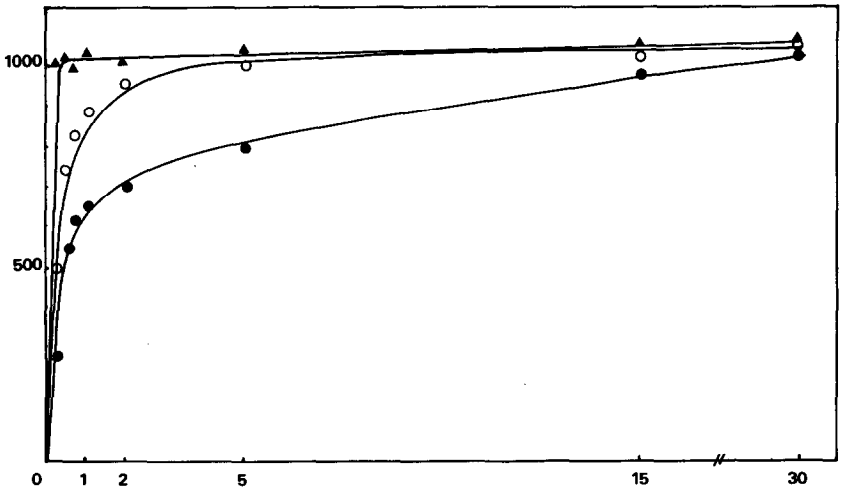

Figure 1 : Rate of uptake of [^{3}H]-3-O-MeG in fibroblasts from 8-, 12- and 16-day chick embryos. The cells were incubated in the presence of 100 µM-[^{3}H]-3-O-MeG at 20 °C. Each point is the average of four separate experiments. Ordinate : cpm/10^6 cells, Abcissa : time in min.
●——● fibroblasts from 8-day embryos, ○——○ fibroblasts from 12-day embryos, ▲——▲ fibroblasts from 16-day embryos.

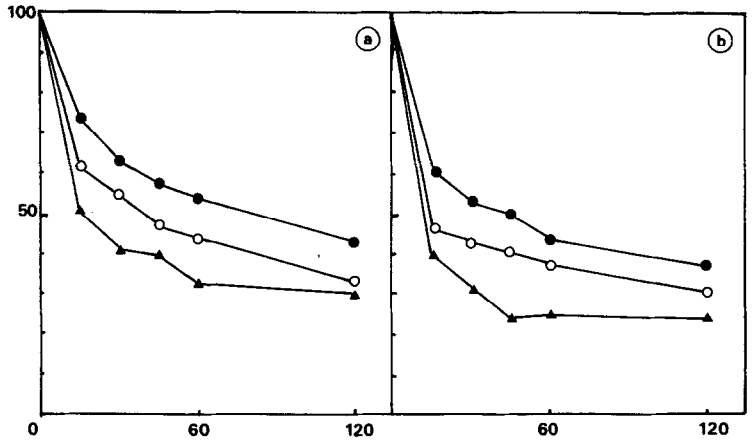

Figure 2 : Efflux of 3-O-MeG from fibroblasts of 8-, 12- and 16-day embryos. The cells were incubated in [^{3}H]-3-O-MeG (1 µCi/ml) for 30 min. at 20 °C, washed with PBS and re-incubated in PBS in the absence, (a), and the presence, (b), of unlabelled 100 mM-3-O-MeG. Each point is the average of four separate experiments.
Ordinate : % cpm/10^6 cells remaining in the cells, Abcissa : time in sec. ●——● Fibroblasts from 8-day embryos, O——O fibroblasts from 12-day embryos, ▲——▲ fibroblasts from 16-day embryos.

concentration. Thus the total uptake of 3-O-MeG was found to increase with time and to reach an equilibrium value between the internal concentration and the outside concentration.

II - 3-O-methylglucose efflux. Cultures were pre-incubated with labelled 3-O-MeG (1 µCi/ml) for 30 min., washed with PBS, incubated, and at varying times the amount of radioactive sugar remaining in the cells was determined. The rate of efflux of 3-O-MeG in fibroblasts from 16-day embryos was about 1.2 times and 1.8 times faster than it was in fibroblasts from 12- and 8-day embryos, respectively (Fig. 2).
Fig. 2 shows that the efflux of intracellular sugar was accelerated by a high concentration (100 mM) of extracellular sugar, this phenomenon has been called accelerative exchange diffusion and constitutes strong evidence that a facilitated diffusion system affects sugar transport in the cells (11, 20).

III - Determination of Km and Vmax of 3-O-MeG transport. The data in Fig. 3, using Lineweaver-Burk plots, show the concentration dependence of the initial rates of 3-O-MeG specific transport in fibroblasts from 8-, 12- and 16-day embryos. To determine initial rates, the cells were incubated at 20 °C for 15 (fibroblasts from 12- and 16-day embryos) or 30 sec. (fibroblasts from 8-day embryos). The 3-O-MeG concentrations ranged from 50 to 2000 µM. The specific carrier-mediated uptake of 3-O-MeG was calculated by subtraction of values

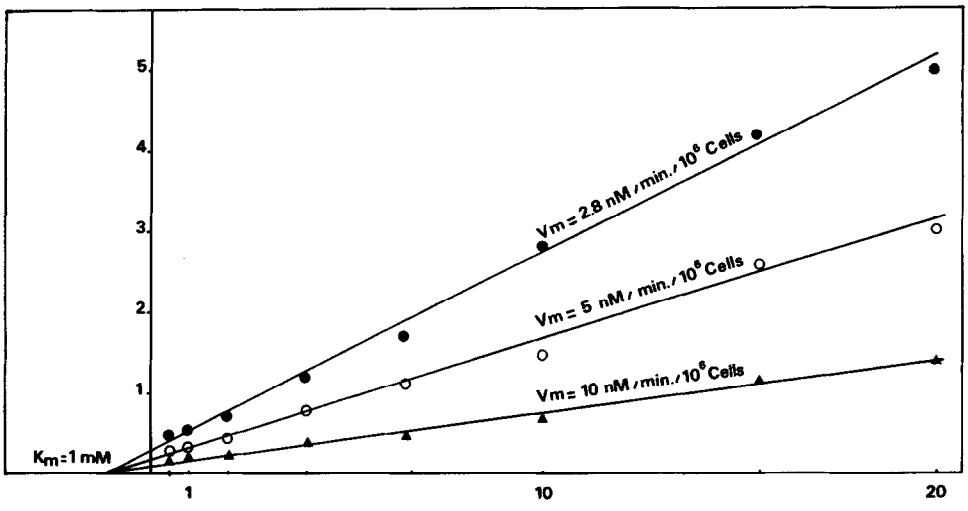

Figure 3 : Lineweaver-Burk plots of 3-O-MeG specific uptake in fibroblasts from 8-, 12- and 16-day chick embryos. The cells were incubated in the presence of [^{3}H]-3-O-MeG for 30 sec. (8-day) or for 15 sec. (12- and 16-day) at 20 °C. The concentrations of [^{3}H]-3-O-MeG ranged from 50 to 2000 µM. Each point is the average of three separate experiments.
Ordinate : $1/V$ (cpm/min./10^6 cells)$^{-1}$ x 10^3, Abcissa : $1/S$ mM^{-1}.
●——● fibroblasts from 8-day embryos, O——O fibroblasts from 12-day embryos, ▲——▲ fibroblasts from 16-day embryos.

for non-specific uptake through diffusion from those for total uptake. The Vmax of the transport system in fibroblasts from 16-day embryos (10 nM/min./10^6 cells) was 3.6- and 2-fold that in fibroblasts from 8- (2.8 nM/min./10^6 cells) and 12-day embryos (5 nM/min./10^6 cells), respectively. No significant difference was found for the Km of the 3-O-MeG transport system in fibroblasts from 8-, 12- and 16-day embryos (ImM) (Fig. 3).

DISCUSSION

It has been postulated that hexose transport may be involved in the control of cell growth (12, 13, 22). Using primary cell cultures, we have found that though the proliferative capacity of fibroblasts from chick embryos exhibited marked differences related to the stage of embryo development as previously reported (19), there was no correlation between these and the differences in the rate of 3-O-MeG uptake. Fibroblasts from older embryos were characterized by a low proliferative capacity and a high rate of 3-O-MeG transport, while in fibroblasts from the younger embryos the proliferative capacity was high and the rate of 3-O-MeG transport low. It appeared that cell proliferation and hexose uptake would be mechanisms that are separately controlled (11, 23-25).

Venuta and Rubin (5) have demonstrated that in secondary cultures of fibroblasts from chick embryos, 3-O-MeG and glucose used the same carrier(s) to move across

the membrane and the uptake of 3-O-MeG could therefore be used as an indicator of the uptake of glucose in these cells.

3-O-methylglucose can enter the cell by means of two types of catalysed process : active transport and facilitated diffusion. Active transport requires energy and is capable of transporting the 3-O-MeG into the cell against a concentration gradient, while facilitated diffusion has neither of these characteristics. Our studies have shown that fibroblasts from 8-, 12- and 16-day embryos were unable to accumulate 3-O-MeG against a concentration gradient and that the efflux of intracellular sugar was accelerated by a high concentration of extracellular sugar. We therefore concluded that 3-O-MeG transport occurred by facilitated diffusion in primary cultures of fibroblasts from the three stages studied and that it was the activation of a facilitated diffusion system that was responsible for the increased rate of 3-O-MeG transport in the fibroblasts from the older embryos. Our results can be compared with those of Venuta and Rubin (5) and of Weber (6) who used secondary cultures of fibroblasts from chick embryos and who found that, after virus-transformation of cells, there was an activation of hexose transport.

Total uptake of 3-O-MeG resulted from both a carrier-mediated process (specific uptake) and a diffusion process (non-specific uptake).

Simple diffusion was studied in the presence of a high concentration (100 mM) of 3-O-MeG in order to block specific uptake without affecting diffusion (20 - 21). The specific uptake (carrier mediated) was then determined by subtraction of the non-specific uptake (diffusion) from the total uptake. In the range of concentrations of substrate used (50-2000 μM), the Lineweaver-Burk plots of the specific uptake of 3-O-MeG was linear which meant that the Vmax and the Km of 3-O-MeG transport could be calculated. We found that the rate of 3-O-MeG transport in our fibroblasts increased along with the stage of the embryo. This increase in the rate of transport could be due either to 1) the unmasking during development of a new carrier with a high affinity for 3-O-MeG or 2) an increase in either the number of carrier molecules with the same affinity for 3-O-MeG or their mobility. In the first case, the Km of the 3-O-MeG transport system in fibroblasts from older embryos would be lower than that in fibroblasts from younger embryos. Our results did not support this. We found that the Km remained unchanged in the fibroblasts from the younger and older embryos which would mean that the carrier molecules at the different stages would be similar. In the second case, the Vmax of the system would increase with aging. Our results agree with this second hypothesis since we observed that the Vmax increased from the 8th to the 16th day of development. This coincides with the results obtained by Venuta and Rubin (5) and Weber (6) with secondary cultures of chick embryo fibroblasts. The most likely explanation would be an increase

in the number of carriers rather than an increase in their mobility from the 8th to the 16th day. Kletzien and Perdue (26) showed that in secondary cultures, the Vmax increase in hexose-starved chick embryo fibroblasts, as compared to cells cultured in standard medium, could be attributed to an increase in the number of carriers since the Km and the activation energy for the transport reaction remained unchanged.

Finally, the embryo stage differences observed in the rate of 3-O-MeG uptake could be due to the more advanced state of differentiation of the fibroblasts from older embryos, as compared to those from the younger embryos, which was maintained in primary culture. Our results are consistent with those of Edström et al. (24) for glioma cells and of Germinario et al. (27) for erythrocytes, both of whom showed that the rate of 3-O-MeG transport was associated with the stage of cell differentiation.

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