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TWO DISTINCT STATES OF SUGAR TRANSPORT SYSTEM IN CULTURES OF BHK CELLS

Y. EILAM and C. VINKLER

Biophysics Section, The Institute of Life Sciences, The Hebrew University, Jerusalem (Israel)

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SUMMARY

The sugar transport of growing and quiescent cultures of BHK-21 cells is studied by the equilibrium exchange method. Two distinct components of sugar transport can be detected. One component displays fast transport rates and is evident in cells at low cell density. The other displays slow transport properties and is typical of quiescent cells. In the course of increase in cell density or following serum-activation of quiescent cells, these two components are present in the same cell-culture. The two components of transport are interpreted as resulting from the presence of two types of cells, one in a "fast" and the other in a "slow" transport state. The transition in each cell from one state of transport into the other appears to be a discrete and sudden event. The gradual change in the cell population results from a change in the number of cells in each state. Cells in the fast transport state show a saturable and a non saturable component of sugar transport. Cells in the slow transport state display only a non saturable component.

INTRODUCTION

It is well established that when untransformed cells in culture grow to confluence and become quiescent the rate of uptake of substrates as sugars, amino acids, uridine and phosphate decreases by several folds [1–6]. The decrease can be reversed however by the addition of a higher concentration of serum [7–10] or by addition of other factors [11]. Cells transformed by virus appear to be always in the rapidly transporting state [11, 12].

The gradual transition from a population of cells which has a fast rate of transport to one which transports slowly and vice versa, has previously been implied from studies performed on net uptake of substrates. However, it is not clear whether the transition results from a gradual change in each individual cell properties or whether the change in every cell is discrete. In the latter case the gradual transition of the population would result from an increase in the number of cells which have altered their transport properties.

In order to resolve between these two features of transport we have applied the technique of "equilibrium exchange" [13]. In this procedure cells are loaded to equilibrium with a certain concentration of a labelled substrate, and the exchange of the isotope into a medium containing the same concentration of the unlabelled substrate is measured. The advantage of this technique relies on the fact that the logarithm of the fraction of isotope remaining in the cell is linear with time. Therefore, in contrast to uptake studies, this technique permits the detection of multiple components of transport in a given population of cells. Evidently this procedure has an absolute requirement for a non metabolized substrate. We have used 3-*O*-methylglucose sugar which enters the cell via the glucose transport system.

From our studies we can infer that the transition in the transport properties as a function of the increase in cell-density or upon activation by serum is the result of a sudden change in the transport properties of each cell. In the course of the alteration, a given population of cells can be clearly seen to be composed of cells which have already altered their transport properties and of cells which have yet to undergo the alteration. This conclusion holds whether the population changes in the direction of increase or decrease in their transport abilities.

MATERIALS AND METHODS

Cell culture. BHK-21 cells were obtained from Dr. I. MacPherson (Imperial Cancer Research Fund Laboratories, London). Cells were cultured at 37 °C at 5 % CO_2 atmosphere in RPMI 1640 medium (Biolab) supplemented with glutamine, 2 mM; antibiotics (Biolab) (streptomycin 400 μg/ml; penicillin 400 unit/ml; neomycin, 4 μg/ml; kanamycin, 5 μg/ml) and 10 % fetal calf serum (Gibco). Unless otherwise indicated, for transport measurement, cells were plated in 5 cm plastic petri dishes (Nunc) at $5 \cdot 10^5$ cells per dish.

Transport measurements by the "Equilibrium Exchange" method. Cells attached to petri dishes were washed twice with phosphate-buffered saline at 25 °C. They were loaded with varying concentrations of ^{3}H-labeled 3-*O*-methylglucose (2–4 μCi/ml) (Amersham) in 1 ml of phosphate-buffered saline, for 1 h at 37 °C. This incubation period is sufficient to load both sparse and confluent cultures to equilibrium even at the highest sugar concentration used (10 mM, not shown). The labelled solution was then removed and the measurement of efflux was initiated by the addition of 5 ml of non-labelled 3-*O*-methylglucose (at 20 °C) at the same concentration as the loading solution. The plates were shaken at 20 °C for different periods of time (20 s–30 min). The flux was terminated by placing each dish on ice and washing five times with 5-ml portions of ice cold phosphate-buffered saline. We found that the number of counts in the loaded cells remained constant between the fourth and the seventh wash. Cells, attached to the dish, were then extracted with 1 ml of 5 % cold trichloroacetic acid and the radioactivity in the solution measured in a Toluene-Triton scintillation fluid.

In every exchange experiment we measured "zero time" and "infinite time" values. The "zero time" value was determined by washing the cells with ice cold phosphate-buffered saline immediately after loading. The "infinite time" value was obtained by incubating the loaded cells with 5 ml of non radioactive 3-*O*-methylglucose for 2 h at 37 °C and then washing as previously described. The "infinite time" counts

were usually very low ($\approx 10\,\%$ of the zero time counts) and represent the non exchangeable component remaining in the cells, probably adsorbed non specifically. They were always substracted from each sample obtained in the corresponding experiment.

RESULTS

The rates of equilibrium exchange at different stages of growth

Equilibrium exchange experiments were carried out on cells which had been grown for different periods after plating, using a concentration of 1 mM 3-*O*-methylglucose. It can be seen from Fig. 1 that the logarithm of the fraction of isotope remaining in the cell is linear with the time of efflux only for cells grown for 22 h (fast rate, Fig. 1A) and for 90 h (slow rate, Fig. 1E). For cells grown from 50–70 h (Fig. 1B–D) the curves indicate the presence of more than one component, which must be interpreted as due to the presence of more than one type of compartment [14]. The data for each of the experiments were examined for their fit to a model consisting of two exponentials in parallel as shown in Eqn. 1, using a computer program based on the non-linear least square method.

$$C_t/C_0 = F \cdot \exp(-k_1 \times t) + (1-F) \times \exp(-k_2 \cdot t) \qquad (1)$$

C_t/C_0 is the number of counts remaining in the cells at time t divided by the number of counts present at time zero (both corrected for the counts present at "infinite time", see Methods). F is the volume fraction of the fast transporting component within the total cellular volume. k_1 and k_2 are two different rate constants. The constants F, k_1 and k_2 as obtained from the data of Fig. 1 are given in Table I. It can be seen that there is a gradual decrease in the fraction of the transport which occurs at the fast rate and a parallel increase in the fraction occurring at the slow rate. The values of the fast and the slow rate constants obtained from all these experiments are of the same magnitude within each of the two categories.

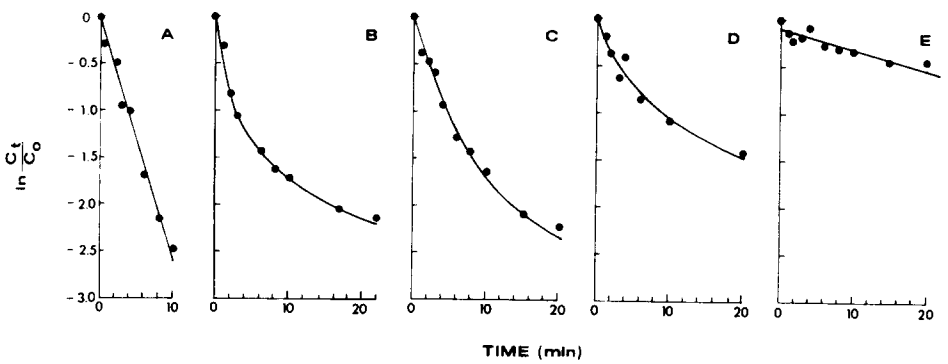

Fig. 1. Equilibrium exchange of 1 mM 3-*O*-methylglucose in BHK cells at different times after plating. C_t/C_0 is the number of counts at time t divided by the number of counts at time zero. Each of the data points is the mean of a duplicate determination. The curves in B, C and D were obtained from the computer-fitting, using the model of Eqn. 1. The lines in A and E were obtained by linear regression. The corresponding kinetic constants are presented in Table I. Cells were plated at $4 \cdot 10^5$ cells/dish and the experiments done at (A) 22 h; (B) 50 h; (C) 55 h; (D) 70 h and (E) 90 h after plating. There was no further change in the slow rate of exchange (E) up to 30 min (not shown).

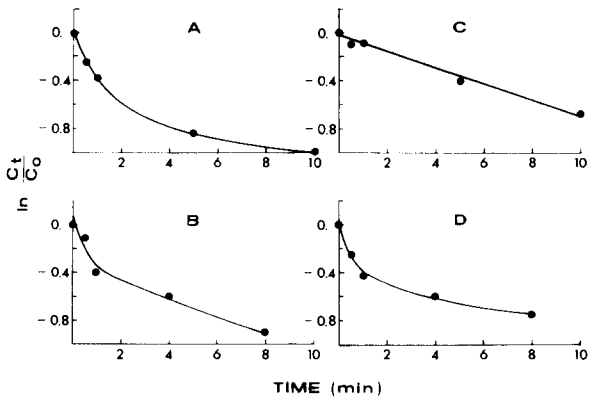

Fig. 2. Equilibrium exchange of 1 mM 3-*O*-methylglucose in BHK cells grown under various conditions. (A) Cells grown with media supplemented with 1 % serum. Experiments done 22 h after plating. (C) as A, 48 h after plating. (B) Cells grown with media supplemented with 10 % serum. Medium and serum changed daily. Experiments done 96 h after plating. (D) Cells grown with media supplemented with 10 % serum. Cells plated at $5 \cdot 10^6$ cells/dish, experiments done 24 h after plating.

The time which elapses before cells become quiescent depends, among other factors, on the concentration of serum in which the cells are grown and on the initial cell density [15]. It is shown in Fig. 2 that serum concentration and cell density affect in the same way the time which elapses before the transition from rapid transport to slow transport. Thus, in cells grown for 22 h in a medium supplemented with 1 % serum, two components of transport were observed (Fig. 2A) whereas, in cells grown for 22 h in 10 % serum only the fast component was observed (Fig. 1A). After 48 h, the cells in 1 % serum reached quiescence and only the slow rate of exchange was observed (Fig. 2C). However, in cells grown with 10 % serum, the presence of the slow component of exchange exclusively was evident only after 90 h (Fig. 1E). In addition, a daily change of medium in growing cultures of cells causes the transition from fast to slow transport state to occur at a higher cell density. At 90 h of growth the number of cells per dish was $12 \cdot 10^6$ and both exponential components were detected (Fig. 2B). Whereas in the control (no daily change of medium), $4 \cdot 10^6$ cells per dish were obtained, but only the slow rate was evident (Fig. 1E). Finally, cells plated at $5 \cdot 10^6$ cells per dish (instead of the usual $5 \cdot 10^5$) showed both components of exchange after 22 h of growth (Fig. 2D), whereas the control cells were still showing the fast rate (Fig. 1A).

The presence of two exponential components of equilibrium exchange (Figs. 1 and 2) clearly indicates that, in all the conditions studied, there is a distinct transition from a fast to a slow state of transport.

The kinetic properties of the two states of the transport system

The experiments described in the previous section were carried out at a concentration of 1 mM 3-*O*-methylglucose. In order to study the kinetic properties of the two transport states, we measured the exchange fluxes at different concentrations of 3-*O*-methylglucose (0.5–8 mM). Cells grown for 24 h after plating showed a single exponential component in equilibrium exchange experiments at all concentrations

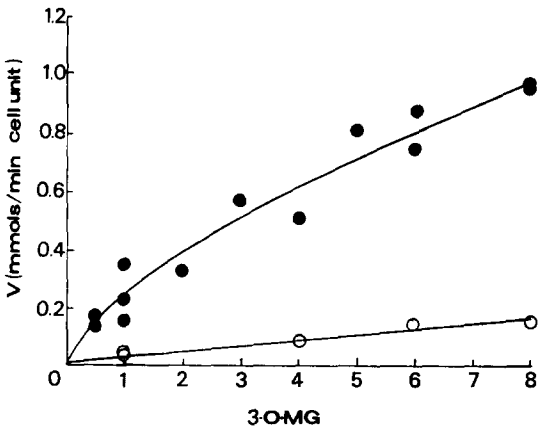

Fig. 3. The rate of equilibrium exchange at different concentrations of 3-*O*-methylglucose (3-O-MG) 22 h and 90 h after plating. At each concentration, the rate of exchange v was obtained by linear regression from the curves of ln (C_t/C_0) versus t (14 measurements each), and multiplying the rate constant obtained by S. (●) Experiments done 24 h after plating. The curve was obtained from the computer-fitting using the model of Eqn. 2 (the parameters given in the text). (○) Experiments done 90 h after plating. The line was obtained by linear regression.

studied. The rate constants were obtained by linear regression from the plot of ln C_t/C_0 vs. t, and the rates of exchange v were obtained from the rate constants*. In Fig. 3 (upper curve) the rates of exchange are plotted versus the concentrations of 3-*O*-methylglucose in cells grown for 24 h. The data indicate a combination of saturable and nonsaturable components of fluxes in the same cell. The saturable component is compatible with a Michaelis-Menten description. The non saturable component may result from simple diffusion or from a parallel transport system which has a K_m lying above the concentration range studied. The kinetic constants were obtained by fitting the data to the model described in Eqn. 2 using the computer program based on the non-linear least square method.

$$v = S \cdot V/(K_m+S)+B \cdot S \tag{2}$$

v is the rate of equilibrium exchange in mmol/min per cell unit**, S is the concentration and B is the rate constant of the non saturable component. The constants obtained by the computer were $K_m = 1.3 \pm 1.6$; $V = 0.4 \pm 0.3$ mmol $\cdot$ min^{-1} per cell unit and $B = 0.08 \pm 0.03$ min^{-1} per cell unit. The same values of kinetic constants were obtained by determining B from the linear portion of equilibrium exchange at high concentrations, 10–20 mM, and the values of B · S substracted from the data at low concentrations.

On the other hand, quiescent cells (90–96 h of growth) displayed only a slow rate of equilibrium exchange at all substrate concentrations. The rate constants were obtained by linear regression from ln C_t/C_0 vs. t. In Fig. 3, lower curve, the rates of exchange are plotted against the concentrations S and a straight line is obtained with a

* In the equilibrium exchange procedure ln $C_t/C_0 = t \cdot V/(K_m+S)$, thus the rate constant, k, equals $V/(K_m+S)$ or v/S [13].

** Cell unit: the number of cells in which the total cell-water volume is 1 litre.

TABLE I

KINETIC CONSTANTS OF THE TWO-EXPONENTIAL CURVES SHOWN IN FIG. 1.

The kinetic constants were obtained by fitting the data to the model of Eqn. 1, using a non-linear least-square computer program.

h after plating	Total number of cells	Fraction of transport of rate constant k_1 (F)	Values of the rate constant k_1 ($min^{-1} \cdot$ cell unit^{-1})**	Values of the rate constant k_2 ($min^{-1} \cdot$ cell unit^{-1})
22	$0.49 \cdot 10^6$	1	0.27* ± 0.01	
51	$1.35 \cdot 10^6$	0.78 ± 0.03	0.42 ± 0.02	0.03 ± 0.008
55	$2.1 \cdot 10^6$	0.59 ± 0.19	0.44 ± 0.14	0.07 ± 0.04
70	$3 \cdot 10^6$	0.49 ± 0.29	0.37 ± 0.25	0.04 ± 0.04
90	$3.5 \cdot 10^6$	0		0.02* ± 0.003

* Obtained by linear regression.

** Cell unit: The number of cells in which the total cell water volume is 1 litre.

slope of 0.02 min^{-1} per cell unit (correlation coefficient = 0.98). No saturable component was detected by our methods.

At an intermediate stage of growth (55 h) we observed that 65 % of the cell population displayed the fast component of transport (Table I) and both exponential components were detected at all substrate concentrations. The curves of C_t/C_0 vs. t, as analysed by the computer program using the two exponential models described in Eqn. 1, yielded two rate constants for each particular concentration of substrate. The corresponding rates of exchange (rate constant $\times S$) were plotted against the substrate concentrations in Fig. 4. It can be seen that the upper curve (fast component) is consistent with the presence of saturable and a non saturable component of trans-

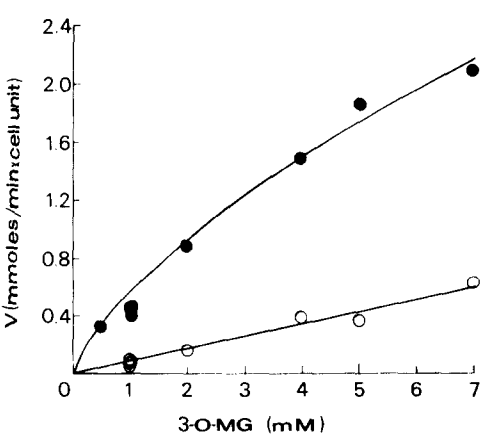

Fig. 4. The rates of equilibrium exchange at different concentrations of 3-*O*-methylglucose (3-O-MG) 55 h after plating. At each concentration, rates of exchange v_1 and v_2 were obtained by fitting the curves of C_t/C_0 versus t, (each composed of 20 measurements), to the model of Eqn. 1, and multiplying the rate constants, k_1 and k_2 by S. The rates of exchange v_1 and v_2 so obtained are given in the upper (fast rates) and the lower (slow rates) curves. The lower line was obtained by linear regression, (correlation coefficient = 0.98). The upper curve has no theoretical significance.

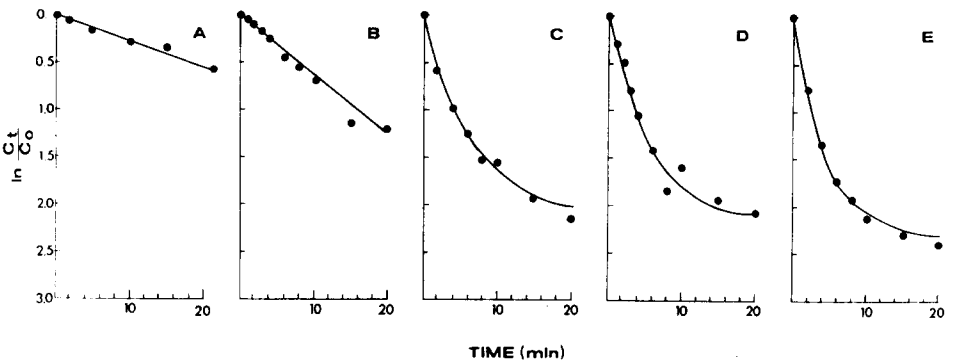

Fig. 5. Equilibrium exchange of 1 mM 3-*O*-methylglucose in cells activated by serum. Cells were grown for 90 h before the addition of a fresh medium containing 25 % serum. Equilibrium exchange experiments were done after: (A) 0 h (control); (B) 0.5 h; (C) 1 h; (D) 2 h and (E) 4 h of incubation with 25 % serum at 37 °C. Each data point is the mean of a duplicate determination. The curves C, D and E were obtained from the computer-fitting, using the model of Eqn. 1. The lines (A and B) were obtained by linear regression. The corresponding kinetic constants are presented in Table II.

port and hence is qualitatively similar to that obtained on day 1 (Fig. 3 upper curve). The lower curve is linear with concentration and hence it is qualitatively similar to that obtained on day 4 (Fig. 3 lower curve). The kinetic constants of the fast rate could not be determined using the non linear least square computer program with the model of Eqn. 2 due to the error inherent in the rate computation by fitting the data to Eqn. 1.

Serum activation

Cells grown for 4 days displayed only the slow rate of equilibrium exchange (Fig. 5A). The same cells were preincubated with fresh medium containing 25 % serum for 0.5, 1, 2 and 4 h, then washed twice with phosphate-buffered saline at 25 °C and loaded for 1 h with 1 mM labelled 3-*O*-methylglucose in phosphate-buffered saline, in the absence of serum. The rates of equilibrium exchange were then deter-

TABLE II

KINETIC CONSTANTS OF THE TWO-EXPONENTIAL CURVES SHOWN IN FIG. 6

The kinetic constants were obtained by fitting the data to the model of Eqn. 1 using a non-linear least-square computer program.

h after addition of 25 % serum	Fraction of transport at rate constant k_1 (F)	Values of the rate constant k_1 ($min^{-1} \cdot$ cell $unit^{-1}$)	Values of the rate constant k_2 ($min^{-1} \cdot$ cell $unit^{-1}$)
0	0	—	$-0.03^{*} \pm 0.002$
0.5	0	—	$-0.07^{*} \pm 0.004$
1	0.67 ± 0.03	-0.46 ± 0.03	-0.05 ± 0.003
2	0.87 ± 0.05	-0.33 ± 0.03	$-0.02 \cdot 10^{-5} \pm 0.03$
4	0.83 ± 0.03	-0.51 ± 0.03	-0.04 ± 0.014

* Obtained by linear regression.

mined as previously described. Following 1 h or more of preincubation with 25 % serum, we detected the presence of two exponential components of transport (Fig. 5C, D, E). The values of the rate constants (k_1 and k_2, Table II) remained of the same order of magnitude within each category as during the increase in cell density (Table I). However, an outstanding feature of these experiments is the reappearance of a major fraction of the fast transport component between 0.5 and 1 h of serum activation (Table II).

Since the serum activation was followed by a 1 h incubation in the absence of serum (loading of substrate) we measured these effects of the serum activation on the transport properties of cells, which were irreversible during this 1 h period.

DISCUSSION

In previous studies it was observed that the rate of uptake of substrates decreases gradually as the cells reach quiescence, or increases gradually upon activation of quiescent cells by addition of serum [5, 10]. These changes can be interpreted in terms of gradual alteration in the transport properties of each cell (model A). However, it is also possible that each cell undergoes a discrete and sudden change in its transport state and that the gradual change in a given population results from the increase in the number of cells which have altered transport properties (model B). These two models have different requirements for the rates of transport during the transition in the transport properties of a given cell population. Model A requires the presence of a main single population of cells in which the transport-rate changes in a gradual fashion. Model B, however, requires the presence of two types of transporting cells, one retaining the unaltered transport rate and the other displaying the altered transport rate. While the two rates remain constant during the transition period, it is the number of cells transporting at the altered rate that shows an increase. In order to distinguish between these two models it is necessary to devise a method that can detect the presence of two types of transporting cells in a given population.

The existence of different components of transport in the same cell (saturable and non saturable) has been detected by both net uptake [4, 16] and equilibrium exchange studies (Fig. 3) by measuring the initial rate of transport at different concentrations of substrates. However the presence of two types of transporting cells (or compartments) in a given population can be observed in the time course of an equilibrium exchange experiment at every concentration, provided that the measurements are followed for a sufficiently long period of time (Figs. 1 and 5). This advantage of the equilibrium exchange procedure relies on the facile separation of linear components of decay by multicompartmental analysis [14]. On the other hand, in the time course of uptake experiments, it is difficult to detect the presence of two different types of transporting cells*.

In order to resolve between the two models, we employed the equilibrium exchange procedure. We have observed a single homogenous population of trans-

* We carried out computer simulations of the amount of substrate taken up (vs. time) by populations which consist of fast transporting and slow transporting cells. Whereas the initial rate of uptake depended on the fraction of population transporting at the slow rate, we could not observe any indication of the presence of two types of transporting cells.

porting cells only at low cell density (fast transport, Fig. 1A) and in quiescent cells (slow transport state, Figs. 1E and 5A). However, during the period of transition in the transport ability of cell populations we detected two exponential components (i.e. compartments) of equilibrium exchange (Figs 1B, C, D and 5C, D, E).

The rate constants of the two components, k_1 and k_2, remained approximately constant during the transition periods, while the fraction of cell populations with altered transport properties increased in the course of transition (Tables I and II). These observations are in line with the requirements of model B, and do not fit the requirements of model A. The two compartments detected by equilibrium exchange are interpreted therefore as resulting from the co-existence of two types of cells, one in the fast and one in the slow transport state (model B).

The alternative interpretation for the presence of two transport components in a cell population would require the existence of two different compartments within each cell. However, in order for this interpretation to hold, it would be required to assume a substantial change in the relative volumes of such compartments during the transition and the disappearance of the reappearance of the fast compartment in quiescence and during serum activation, respectively. It will be required also that changes in the relative volumes of the compartments will have to occur at exactly the same rate in all the cells present in unsynchronized cultures. It may be difficult however to support this interpretation on a biological ground.

Assuming that we are measuring the transport properties of two types of cell, we plot, in Fig. 6, the data calculated from Table I, which show the change in the number of each type of cell after plating. It can be seen that the number of cells

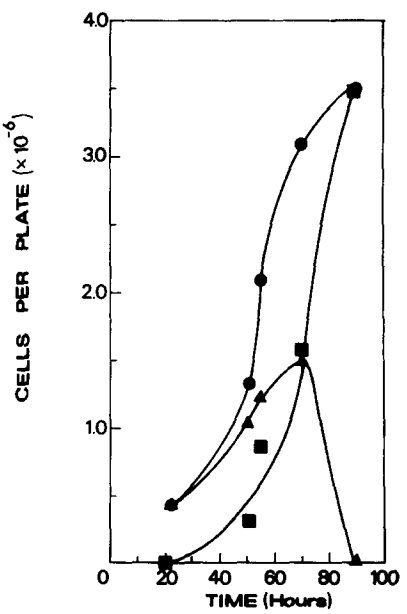

Fig. 6. Number of cells transporting at the 'fast' and 'slow' rate at different times after plating. Data were calculated from Table I. ●, Total number of cells in the plate; ▲, number of cells transporting at the fast rate; ■, number of cells transporting at the slow rate.

transporting at the slow rate increases roughly in parallel with the total number of cells, displaying a delay of approximately 22 h. This may indicate that the change from the fast to the slow transport state occurs in each cell after an average period of 22 h.

Since only two states of transport are detectable, the change from one state to the other, in either direction, should be considered a discrete and sudden event. Cells at a possible intermediate state of transport, if present, are probably an undetectable minority.

The kinetic study of the fast transport state reveals the presence of a saturable high affinity component and a non saturable linear component, both present in the same cell. The kinetic study of the slow transport state displays merely a non saturable component. Thus the event of transition during increase in cell density consists of "switching off" the saturable component and perhaps also of a concomitant reduction in the transport rate of the non saturable component.

Our findings are in line with the recent hypothesis suggested by Pardee et al. [17]. In their hypothesis the membrane can exist in two states, a P state, typical of growing cells and a Q state, typical of quiescent cells. The fast and the slow states of the sugar transport system, as detected in the present study, may be considered part of the P state and the Q state of the membrane, respectively. Their general scheme of growth-control in untransformed cell culture predicts that the switching of cells to a state of growth or to a state of non growth occurs as a result of an autocatalytic sequence of events. The change in the transport-rate of nutrients is considered to be an important component in this sequence [17].

The discrete and sudden transition from one state of transport to the other, as detected in the present study, is evidently in line with these predictions.

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