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Hexose Transport Regulation in Cultured Hamster Cells

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Hamster (nil) cells maintained overnight in culture medium containing cycloheximide and either glucose or fructose exhibit strikingly different rates of hexose transport and metabolism (i.e., uptake). Pretreatment of cultures with sulfhydryl reagents makes it possible to determine initial transport rates for a physiological sugar such as galactose which is a catabolite in hamster cells. Using galactose transport as a model, hexose uptake enhancements can now be shown to be due almost entirely to increases in the rate of the transport step. The transport regulation can best be accounted for by a model comprised of 2 antagonizing mechanisms. This model involves turnover of transport carriers as well as inhibitory units ("regulators"). The experimental as well as the theoretical model may also apply to the well-known uptake enhancements observed in oncogenically transformed cells.

Key words: cell culture, hexose transport, N-ethylmaleimide, derepression, catabolite inactivation, regulatory factor

The distinction between the transport step for natural (or metabolizable) hexoses and their subsequent metabolism has been difficult to determine experimentally. This is due in part to the uncertain degree to which metabolizable sugars are phosphorylated in vivo by their kinases and in part to the fact that nonmetabolizable analogs such as 3-O-methyl-D-glucose (3-O-meG) may not truly represent their natural counterparts (1, 2). D-Galactose is a natural analog of D-glucose and, relative to glucose metabolism, has a much simpler pattern of metabolic products (3–5). Using D-galactose as a model ligand for hexose transport, it was previously reported that studies of uptake (i.e., transport plus metabolism) enhancements caused by oncogenic virus transformation or glucose deprivation were comparable with similar studies using glucose, 2-deoxy-D-glucose (2-dG) or 3-O-meG in other cell types (3, 6). Using low concentrations of N-ethylmaleimide (NEM), we have been able to completely inhibit the galactokinase (ATP:D-galactose-1-phosphotransferase, E.C. 2.7.1.6) activity of intact hamster cells without interfering with galactose transport. Thus the physiological utilization of galactose by cultured cells can be arrested at the first step after transport, making possible direct studies of hexose transport and transport regulation. As a result of this finding, previously observed changes in galactose uptake caused by extended maintenance of cells in culture media devoid of glycolytic sugars (3, 6–8) can now be unequivocally shown to be due to changes in the rate of the transport step. The changes

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in hexose transport rates appear to be due to the synthesis and activation of the hexose carriers counteracted by a catabolite inactivation-like mechanism (9) mediated by a regulatory factor. In addition, de novo synthesis of the regulatory factor is required for inactivation of carriers.

MATERIALS AND METHODS

Cycloheximide and N-ethylmaleimide were purchased from Sigma Chemical Company (St. Louis, Missouri) and cytochalasin B was from Aldrich Chemical Company, Inc. (Milwaukee, Wisconsin). Culture media and sera (fetal calf and dialyzed fetal calf) were obtained from Grand Island Biological Company (Grand Island, New York) and Microbiological Associates (Bethesda, Maryland). Sugar-free culture medium or medium containing fructose contained all components of Dulbecco's modified Eagle's medium (10) except glucose. Radioisotopes of the highest specific activity available were obtained from New England Nuclear Corporation (Boston, Massachusetts).

Culture conditions for the growth and maintenance of hamster fibroblasts (nil strain courtesy of Ms. M. T. Gammon and Dr. K. J. Isselbacher of the Gastrointertinal Unit, Massachusetts General Hospital) were described previously (4, 7, 8). In order to inhibit galactokinase activity, cell monolayers were washed with Dulbecco's phosphate-buffered saline (D-PBS) and preincubated at 37°C for 15 min in D-PBS containing 0.5 mM NEM. Transport of 0.1 mM D-[^{14}C]galactose was for 10 sec at 25°C. Simple diffusion and/or nonspecific hexose adsorption was monitored simultaneously with galactose transport by including 0.1 mM L-[^{3}H]glucose in the assay medium (all transport assays were double label experiments). In 10 to 15 sec immediately following the transport assay, the cells were washed 5 times with 2 ml D-PBS containing 21 μM cytochalasin B and then extracted with 70% ethanol. The ethanol extract was counted as previously described (4, 7, 8), and the data corrected for machine background and channel overlap. Transport of galactose is defined as pmole D-galactose per milligram of cell protein minus pmole L-glucose per milligram cell protein per unit time (usually 10 sec).

RESULTS

Of 3 sulfhydryl reagents tested, only NEM blocked the in vivo activity of galactokinase (Fig. 1, panel II) without affecting transport (Table I). The effective concentration range of NEM was between 0.2 mM and 1.0 mM. Below 0.2 mM NEM, galactokinase activity often contributed to transport measurements and above 1.0 mM NEM cells became progressively more permeable to L-glucose. Although mercuric chloride also inhibited the in vivo activity of galactokinase (Fig. 1, panel IV), it opened the cells to permeation of L-glucose to the extent that there was no net difference between facilitated diffusion and simple diffusion/adsorption (Table I). Treatment of the cells with parahydroxymercuribenzoate (pHMB) resulted in significant inhibitions of galactose transport (Table I). The presence of uridine di-phosphate-hexose (UDP-hexose) and absence of galactose-1-phosphate as well as free galactose in the pHMB-treated cells (Fig. 1, panel III) is like the metabolic pattern of the glucose-fed control (Fig. 1, panel I). These show that the galactose that has entered the cells is immediately processed to UDP-hexose by the activity of at least 2 enzymes (4). The presence, therefore, of UDP-hexose coupled with the absence of free galactose in the extracts

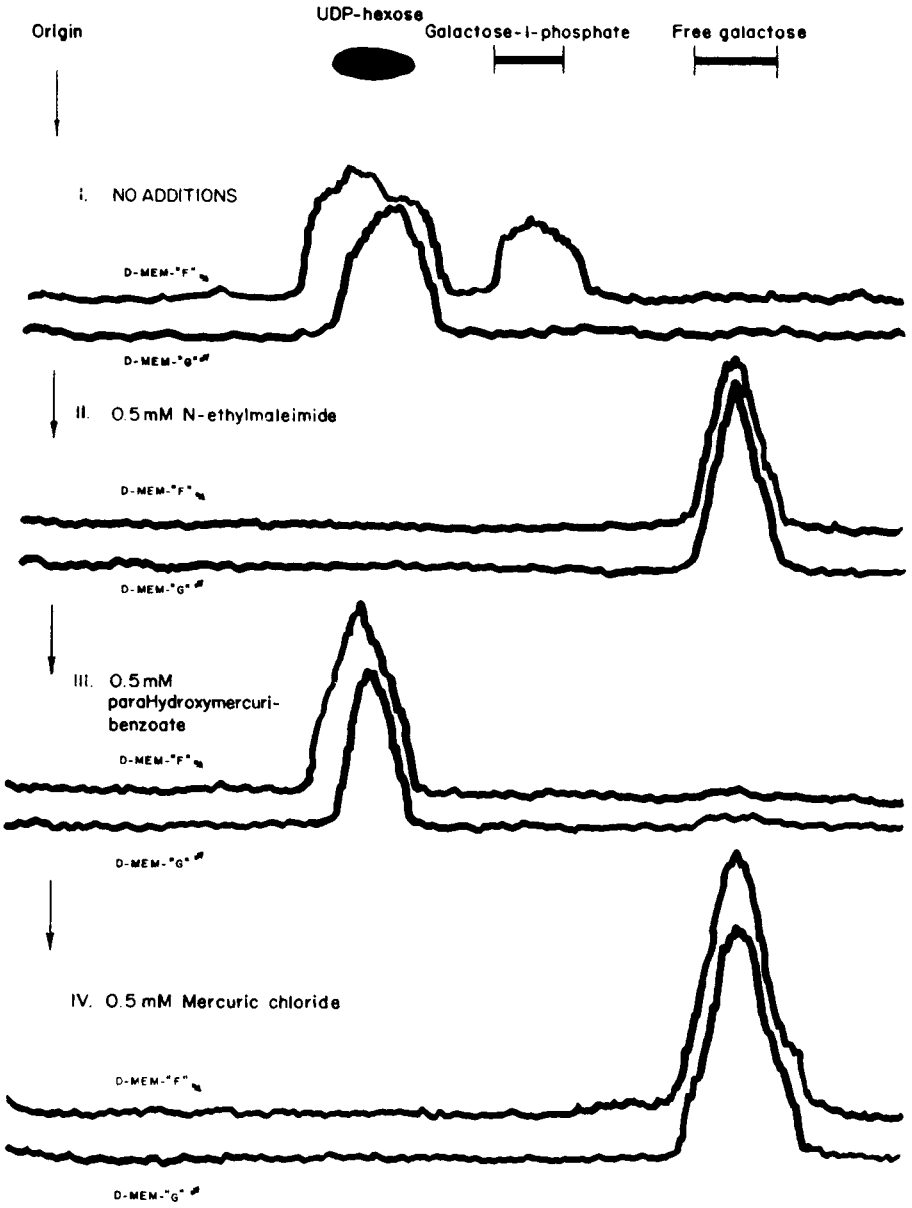

Fig. 1. Confluent monolayers of hamster fibroblasts were washed with sterile Dulbecco's phosphate-buffered saline (PBS) and then maintained in Dulbecco's modified Eagle's minimal essential medium (D-MEM) containing either 22 mM D-glucose (D-MEM-"G") or 22 mM D-fructose (D-MEM-"F") for 18–24 h. The monolayers were then washed with PBS and preincubated for 15 min at 37°C in PBS or PBS containing 0.5 mM NEM, 0.5 mM pHMB, or 0.5 mM $HgCl_2$. Following the preincubation the medium was changed to include 0.1 mM D-[^{14}C]galactose with the above reagents and the cells were allowed to take up the galactose for 10 min. The monolayers were then washed with PBS containing 21 μM cytochalasin B and then approximately equivalent amounts of radioactivity were chromatographed as described in detail previously (4).

TABLE I. Effect of Sulfhydryl Reagents on Hexose Transport

Experiment number	Culture condition[a] (Dulbecco's MEM containing 22 mM)	Preincubation[b] (15 min/37°C)	Hexose transport[c] (pmole/mg protein/10 sec) 3-O-meG	D-galactose	L-glucose	Net transport (D-[^{14}C]gal-L-[^{3}H]glc)[d]
1	Glucose	no additions	–	8 ± 0	3 ± 1	5 ± 1
		NEM	–	13 ± 2	7 ± 2	5 ± 1
	Fructose	no additions	–	112 ± 11	4 ± 2	109 ± 10
		NEM	–	87 ± 6	5 ± 1	82 ± 6
2	Glucose	no additions	–	8 ± 0	4 ± 0.4	4 ± 0.8
		pHMB	–	6 ± 2	4 ± 0.7	1 ± 1
		$HgCl_2$	–	43 ± 6	44 ± 6	0
	Fructose	no additions	–	102 ± 8	5 ± 0.4	92 ± 11
		pHMB	–	18 ± 3	8 ± 1	10 ± 2
		$HgCl_2$	–	44 ± 1	46 ± 3	0
3	Glucose	no additions	7 ± 1	–	–	–
		NEM	15 ± 1	–	–	–
	Fructose	no additions	110 ± 2	–	–	–
		NEM	110 ± 8	–	–	–

[a] Confluent monolayers of hamster fibroblasts were maintained for 18–24 h in Dulbecco's modified Eagle's medium and then washed with Dulbecco's phosphate-buffered saline (PBS).

[b] Washed cells were preincubated in PBS or PBS containing 0.5 mM sulfhydryl reagent as indicated for 15 min at 37°C.

[c] Preincubated cells were assayed in the presence of 0.5 mM sulfhydryl reagent for transport of 0.1 mM hexose as described in METHODS. Values are the mean ± standard deviation of triplicate samples.

[d] Double label experiments, see METHODS. Experiment 3 was a single label experiment; corrections for simple diffusion adsorption were not made in these cases. In view of corrections for experiment 1, the changes in 3-O-meG transport by glucose-fed cells after treatment with NEM are probably not significant.

indicates that, under the conditions of the experiment, the galactokinase was not noticeably affected by pHMB. Table I also shows that there are no adverse effects of NEM on 3-O-meG transport. Thus these data show that NEM has no effect on the transport of either the metabolizable sugar (galactose) or the nonmetabolizable sugar (3-O-meG) and therefore treatment of whole cells with NEM under carefully controlled conditions can be used to dissociate hexose transport from hexose metabolism.

In NEM-treated cells, D-glucose, 2-dG, and 3-O-meG compete with D-galactose equally well (Fig. 2). The concentration of these sugars that results in 50% inhibition of galactose transport is approximately 5 mM. This value is in close agreement with the estimated K_m for D-galactose, 3.8–5.6 mM (Table II). Table II also shows that galactose transport changes (enhancements observed after prolonged maintenance of cells in media containing no sugar or fructose and losses of transport activity seen after maintenance in media containing both glucose and cycloheximide) are due to changes in V_{max}.

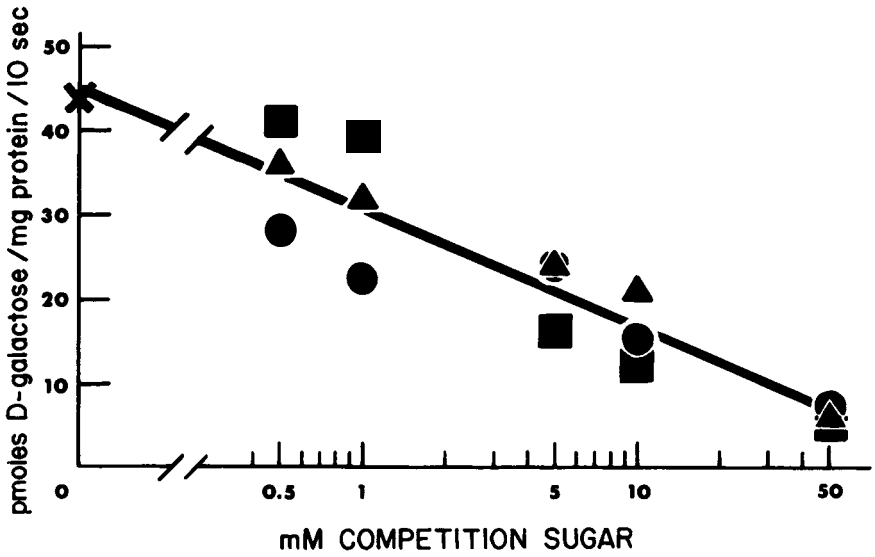

Fig. 2. Monolayers, maintained for 24 h in D-MEM containing fructose, were preincubated for 15 min at 37°C in PBS containing 0.5 mM NEM to inhibit galactokinase activity. Following the NEM treatment, the cells were assayed for 0.1 mM D-galactose transport in the presence of increasing concentrations of D-glucose (•), 2-deoxy-D-glucose (■), or 3-O-methyl-D-glucose (▲).

TABLE II. Galactose Transport Kinetics; K_m and V_{max}

Culture condition	K_m[a]	V_{max}[b]
Glucose	3.8 (1.0)[c]	0.98 (1.0)
Glucose + cycloheximide	5.6 (1.4)	0.27 (0.3)
Fructose	4.4 (1.2)	13.5 (14)

[a] K_m = mM D-galactose
[b] V_{max} = nmole D-galactose transported/mg protein/10 sec
[c] Relative values in parentheses

DISCUSSION

Galactose and Sugar Transport

The use of D-galactose as a probe in studying the regulation of hexose transport gains advantage from the observations that D-galactose is a natural analog of D-glucose, D-galactose supports the growth of animal cells (11), and D-galactose is believed to enter animal cells through the glucose transport system (12). In addition, compared with the multiple isozyme hexokinase (ATP: D-hexose-6-phosphotransferase, E.C. 2.7.1.1) system which phosphorylates a wide range of hexoses without being completely inhibited by sulfhydryl reagents (1), galactokinase restricts its phosphorylation to galactose (or galactosamine) and is completely inhibited by such sulfhydryl reagents as those that can penetrate the plasma membrane of intact cells. Taken together the results of the experiments presented in this paper demonstrate that transport of metabolizable sugars can in fact be measured independently of metabolism. Although other interpretations may apply, the simplest explanation for the results of kinetics measurements at approximate initial rates is that it is the number of active carriers that increases in response to nonglycolytic conditions. Similarly, the decreases in transport in response to inhibitors of protein synthesis during maintenance under glycolytic culture conditions (1, 7, 8) appear to be due to decreases in the number of active carriers.

Since doubts have been expressed concerning the use of 3-O-meG in transport studies (1, 2), this evidence lends validity to the use of 3-O-meG as a reliable indicator of hexose transport. That is, by direct comparison the nonmetabolizable glucose analog, 3-O-meG, is shown to be transported at the same rate as the metabolizable sugar, galactose, and 3-O-meG is as good an inhibitor of galactose transport as glucose and 2-dG. This use of 3-O-meG for hexose transport studies may apply, however, only to the predominant low-affinity transport system for glucose since a high affinity system for glucose does not appear to transport 3-O-meG (1). Nevertheless, changes in hexose uptake detected by the use of glucose, galactose, 2-dG, or 3-O-meG (1, 3, 4, 6–8) can now be attributed to changes in the transport step.

Regulation of Hexose Transport: Derepression/Synthesis vs Inactivation/Turnover, A Model

How is transport of hexose regulated in animal cells? Although the mechanism of regulation is not clear, it is useful to consider a model which accounts for several experimental observations and suggests new experimental approaches. The initial observations of Amos and his co-workers (13) suggesting that derepression of carrier synthesis could account for enhancements of hexose uptake caused by sugar starvation were confirmed by Kletzien and Perdue (14). Based on their recent experimental data Christopher et al. (7, 8) proposed that inactivation and/or turnover of carriers was also involved in regulation and, in addition, a regulatory factor (which promoted the inactivation and/or turnover of the hexose carrier) was itself subject to inactivation (7, 8). Hexose transport control in at least hamster and chick cells appears therefore to be dependent upon 2 opposing forces, namely, synthesis of carriers vs inactivation of carriers. The features of this model are shown in Fig. 3.

During growth under normal culture conditions (Fig. 3A and upper left panel), synthesis and inactivation of the carrier to a functional state are offset by inactivation and/or turnover of the carrier. When protein synthesis in cells maintained in medium containing glucose is blocked by cycloheximide (Fig. 3A and lower left panel), a short period of

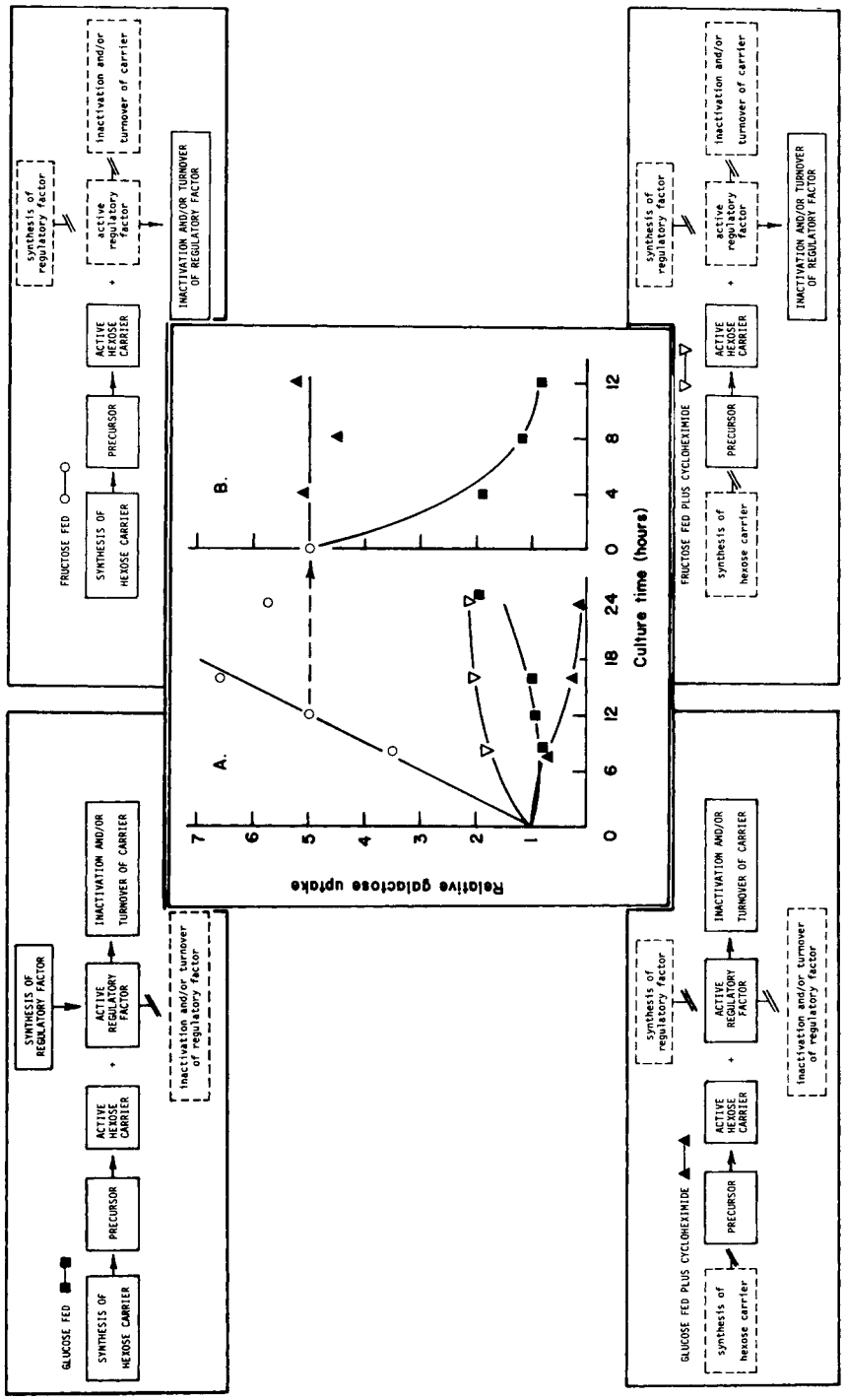

Fig. 3. Parts A and B are modified from Figs. 2 and 4 of Ref. 7. Panels on the periphery of these figures illustrate a scheme of hexose carrier control for each of the experimental observations shown in A. The upper left panel illustrates the hypothetical dynamics of carrier synthesis vs inactivation for cells maintained in medium containing glucose (filled squares in A). The lower left panel illustrates the effects of cycloheximide in medium also containing glucose (filled triangles in A). The upper right panel illustrates regulation in cells maintained in medium containing no sugar or fructose (open circles in A) and the lower right panel illustrates the effects of cycloheximide in the no sugar or fructose-fed cells (open triangles in A). Changes in transport activity depicted in B contribute to the concept of a regulatory factor which appears to become inactive when cells are maintained in media containing no sugar or fructose. The downward regulation of transport by sugar-starved or fructose-fed cells after the addition of glucose to the medium (filled squares in B) is prevented when cycloheximide is added simultaneously with the glucose (filled triangles in B). Cycloheximide concentration was 36 μM; glucose concentration was 11 mM (7).

steady state exists where the apparent rate of activation of the putative precursor carrier is counteracted by the inactivation process. Upon exhaustion of the "precursor pool," further time-dependent inactivation of functional carriers leads to dramatic losses of activity since no new carriers can be synthesized. However, treating the cells with cycloheximide in the absence of glucose or in media containing fructose (Fig. 3A and lower right panel) leads to an increase in transport rates. That the increase under these conditions is immediate and cycloheximide independent further suggests a movement of preformed carriers from an inactive to an active state. This increase in the absence of glucose under otherwise identical culture conditions as those depicted in the lower left panel of Fig. 3 is striking and suggests that glucose (or its catabolites) is required for inactivation/turnover of the carrier. This is supported by the observation that transport rate increases are linear with respect to time when synthesis of carriers continues either in the absence of sugars or in the presence of nonglycolytic sugars such as fructose (Fig. 3A and upper right panel). These observations bear a remarkable resemblance to those described to support the model of the catabolite inactivation mechanism of control in the eukaryotic microorganism, Saccharomyces (9).

Is the Regulatory Factor a Protease?

Some evidence suggests that the regulatory mechanism involved in the inactivation/turnover may be a proteolytic process. High concentrations of inhibitors of protein synthesis (cycloheximide or puromycin) inhibit the process of inactivation (7, 8). The reactivation of carrier activity requires de novo synthesis of carriers. Following glucose plus cycloheximide treatment, the recovery of activity during maintenance of the cells in medium containing fructose (8) is characterized by an initial 2-h lag period and can be prevented by 7.1 μM cycloheximide (unpublished results). If proteolysis should turn out to be the principal mechanism of hexose transport inactivation, it is all the more interesting that de novo synthesis of a protease (or an unknown cofactor for proteolysis) is also required. This interpretation comes from the observation that the addition of glucose to cells fully "derepressed" by prior maintenance in media containing no sugar results in a rapid inactivation of existing carriers (Fig. 3B). Yet the simultaneous addition of low levels of cycloheximide with the glucose does not lead to inactivation (Fig. 3B). From Fig. 3A it can be seen that cycloheximide, at the concentrations used, does not block inactivation/turnover in cells that have been continually maintained in media containing glucose. This indicates that the inhibition of the inactivation/turnover process cannot be due to a direct action of cycloheximide (e.g., inhibition of proteolysis) since loss of carrier activity was strikingly evident by 12 h (Fig. 3A).

Is the Regulatory Factor an Allosteric Effector?

Regulation may depend upon the synthesis and action of a regulatory unit of the hexose carrier system. In Fig. 4 a simple model of hexose transport control shows a labile factor interacting with the carrier in a way that does not compete with but impedes the movement of the ligand. In the figure the activity of the triangular shaped regulatory factor is illustrated as being lost when cells are maintained under nonglycolytic conditions (Fig. 4A) and as being present in cells under glycolytic culture conditions (Fig. 4B).

This model might also accomodate the well known effect that transformation has on hexose transport control. In Fig. 4C a defective regulatory unit, induced by a transformation event, is shown only partially blocking transport. In Fig. 4D an alternative model suggests that a transformation induced factor could compete with the regulatory factor for

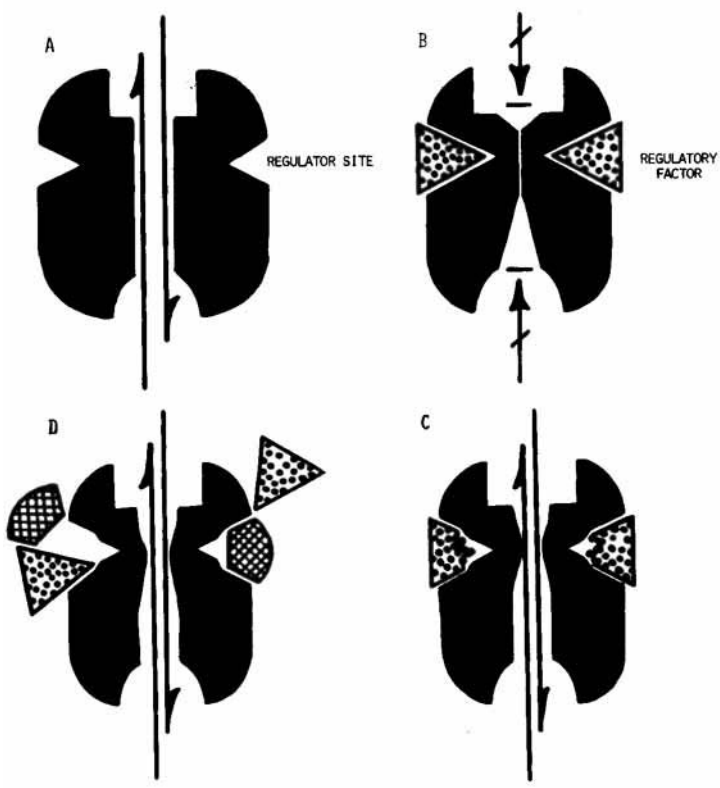

Fig. 4. Simplified, hypothetical model of the interaction of a hexose carrier (filled oval with transport marked by arrows) regulatory factors (stippled). A) Carrier of cells deprived of glycolytic sugars having little or no regulatory factor activity (regulator site is unfilled; transport, influx and efflux, is open). B) Carrier of cells maintained in medium containing glucose (regulatory factor association with regulatory sites of carrier shuts off transport). C) Carrier of transformed cells maintained in media containing glucose and having defective regulatory factors or carriers with defective regulator sites. D) Carrier of transformed cells (glucose-fed) with regulator sites being competed for by normal regulatory factors (stippled) and transformation-specific products (cross hatched). In both C and D transport is only partially regulated.

the regulatory site. These models also could account for further enhancements of hexose transport by sugar-starved, transformed cells (4, 5, 16) by allowing for the inactivation of regulatory activity that is normally caused by nonglycolytic culture conditions. Hence, labile factors and carriers must not be overlooked in efforts to understand the role of transformation in hexose transport control.

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