Counter-transport in chick embryo fibroblasts

A significant factor in measurement of glucose entry

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Enhanced rates of carrier-mediated 3-*O*-methyl-D-glucose (0.1 mM) transport were observed in primary cell cultures of chicken embryo fibroblasts deprived of glucose for 1 day. The addition of 5.5 mM-glucose, glucosamine or 2-deoxy-D-glucose for 15 min (37°C) to glucose-starved cultures followed by washing and immediate measurement of 3-*O*-methyl-D-glucose transport resulted in an apparent further stimulation of transport. Transport stimulation increased with increasing concentrations of the added pre-incubation sugar and was observed at test concentrations ranging from 0.1 mM- to 10 mM-3-*O*-methyl-D-glucose. This enhancement occurred when the preloaded sugar was rapidly effluxing from cells and was eliminated by allowing cultures to incubate in buffer without sugar for 30 min (37°C) after the removal of hexose and before measuring transport. A transient overshoot in the cumulative uptake of 3-*O*-methyl-D-glucose was observed in glucose-starved cultures that were pre-incubated in the presence of 55 mM-glucose or -glucosamine for 15 min (37°C). These data suggest that counter-transport accounts for the apparent enhancement of glucose-transport capability observed in glucose-starved cells when they are briefly re-exposed to hexose.

The way in which cellular transport systems respond to available extracellular substrate concentrations may be a vital mechanism for ensuring metabolic homeostasis. In this laboratory, Martineau *et al.* (1972) first reported that hexose starvation resulted in a marked (15–30-fold) increase in glucose entry into cultured chick embryo fibroblasts. Additional studies on this cell system (Musliner *et al.*, 1977; Amos *et al.*, 1977) showed that entry of the non-metabolized glucose analogue, 3-OMG, was also enhanced by glucose deprivation and that inhibitors of protein synthesis prevented this increase. In addition, short-term exposure (15 min) of starved cells to 5.5 mM-glucose resulted in a rapid return of glucose uptake to levels observed in glucose-fed cultures. This glucose-induced carrier inactivation was prevented by the inclusion of cycloheximide. These results led Amos *et al.* (1977) to conclude that (1) a carrier-inactivator protein was involved in the adaptive response and (2) the amount of putative inactivator protein was positively regulated by glucose or a metabolite of glucose acting at the post-transcriptional level. Additional

studies on the adaptive regulation of glucose transport using cultured hamster cells (Christopher, 1977) and rat mammary tumour cells (Gay & Hilf, 1980) have also been interpreted to involve a glucose-carrier inactivator or repressor protein.

In this paper the starvation-induced derepression of glucose transport was examined further in CEF with 3-OMG as the transported substrate. The data illustrate that, depending on experimental conditions, counter-transport can be a factor influencing transport measurements and verify that the adaptive response involves the regulation of glucose-carrier activity.

Materials and methods

Cell cultures

Primary and secondary CEF were prepared from trypsin-treated embryos as described by Christopher *et al.* (1976). Dissociated cells (1×10^6) were inoculated into Costar six-well dishes (35 mm diameter) and grown in 2 ml of MEM (GIBCO) with Hanks' salts, L-glutamine and non-essential amino acids without NaHCO_3 and supplemented with 3% calf serum (GIBCO) and 100 units of penicillin/ml and 100 μg of streptomycin/ml. Since cells were grown in the absence of CO_2 and

Abbreviations used: 3-OMG, 3-*O*-methyl-D-glucose; 2-DOG, 2-deoxy-D-glucose; CEF, chick embryo fibroblasts; BSS, balanced salt solution; MEM, Eagle's minimal essential media.

bicarbonate to maximize the adaptive response of hexose transport to glucose starvation (Amos *et al.*, 1976), all media were supplemented with 10 mM-Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid] buffer (pH 7.4). In all experiments dissociated cells were allowed to attach for 1 day in MEM containing glucose and calf serum preceding any experimental manipulation. This time after the 24 h attachment period has been defined as day 0 on the growth curves. In glucose-starvation experiments, cells were washed free of standard growth medium containing 3% calf serum + 5.5 mM-glucose and transferred to medium containing 3% dialysed calf serum + 5.5 mM-fructose or 5.5 mM-glucose as the control. Results are expressed in terms of cellular protein as determined by the method of Lowry *et al.* (1951).

Initial rates of 3-OMG influx

Medium was aspirated from the plates and monolayers were washed three times (20°C) with 2 ml of BSS, pH 7.4 (137 mM-NaCl/5 mM-KCl/0.8 mM-MgSO₄/1 mM-CaCl₂/0.22 mM-Na₂HPO₄/0.44 mM-KH₂PO₄ and 20 µg of Phenol Red/ml). The transport assay was started by adding 0.8 ml of BSS containing 3 µCi of 3-*O*-methyl-D-[³H]glucose (sp. radioactivity 80.0 Ci/mmol; NEN) and the appropriate concentration of unlabelled 3-OMG to monolayers containing 0.6 ml of BSS (20°C). Transport was rapidly halted within 2 s by dunking the multiwell plate in a beaker containing 3 litres of ice-cold iso-osmotic NaCl; residual radiolabelled sugar was removed by successively dunking and swirling the plates in three additional cold (4°C) NaCl washes. Plates were inverted to allow draining; monolayers were extracted with 1 ml of 5% trichloroacetic acid at 4°C for 2 h, and the acid-soluble fraction was counted for radioactivity in 10 ml of liquiscint (National Diagnostic) in a liquid-scintillation counter. The time between the removal of the growth medium and starting the transport assay was approx. 1 min. Transport measurements performed at 10 s intervals over a period of 2 min showed that net 3-OMG influx was linear for 60 s in cultures both repressed and derepressed for hexose transport (results not shown). In cultures that had been preloaded with hexose before performing the transport assay, uptake was linear for only 30 s (see Fig. 5). Initial rates of 3-OMG transport were therefore estimated by exposing monolayers to labelled 3-OMG for 60 s or less (at 20°C), depending on the history of the particular cell cultures being assayed. Carrier-mediated entry was estimated by subtracting entry observed in the presence of 10 µg of cytochalasin B/ml (Sigma) from the total rate of entry in the absence of the transport inhibitor.

Cumulative uptake and efflux of labelled hexoses

Uptake of other sugars was performed as

described above using 0.5 µCi of D-[U-¹⁴C]glucose (sp. radioactivity 300 Ci/mol), D-[U-¹⁴C]glucosamine (sp. radioactivity 250 Ci/mol) and 2-deoxy-D-[1-¹⁴C]glucose (sp. radioactivity 57 Ci/mol; NEN) and the appropriate concentration of unlabelled sugar.

Estimates of sugar efflux were obtained as follows: 0.1 ml of BSS containing 0.5 µCi of ¹⁴C-labelled glucose, glucosamine or 2-DOG and the appropriate concentration of unlabelled sugar to give a final concentration of 5.5 mM was added to the cell culture medium for 7 min (37°C). At that time uptake was either terminated by washing in cold NaCl as described for 3-OMG transport or the medium containing radiolabelled sugar was removed and the monolayer was washed three times with 2 ml each of BSS (20°C) and re-incubated in 2 ml of BSS at 37°C for various periods of time. Efflux of sugar was halted by four cold NaCl washes, as described.

ATP assay

Cell monolayers were extracted with 0.4 M-HClO₄ (ice-cold) and neutralized with KOH. Levels of ATP were determined by using the luciferase assay as described by Cole *et al.* (1967) with the modification of Stanley & Williams (1969) using a Beckman LS 230 liquid-scintillation counter with the coincidence switch off.

Results

Enhanced transport in glucose-starved cells exposed to various sugars

An approximate 4-fold increase in the rate of 3-OMG transport was observed when cultures grown for 2–3 days on standard growth media (MEM + 3% calf serum + 5.5 mM-glucose) were transferred to media supplemented with 3% dialysed calf serum + 5.5 mM-fructose for 1 day (Fig. 1). A comparable transport enhancement was observed in cells maintained in the absence of any sugar for 1 day, whereas cells kept in 5.5 mM-glucosamine exhibited initial rates of 3-OMG transport similar to those of cells kept in 5.5 mM glucose for the 1-day pre-incubation period (results not shown). The addition of 5.5 mM-glucosamine to cells pre-incubated in fructose-containing media resulted in an additional increase in 3-OMG transport, whereas no such effect was seen in cultures maintained in glucose (Fig. 1). This apparent stimulation was highest at 15 min and persisted for 6 h provided glucosamine remained in the incubation medium. The glucosamine-induced increase in 3-OMG transport was (1) greater with 55 mM- than with 5.5 mM-glucosamine, (2) not influenced by the presence of cycloheximide (10 µg/ml), (3) seen as early as 5 min after the addition of glucosamine and (4) was observed using 0.1 mM-¹⁴C-labelled D-glucose as the transported substrate (results not shown). It should

Fig. 1. Effect of 5.5 mM-glucosamine on 3-OMG transport in glucose-starved CEF

Primary CEF were grown for 2 or 3 days in MEM + 5.5 mM-glucose + 3% calf serum and switched to MEM + 3% dialysed calf serum + 5.5 mM-glucose (●) or 5.5 mM-fructose (■) for 1 day and assayed for initial rates (60 s, 20°C) of cytochalasin B (10 µg/ml)-inhibitable 3-OMG (0.1 mM) transport. Some of these cultures were supplemented with 5.5 mM-glucosamine (▲) for 15 min or 1, 3 or 6 h (37°C) before washing and measurement of transport. Results are means ± S.E.M. for seven experiments.

be noted that in these experiments preincubation with glucosamine was performed at 37°C, whereas cells were washed three times with 2 ml of BSS (20°C), immediately after which the transport assay was performed employing 0.1 mM-3-OMG at 20°C.

It was of interest to ascertain whether the glucosamine effect was dependent on the concentration of 3-OMG employed in the transport assay. Cell cultures were exposed to glucosamine (5.5 mM) for 7 min (37°C) and assayed for transport using 0.1, 1, 5 and 10 mM-3-OMG (Table 1). Cells grown in the presence of fructose for 1 day showed enhanced rates of transport at all concentrations tested when compared with similarly tested cells grown in glucose for 1 day. The addition of 5.5 mM-glucosamine to fructose-grown cultures resulted in a 30% increase in the rate of transport at 0.1 mM-3-OMG, whereas at the higher substrate concentrations (1–10 mM-3-OMG) transport enhancement was in the range 7–10%.

To determine whether the glucosamine-induced increase in 3-OMG transport was (1) dependent on the age of the cultures and (2) specific for glucosamine, CEF were deprived of glucose for 24 h at daily intervals after attachment to the multiwell plates and transport measurements were performed before and after the addition of 5.5 mM-glucose, -glucosamine or -2-DOG for 15 min at 37°C (Fig. 2).

As illustrated in Fig. 2(a) cells maintained in 3% calf serum + 5.5 mM-glucose throughout the time in culture grew for 5 days after plating (media changed on days 1 and 3). Growth of cells switched for MEM containing 3% dialysed calf serum and either glucose or fructose for 1 day was not significantly different from the growth observed in medium containing 3% calf serum and glucose (see the legend to Fig. 2).

Table 1. Effect of glucosamine on rates of 0.1, 1, 5 and 10 mM-3-OMG transport

Primary CEF grown for 2 days in standard growth media were transferred to media containing 3% dialysed calf serum + 5.5 mM-glucose or 5.5 mM-fructose for an additional day. Some of the fructose-grown cultures were exposed to 5.5 mM-glucosamine for 7 min (at 37°C) before washing and measuring 3-OMG transport at the indicated concentrations; 60 s (at 20°C) 3-OMG transport was corrected for entry not inhibited by cytochalasin B (10 µg/ml) to give estimates of carrier-mediated transport. Results are means ± S.E.M. for determinations performed on three plates.

Sugar present for 1 day preconditioning at 5.5 mM	7 min exposure to 5.5 mM-glucosamine	[3-OMG] used in assay (mM)	Rates of 3-OMG transport (nmol/min per mg)	Rate (+ glucosamine)	
				Rate (– glucosamine)	
Fructose	–	0.1	0.340 ± 0.005	1.30	
	+	0.1	0.440 ± 0.013		
	–	1	2.84 ± 0.14	1.10	
	+	1	3.13 ± 0.08		
	–	5	9.85 ± 0.29	1.09	
	+	5	10.77 ± 0.27		
	–	10	15.75 ± 0.41	1.07	
	+	10	16.85 ± 0.71		
	–	0.1	0.147 ± 0.005		
	–	1	0.711 ± 0.046		
Glucose	–	5	2.17 ± 0.14		
	–	10	4.00 ± 0.29

Fig. 2. Adaptive response and stimulation of 3-OMG transport by glucose, glucosamine and 2-DOG during growth in culture

(a) Protein content of primary CEF as a function of time in culture. Cells were maintained in MEM + 3% calf serum + 5.5 mM-glucose (■) throughout the time in culture (media changed on days 1 and 3). Some of the cultures were switched from the above media to media containing 3% dialysed calf serum + 5.5 mM glucose (▲) or 3% dialysed calf serum + 5.5 mM-fructose (●) on days 0, 1, 2, 3 and 4 and protein content was determined 1 day later. Each point represents the mean of determinations performed on three to six plates; S.E.M. did not exceed $35 \mu\text{g}$ of protein. (b) Rates of 3-OMG (0.1 mM) transport in cultures grown in calf serum + glucose (■) or in cultures switched from this medium to dialysed calf serum + glucose (▲) or dialysed calf serum + fructose for 1 day (●) (see the legend to a). Each point represents the mean of transport, performed on three to eight plates; S.E.M. did not exceed $\pm 30 \text{ pmol/min per } 10^6 \text{ cells}$. (c) Rates of 3-OMG transport in cultures switched to dialysed calf serum + fructose for 1 day (●) (same as b) and in similarly treated cells to which 5.5 mM-2-DOG (■), -glucosamine (○) or -glucose (▲) had been added for 15 min (at 37°C) before washing and transport measurement. Each point represents the mean $\pm$ S.E.M. for transport performed on three plates; S.E.M. is not shown unless greater than $30 \text{ pmol/min per } 10^6 \text{ cells}$.

Basal levels of 3-OMG transport (CEF grown in 3% calf serum + 5.5 mM-glucose) were relatively constant for the first 3 days in culture, fluctuating between 76 and $100 \text{ pmol/min per mg}$ of protein on days 1 and 3 respectively (Fig. 2b). A 2–3-fold drop in transport activity was observed on days 4 and 5 in these cultures. Derepression of 3-OMG transport by glucose starvation was observed on days 2–5. No glucose starvation-induced increase in transport was observed on day 1. Strangely enough maximum enhancement of glucose transport results from a change to fresh medium on day 0, irrespective of sugar supplement. This effect was seen only on day zero and occurred with dialysed or normal serum (results for normal serum not shown). It should be noted that in this experiment cells maintained in 3% calf serum + glucose were changed to fresh medium on days 1 and 3 with no detectable increase in transport on days 2 and 4 respectively.

Fig. 2(c) illustrates that the addition of 5.5 mM-2-DOG, -glucose or -glucosamine (15 min, 37°C) to cells deprived of glucose for 1 day, resulted in increased 3-OMG transport on washing cultures free

of these sugars and immediately measuring transport. Only the addition of 5.5 mM-glucose to day-4 starved cultures failed to show a significant transport enhancement. In other experiments (results not shown) the addition of 55 mM-glucose to starved cultures resulted in an increase in 3-OMG transport when 5.5 mM-glucose was not effective in causing a transport stimulation.

ATP levels in cells treated with glucose, glucosamine or 2-DOG

In this experiment (Table 2) cells grown in the presence of fructose for 1 day had an approx. 2-fold higher level of ATP than cells grown in glucose for 1 day (values in parentheses in Table 2). Glucose-grown cells to which 5.5 mM-glucosamine or 2-DOG had been added for 15 min (37°C) showed no significant change in ATP levels compared with non-treated cells. In contrast with this, supplementing the fructose-containing media with glucosamine or 2-DOG resulted in a 40% and 90% decrease in ATP levels compared with non-treated cells. The addition of glucose to cells growing on

Table 2. Influence of added glucose, glucosamine or 2-DOG on ATP levels of cells grown in media containing fructose or glucose

Confluent CEF were switched to media containing 3% dialysed calf serum + 5.5 mM-glucose or 5.5 mM-fructose for 1 day. At this time the indicated sugars were added directly to the media for 15 min (37°C) to give a final concentration of 5.5 mM added sugar. ATP levels were determined on cell cultures after the 15 min exposure to added sugar or after cells had been washed three times with BSS (2 ml at 20°C) and incubated in 2 ml of BSS for an additional 30 min at 37°C. Determinations were performed on three plates and results are mean percentage ATP values $\pm$ S.E.M. compared with ATP levels of cells grown in either fructose or glucose with no additions or washings. The ATP levels in parentheses represent the 100% values to which percentages were standardized.

Preconditioning sugar at 5.5 mM for 1 day	Additional sugar at 5.5 mM for 15 min	ATP (%)	
		Immediately after exposure to additional sugar	30 min after washout of additional sugar
Glucose	None	100 $\pm$ 11	(8.7 nmol/mg) 87 $\pm$ 3
	Glucosamine	123 $\pm$ 12	103 $\pm$ 8
	2-DOG	93 $\pm$ 20	82 $\pm$ 1
Fructose	None	100 $\pm$ 4	(17.5 nmol/mg) 75 $\pm$ 13
	Glucose	94 $\pm$ 6	73 $\pm$ 15
	Glucosamine	57 $\pm$ 5	47 $\pm$ 1
	2-DOG	11 $\pm$ 2	9 $\pm$ 1

fructose had no significant effect on ATP concentration. Because later transport experiments (Figs. 3 and 4) would involve washing the monolayer and a 30 min pre-incubation in BSS (37°C) ATP levels were also determined under these conditions. In the presence of glucose, only the 2-DOG-pretreated cells revealed a slight decrease in ATP levels on incubation for 30 min in BSS. Non-treated and glucose-supplemented fructose-grown cells displayed an approx. 25% loss of ATP during this time, whereas incubating fructose-grown cells pretreated with glucosamine or 2-DOG had little further effect on lowering ATP.

Reversal of transport stimulation on removal of glucose

The experiment shown in Fig. 3 was designed to ascertain whether the time interval between removing the added sugar and measuring transport influenced the observed transport stimulation. In this experiment, cultures were maintained for 1 day in medium containing 5.5 mM-fructose after which cells were exposed to either 5.5 mM- (Fig. 3a) or 55 mM- (Fig. 3b) glucose for 15 min, 1 or 3 h. After exposure to glucose, cells were washed and assayed for 3-OMG transport immediately (zero time) or were washed and allowed to incubate in 2 ml of BSS (37°C) for 5, 15 or 30 min before measurement of transport. Transport was enhanced approx. 2-fold in cells kept in fructose for 1 day compared with cultures kept in glucose (see Figs. 3a and 3b). Transport stimulation by both 5.5 mM- and 55 mM-glucose was greatest after the 15 min pre-incubation and was not evident after the 3 h pre-incubation if

tested immediately after washing (zero time in buffer on Figs. 3a and 3b). The apparent transport enhancement by 15 min pre-incubation decreased as a function of time in sugar-free BSS. Exposure to 5.5 or 55 mM-glucose for 1 h followed by 15 min or 30 min incubation before measuring transport actually resulted in reduced transport, below that of control glucose-starved cells. Cultures exposed to glucose for 3 h exhibited reduced rates of transport after only 5 min in BSS and transport in these cells at 15 and 30 min was comparable with that observed in cultures kept in glucose for 1 day. Thus it appears that the glucose-induced stimulation observed in starved cultures can be reversed by allowing cells to incubate in BSS; furthermore, transport inactivation can be observed under these conditions.

Correlation between transport stimulation and sugar efflux

The data thus far are consistent with a role for counter-transport or trans-stimulation in the observed hexose stimulation of 3-OMG transport in glucose-starved cultures.

Since in these experiments counter-transport should be dependent on the concentration of sugar available for efflux, glucose-fed and starved cultures were monitored for uptake and efflux of ^{14}C -labelled glucose (Fig. 4d), 2-DOG (Fig. 4e) and glucosamine (Fig. 4f). In addition, initial rates of 3-OMG transport were determined in cells preloaded with these sugars (5.5 mM, 7 min 37°C) and subsequent rates of transport were followed as a function of time after the removal of these preloading sugars (Figs. 4a–4c).

Fig. 3. Effect of incubation time in BSS on the transport stimulation induced by the addition of 5.5 mM- or 55 mM-glucose in cells starved of glucose for 1 day (a) Rates of 0.1 mM-3-OMG transport were determined in primary CEF grown for 3 days in MEM + 3% calf serum + 5.5 mM-glucose, which were switched to media supplemented with 3% dialysed calf serum + 5.5 mM-glucose or 3% dialysed calf serum + 5.5 mM-fructose for 1 day. To some of the glucose-starved cultures 5.5 mM-glucose was added for 15 min (▲), 1 h (■) or 3 h (●) before removing glucose, washing three times with BSS (2 ml) and measuring 3-OMG transport immediately after glucose wash out (zero min in BSS). Cells exposed to 5.5 mM-glucose were also washed and allowed to incubate in 2 ml of BSS (at 37°C) for 5, 15 or 30 min before measuring transport. (b) The experiment was conducted as described in the legend to (a) except that cells were exposed to 55 mM-glucose for 15 min (▲), 1 h (■) or 3 h (●) before washing and measuring transport as a function of incubation time in BSS (37°C). Each point or line represents mean $\pm$ S.E.M. for determinations performed on three to eight plates.

Cells maintained in fructose for 1 day displayed a 3–4-fold higher rate of 3-OMG transport than cultures kept in glucose for 1 day (Figs. 4a–4c at zero min). Fructose-grown cells incubated with glucose, glucosamine or 2-DOG all showed enhanced rates of 3-OMG transport on immediate measurement of transport (Figs. 4a–4c, at 7 min). These increases were 232, 516, and 125 pmol of

3-OMG/min per mg of protein for glucose-, 2-DOG- and glucosamine-treated cells respectively. In all cases transport enhancement was reversed by washing the cultures free of the sugar-supplemented media and incubating the cells for 5 min in BSS (37°C) (Figs. 4a–4c, at 12 min). In contrast, the addition of glucosamine or 2-DOG had little effect on the transport rates of cells maintained in glucose.

In the parallel experiment (Figs. 4d–4f) fructose-grown cultures accumulated more ^{14}C -labelled glucose, 2-DOG or glucosamine than similarly treated glucose-grown cells during the initial 7 min preloading. The fructose-grown cultures so treated and then washed and subsequently incubated in BSS for 5 min lost 42, 57 and 17 nmol of the labelled glucose, 2-DOG and glucosamine/mg of protein respectively (Figs. 4d–4f, at 12 min). Compared with cells grown in fructose, cultures maintained in glucose not only accumulated less of these sugars but also effluxed less on removal of media and incubation in BSS. These observations revealed that the apparent stimulation of 3-OMG influx in preloaded cells was occurring during a time of net sugar efflux. In fact, the magnitude of transport stimulation appeared to be directly related to the sugar remaining free (unphosphorylated) and available for efflux.

The experiment shown in Fig. 5 was designed to verify the role of trans-stimulation by monitoring the cumulative uptake of 3-OMG in cultures that had first been starved of glucose for 1 day followed by exposure to 55 mM-glucose or -glucosamine for 15 min (37°C). Glucose-starved cultures displayed a higher initial rate of 3-OMG transport (at 10, 30 and 60 s) than cultures fed on glucose for 1 day (at 1 and 2 min). Glucose-starved cultures exposed to 55 mM-glucose or -glucosamine for 15 min showed an enhanced initial rate of 3-OMG transport (at 10 and 30 s) compared with glucose-starved cultures not exposed to hexose. The cells exposed to either sugar transiently accumulated 3-OMG against a concentration gradient (at 5 min) compared with the final equilibration value (at 35–60 min), whereas no overshoot was observed in cultures that were not incubated with glucose or glucosamine.

Discussion

Experiments designed to follow the kinetics of hexose-transport 'down regulation' in CEF failed to produce the projected result reported previously in CEF (Amos *et al.*, 1977) and in other cell types (Christopher, 1977; Gay & Hilf, 1980). In principle, the presence of certain sugars in the growth medium (glucose, glucosamine or mannose) was found to maintain a low level of glucose-transport activity in cultured cells. CEF that had been deprived of hexose for extended periods of time or in which fructose had

Fig. 4. Correlation between hexose-induced 3-OMG influx stimulation and rates of sugar efflux

Confluent primary CEF were grown in medium containing 3% dialysed calf serum + 5.5 mM-glucose (●) or 5.5 mM-fructose (■) for 1 day. Rates of 0.1 mM-3-OMG transport (a–c) were determined in cell cultures before (zero min) and after a final concentration of 5.5 mM-glucose (a), 2-DOG (b) or glucosamine (c) had been added directly to the medium for 7 min (37°C) followed by washing and immediate measurement of transport. After the 7 min pre-incubation some of the cultures were washed three times with BSS (2 ml, 20°C) and incubated for an additional 5, 15 or 30 min in 2 ml of BSS (37°C) before transport measurement (at 12, 22 and 37 min in the Figure respectively). Each point represents the mean $\pm$ S.E.M. for transport measurements performed on three to six plates. In a parallel set of experiments (d–f) cultures were monitored for cumulative uptake (0–7 min) and efflux (12–37 min) of 5.5 mM- 14 C-labelled glucose (d), 2-DOG (e) or glucosamine (f). Each point represents the mean of determinations performed on three to six plates; S.E.M. did not exceed 5 nmol of sugar/mg of protein in these experiments. Initially radiolabelled sugar and the appropriate concentration of unlabelled sugar to yield a final concentration of 5.5 mM were added to the cell cultures for 7 min (at 37°C). In some of these cultures uptake was halted at 7 min by washing in cold NaCl as described in the Materials and methods section. Other cultures were washed three times with 2 ml of BSS (at 20°C) to remove the external sugar, then were allowed to incubate in 2 ml of BSS (at 37°C) for the indicated times after which efflux was halted by washing in cold NaCl. It should be noted that in the case of glucose-grown cells, 5.5 mM-glucose was present in the medium and no exogenous glucose was added (● in a). Similarly, only trace amounts of 14 C-labelled glucose were added to glucose-grown cells in (d).

been substituted for glucose in the culture medium had attained high levels of hexose transport. The re-addition of glucose to such starved cells resulted in a reduction in transport activity over a period of

time. This 'down regulation' was considered to reflect carrier inactivation and it was speculated that an intermediate of carbohydrate metabolism produced by effective concentrations of glucose, man-

Fig. 5. Transient uptake of 3-OMG against its concentration gradient

Cumulative uptakes of 0.1 mM-3-OMG were assayed in primary CEF grown for 2 days in MEM + 3% calf serum + 5.5 mM-glucose and switched to media supplemented with 3% dialysed calf serum + 5.5 mM-glucose (●) or 3% dialysed calf serum + 5.5 mM-fructose (▲) for 1 day. Some of the glucose-deprived cultures were pre-incubated with 55 mM-glucose (■) or 55 mM-glucosamine (○) for 15 min (at 37°C) before washing and uptake determinations. Each point represents either the average of measurements performed on two plates, with bars donating range, or the mean $\pm$ S.E.M. of determinations performed on three to six plates.

nose or glucosamine was responsible for reducing glucose transport. Efforts at repeating the aforementioned experiments using 3-OMG as a non-metabolizable transport probe resulted, in our hands, in an apparent increase in the initial rates of influx.

In this paper the addition of 5.5 mM-glucose, -glucosamine or -2-DOG (15 min, 37°C) to cells grown in media containing fructose for 1 day, followed by washing (three times with 2 ml BSS at 20°C), resulted in an apparent increase in the initial rates of influx. We have concluded that this newly observed enhancement of transport is a reflection of counter-transport due to the accumulation of unphosphorylated sugar in the cells.

Enhancement was not observed in cells grown under conditions repressive for transport (in media with glucose or glucosamine) and was eliminated by allowing cultures to incubate in buffer (30 min at 37°C) after the removal of added sugar and before assaying for transport activity. The transport influx stimulation was more apparent at lower concen-

trations of test 3-OMG and occurred during a time of net sugar efflux (Fig. 4). In fact, in these experiments (Figs. 4a-4f) the quantity of labelled sugar available for efflux, presumably non-phosphorylated sugar, was roughly proportional to the magnitude of 3-OMG transport stimulation. Apparently, counter-transport could account for this stimulation and incubation in buffer had permitted sufficient efflux of free hexose to abolish this effect. Fig. 5 illustrates the characteristic counter-transport overshoot in 3-OMG accumulation above the final equilibration value in glucose-starved cells that were preloaded with glucose or glucosamine.

In these experiments, the transient accumulation of 3-OMG against its concentration gradient can be explained by the ability of intracellular free hexose to compete for efflux, although initially having no influence on rates of influx. In addition to this isotope-trapping effect, enhanced mobility of loaded hexose carriers has been proposed to be a common feature of glucose-transport systems in a variety of cell types (Plagemann *et al.*, 1981). These two mechanisms probably account for the observed increased initial rates of 3-OMG entry.

The magnitude of counter-transport stimulation is a function of the relative concentrations of intracellular and extracellular substrate available for efflux and influx respectively. The intracellular concentration is dependent on (1) the preloading conditions, including the temperature, duration and concentration of hexose employed, (2) the kinetic parameters of the carrier protein with respect to the particular sugar used in the preloading, which in turn is dependent on the prehistory of the cell (in these experiments glucose starvation has enhanced transport capacity), (3) the extent of metabolism of the sugar employed influencing the concentration of free hexose available for efflux and (4) the conditions under which the extracellular sugar employed in the preloading is removed from the cells, including the duration and temperature at which the washing is performed. The influence of this last factor is shown in Figs. 3(a) and 3(b) in which the counter-transport stimulation decreases as a function of efflux time. The eventual carrier inactivation observed at 1 and 3 h after re-addition of glucose (5.5 and 55 mM) followed by an efflux period of 30 min in BSS represents glucose-induced carrier inactivation, as previously reported (Amos *et al.*, 1977). It is apparent that at earlier efflux times this carrier inactivation was overshadowed by the counter-transport phenomenon.

The previously reported (Amos *et al.*, 1977) stimulation of glucose uptake on the addition of 2-DOG to glucose-starved cultures is shown here to be due to counter-transport. In these experiments the addition of 2-DOG and, to a lesser extent, glucosamine to starved cultures rapidly depleted intra-

cellular ATP pools and allowed for the accumulation of sufficient concentrations of free intracellular sugar to stimulate counter-transport. Plagemann & Erbe (1973) have reported that glucosamine (5 mM) rapidly decreased ATP levels in Novikoff rat hepatoma cells and resulted in high levels of intracellular glucosamine. Similarly, incubating cells with 2-DOG has been reported to deplete ATP levels, lower the rate of phosphorylation (Graff *et al.*, 1978) and result in a progressive increase in the intracellular concentration of free deoxyglucose (Renner *et al.*, 1972). However, in this paper, 5.5 mM-glucose was also effective in eliciting counter-transport stimulation without affecting ATP levels and it seems likely that glucose-starved cultures have a transport capacity greatly exceeding that of phosphorylation and subsequent metabolism. The addition of 2-DOG or glucosamine (5.5 mM) had no detectable influence on the transport rates or ATP levels of cells grown in glucose and exposed to these sugars in the presence of glucose. These results undoubtedly reflect the ability of glucose to inhibit the uptake of glucosamine and 2-DOG, whereas fructose is not a substrate for the glucose carrier. In addition the counter-transport stimulation was only observed in cells that had been starved of glucose, i.e. grown in media containing fructose for 1 day (Figs. 4a–4c). This finding reflects the carrier activation of glucose-starved cells, which have enhanced rates of carrier-mediated hexose influx and efflux (Figs. 4d–4f).

Preliminary experiments indicate that uncouplers of oxidative phosphorylation that lower ATP levels will enhance transport capacity only in the presence of glucose. This result may be explained, in part, by the involvement of counter-transport. Overall, these results warn that experimental conditions and

cellular prehistory should be closely monitored in transport studies in which environment conditions have been altered.

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