Purines as 'hyper-repressors' of glucose transport

A role for phosphoribosyl diphosphate

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Under selected conditions the rate of glucose transport and the intracellular phosphoribosyl diphosphate (*PPRibP*) concentrations of chick-embryo fibroblasts are inversely correlated. This relationship holds when cells are incubated with mannose, fructose, xylose or various concentrations of glucose. The metabolic inhibitors 2,4-dinitrophenol, rotenone and Methylene Blue increased glucose transport and decreased *PPRibP*. The addition of any pyrimidine or purine base or ribonucleoside dramatically depleted *PPRibP* pools, regardless of the carbon source. Addition of guanine (10 μ M) or hypoxanthine (100 μ M) decreased transport in glucose-grown chick cells to barely detectable values, but did not affect increases observed in cells derepressed by substitution of xylose for glucose. Guanosine, inosine and the purine analogues 6-thioguanine, 6-thioguanosine, 8-azaguanine and 6-methylmercaptapurine riboside sharply decreased transport in glucose-grown cells and blocked the increase in transport resulting from the replacement of glucose by fructose or xylose in the culture medium.

Previous work in this laboratory has provided evidence that the glucose carriers of cultured chick fibroblasts are 'down regulated' by one or more intermediates of hexose metabolism (Martineau *et al.*, 1972; Musliner *et al.*, 1977; Amos *et al.*, 1977). In those experiments, cells grown in the presence of certain sugars (e.g. glucose, glucosamine or mannose) were shown to maintain low or repressed rates of transport. Cultures deprived of hexose or grown in media supplemented with fructose or xylose exhibited enhanced rates of carrier-mediated hexose influx. The evidence derived from the cited experiments led to the formulation of a model in which steady-state transport activity is viewed as a function of the relative rate of carrier synthesis and incorporation into the plasma membrane and the rate of carrier inactivation (Amos *et al.*, 1977). In the proposed scheme a particular product of sugar metabolism is hypothesized to play a role in promoting carrier inactivation, resulting in lowered transport activity. Results from additional studies using cultured hamster fibroblasts (Christopher, 1977) and rat mammary tumour cells (Gay & Hilf, 1980) lend support to the principal elements of the model. The present study was directed towards

identifying the specific repressor metabolite(s) hypothesized in the model.

Certain metabolic pathways and intermediates seem unlikely candidates for sustaining this role. The involvement of glycolytic intermediates as transport repressors is improbable, since a mutant strain of Chinese-hamster fibroblast, defective in phosphoglucose isomerase, hence exhibiting drastically curtailed glycolytic rates, proved as responsive to glucose as was the wild-type parental cell (Pouyssegur *et al.*, 1980). More recently, Ullrey *et al.* (1982) have shown that glucose, but not glucosamine, is capable of repressing transport in the isomerase-less strain, whereas both sugars are equally effective in the wild type. In the mutant, glucose flows predominantly through the pentose cycle, whereas glucosamine is primarily routed through glycolysis and the tricarboxylic acid cycle. These data implicate the hexose monophosphate shunt and/or an intermediate of this pathway as a potential regulator of transport.

Several studies have re-emphasized the vital role of exogenous glucose in generating ribose 5-phosphate and thus *PPRibP* in cultured cells under 'normal' conditions (Reitzer *et al.*, 1980; Wice *et al.*, 1981; Wice & Kennell, 1982). Since *PPRibP* is critical for sustaining nucleic acid synthesis, and hence cell growth, the ribose phosphate pool could

Abbreviation used: *PPRibP*, phosphoribosyl diphosphate.

be viewed as a potentially important feedback signal in the repressive regulation of glucose transport.

Given the existing evidence, we initially elected to monitor the steady-state concentrations of *PPRibP* in cultured chick fibroblasts under a variety of environmental conditions known to affect glucose transport. As reported here, under certain conditions low transport rates accompanied high concentrations of *PPRibP*, and conditions eliciting transport enhancement were coincident with decreased *PPRibP* pools. Of particular interest is the evidence that purine nucleosides, in particular guanosine and analogues of guanosine, are hyper-repressors of glucose transport despite depleting intracellular *PPRibP*.

Materials and methods

Cell cultures

Primary chick fibroblasts were prepared from homogenized trypsin-treated embryos as described by Amos & Moore (1963). Dissociated cells (1×10^6) were inoculated in tightly sealed Kimble glass scintillation vials and grown for 2 days in 1 ml of Eagle's minimal essential medium (Gibco) with Hanks' salts, L-glutamine and non-essential amino acids without NaHCO_3 . The Eagle's medium was supplemented with dialysed calf serum (3%), penicillin (100 units/ml), streptomycin ($100 \mu\text{g/ml}$) and Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid] buffer (pH 7.4, 10 mM). In all experiments, cultures were grown for 2 days without a change of medium before any experimental manipulation and contained 2×10^6 – 3×10^6 cells/vial at the start of the experiments. Data are expressed in terms of cell number, which was determined by releasing cells with trypsin (Gibco; 0.25%)/EDTA (0.1%) solution and counting the released cells in a Coulter counter. Nucleosides, analogues and inhibitors were obtained from Sigma.

Initial rates of 3-O-methyl-D-glucose influx

Medium was aspirated from the vials and cells were washed (20°C) with $3 \times 2 \text{ ml}$ of balanced salt solution, pH 7.4 (137 mM- NaCl /5 mM- KCl /0.8 mM- MgSO_4 /1 mM- CaCl_2 /0.22 mM- Na_2HPO_4 /0.44 mM- KH_2PO_4 and $20 \mu\text{g}$ of Phenol Red/ml). Transport was initiated by adding 0.1 ml of balanced salt solution containing $3 \mu\text{Ci}$ of 3-O-methyl-D-[3- ^3H]-glucose (sp. radioactivity 80.0 Ci/mmol; NEN) and $1 \mu\text{mol}$ of unlabelled 3-O-methylglucose to vials containing 0.9 ml of balanced salt solution (20°C). Transport was halted as described by Gay & Hilf (1980), by rapidly washing with $5 \times 8 \text{ ml}$ of ice-cold iso-osmotic NaCl ; 10 ml of Liquiscint (National Diagnostic) was added directly to the vials, and

radioactivity was counted in a Beckman scintillation counter. Initial rates of carrier-mediated 3-O-methylglucose transport were measured at 60 s or less (Gay & Amos, 1982) and corrected for diffusional entry by subtracting entry observed in the presence of $10 \mu\text{g}$ of cytochalasin B/ml.

Determination of *PPRibP*

This was done by the method of Michelson & Villadsen (1979), with some modifications. Cultures were washed with $3 \times 5 \text{ ml}$ of ice-cold iso-osmotic NaCl and placed on ice. Residual NaCl was removed by aspiration, and 0.3 ml of ice-cold 50 mM-Tris base, pH 8.0, containing 2 mM-EDTA was added to each vial. Cells were subjected to extraction in a boiling-water bath for 30 s, after which vials were placed immediately on ice. Extracts could be used immediately or stored at -80°C for as long as 4 months without detectable loss of *PPRibP*. Portions ($93 \mu\text{l}$) of 17.8 mM-Tris base buffer (pH 8.0) containing 44 mM- MgCl_2 , $3 \mu\text{Ci}$ of [5- ^3H]orotic acid (sp. radioactivity 20.0 Ci/mmol; NEN) and 0.013 unit of orotidine 5'-phosphate pyrophosphorylase and orotidine 5'-phosphate decarboxylase mixed-enzyme preparation (Sigma) were added to 5 ml snap-top shell vials. To these reaction vessels $100 \mu\text{l}$ portions of the cell extract or extract diluted with the Tris/EDTA buffer were added; vials were capped and allowed to incubate for 1 day at room temperature. At this time, $7 \mu\text{l}$ of Tris base/ MgCl_2 reaction buffer containing 1.1 units of bovine intestine alkaline phosphatase (Sigma) was added and vials were incubated for 1 day further (at 20°C). Labelled uridine was separated from unchanged [^3H]orotate by spotting $5 \mu\text{l}$ of the phosphatase-treated reaction mixture together with $1 \mu\text{l}$ of 100 mM-uridine and running on polyethyleneimine-cellulose plates (Schleicher & Schull) in butanol-1-ol/methanol/water/ 14.8 M-NH_3 (60:20:20:1, by vol.) (Randerath & Randerath, 1967). The uridine spot ($R_F = 0.37$), located by u.v. light, and the origin, containing unchanged [^3H]orotate ($R_F = 0.00$), were extracted with $400 \mu\text{l}$ of 2 M-NH_3 (1 h, 20°C); then 10 ml of Liquiscint was added and samples were allowed to equilibrate with shaking on a rotary table (37°C) for 1 day before radioactivity counting. The percentage conversion (c.p.m. of uridine/c.p.m. of uridine + c.p.m. of orotate) was multiplied by the 150 pmol of orotate present in the reaction vessel to give estimates of *PPRibP* content present in the extracts. Under these conditions total radioactivity extracted from the t.l.c. plates was typically 30 000–40 000 c.p.m. Conversion of [^3H]orotate into [^3H]uridine was determined to be linear from 1 to 120 pmol of *PPRibP* by using a *PPRibP* standard (P-L Biochemicals). Extract dilutions were made accordingly.

Results

Kinetics of 3-O-methylglucose transport

Since the substrate of physiological importance for hexose transport *in situ* is D-glucose, ideally transport measurements should use that substrate. Complications arising from the metabolism of D-glucose by the cells have led to the substitution of 3-O-methylglucose, 2-deoxy-D-glucose (Graff *et al.*, 1978) or D-xylose (Romano & Connell, 1982) for glucose in efforts to separate metabolism from the uptake equation. 3-O-Methylglucose is a less polar compound than glucose, and by competition experiments has been shown to possess significantly altered affinity for the putative hexose carrier when compared with the natural substrate, D-glucose. Efflux of 3-O-methylglucose from the cells is an important consideration in measurements of transport with that compound. In Fig. 1 the kinetics of 3-O-methylglucose transport under the conditions of assay described in the Materials and methods section are presented. The experimental points in Fig. 1(a) come from a single experiment with cells grown on glucose at the normal concentration (5.5 mM). Each time point is in quadruplicate. Uptake was terminated by addition of ice-cold balanced salt solution, followed by four additional

rapid rinses with the same cold buffer. Fig. 1(b) presents data obtained from cells derepressed by incubation for 24 h without a carbon source. In both cases the results are representative of those obtained in other experiments of this series and reported in previous publications from our laboratory (Musliner *et al.*, 1977; Gay & Amos, 1982). The uptake of 3-O-methylglucose appears to be linear with time for at least 30 s and as long as 1 min. We have therefore utilized the 30 s reading in estimating relative rates of transport. In comparable experiments the addition of 10 μ g of cytochalasin B/ml to the wash fluid for all five rinsings did not alter the apparent retention of substrate observed in the absence of cytochalasin B.

Glucose concentration

Among the clearly established effectors of glucose transport are: (1) the concentration of glucose in the medium (Amos *et al.*, 1977; Christopher, 1977; Gay & Hilf, 1980); (2) the replacement of glucose by other sugars, repressive or non-repressive for transport (Amos *et al.*, 1977); and (3) the presence of metabolic inhibitors that alter carbohydrate utilization (Kalckar *et al.*, 1980). Primary cultures establish steady-state rates of glucose transport as an inverse function of the external glucose concentration (Fig. 2a); 2-day-old cultures, washed and

Fig. 1. Kinetics of uptake and retention of 3-O-methylglucose

Primary chick fibroblasts were grown in Eagle's minimal essential medium (+3% dialysed calf serum) for 2 days before a change of medium. One set of cells received fresh medium containing glucose (5.5 mM), the other the same medium without glucose. After 24 h incubation at 36°C, the cells were assayed for 3-O-methylglucose retention after 10–60 s exposure to 0.1 mM-3-O-methyl-D-[³H]glucose (3 μ Ci/ml of assay medium). Five rapid rinsings in ice-cold buffer followed exposure to radiolabelled 3-O-methylglucose. In panel (a) the results obtained in quadruplicate for each time point are plotted as actual radioactivity (c.p.m.) corrected for simple diffusion. The monolayers represented in (a) received glucose during the 20 h incubation. For panel (b), quadruplicate assays per time point were averaged; bars indicate S.E.M.. The cells used to obtain the data in panel (b) were starved of glucose during the 24 h incubation. For (a) the vessel contained 225 mg of protein, and for (b) 100 μ g of protein.

Fig. 2. Relationship between glucose transport and PPRibP contents as a function of the glucose concentration in the preincubation media

(a) Cultures grown in standard growth media for 2 days were washed and incubated in fresh Eagle's minimal essential medium containing 3% dialysed calf serum and various concentrations of glucose for 12 h. At this time rates of 3-O-methylglucose influx (●) and concentrations of PPRibP (■) were measured. Each point represents the mean $\pm$ S.E.M.

incubated in fresh medium containing increasing glucose concentration (0–55.5 mM) for 12 h at 37°C exhibited decreasing rates of 3-O-methylglucose transport. Intracellular PPRibP pools responded positively to increasing glucose. A plot of PPRibP concentration versus transport (Fig. 2b) depicts more explicitly the inverse quantitative relationship between these two parameters. Linear-regression analysis of these data (broken line) suggests that this inverse correlation is linear over the range of glucose concentrations used.

Sugar substitution

Substitution of D-fructose or D-xylose for D-glucose resulted in concomitant changes in transport and PPRibP pools (Fig. 3). Exposure for 1 day to D-xylose or to sugar-free medium produced the lowest concentrations of PPRibP, approx. 10% of that detected in cells grown in glucose-containing medium. For cells maintained in fructose and xylose, glucose transport rates were respectively 4- and 7-fold higher than those observed in controls (incubated with glucose). When fructose was provided as the maintenance sugar, PPRibP concentration was approx. 40% of that in glucose-grown cells, and transport was enhanced 2.5-fold over control values. Mannose was as effective as glucose in restricting transport and in supporting high PPRibP concentrations. Again the inverse correlation between PPRibP pools and transport is clearly demonstrated.

Metabolic inhibitors

The effects of certain metabolic inhibitors reported or expected to alter PPRibP concentrations in cultured cells were also assessed. In Table 1 glucose transport and PPRibP concentrations are reported in cells incubated for 1 day in glucose-containing medium when exposed to: (1) 2,4-dinitrophenol, an uncoupler of oxidative phosphorylation, reported to lower PPRibP concentrations (Henderson & Khoo, 1965a); (2) Methylene Blue, an artificial hydrogen acceptor, which increases PPRibP in some cells (Hershko *et al.*, 1969); this reagent can also act as an uncoupler of oxidative phosphorylation and lower PPRibP concentrations (Smith & Buchanan, 1979);

for transport or PPRibP determined on three or six vials respectively. (b) Same data as in (a) plotted as [PPRibP] versus glucose transport. Starting at the lower right-hand side of the figure and following the solid line connecting the points, the concentrations of glucose used were (mM): 55.5, 11.1, 5.5, 1.1, 0.55, 0.11, 0.055, 0.011 and 0. Broken line is the best-fitting straight line as determined by linear-regression analysis; $r = 0.98$.

Fig. 3. Relationship between glucose transport and PPRibP contents in cells incubated in various sugars for 1 day

Cultures grown for 2 days in standard growth media containing 3% dialysed calf serum + 5.5 mM-glucose were washed and switched to media containing dialysed calf serum and 5.5 mM-glucose (O), -mannose (□), -fructose (▲), -xylose (■) or no sugar (●) for 1 day. At this time initial rates of carrier-mediated 3-O-methylglucose (0.1 mM) transport (at 20°C) and intracellular concentrations of PPRibP were determined as described in the Materials and methods section. Each point represents the mean $\pm$ S.E.M. for 6–23 separate experiments. [Cell numbers were not significantly different, varying from 330 ± 20 ($\times 10^3$) to 385 ± 10 ($\times 10^3$) per vial.]

(3) rotenone, reported to inhibit the oxidative branch of the pentose cycle (Kather *et al.*, 1972b) and to block glucose utilization in fat-cells (Kather *et al.*, 1972a), which was expected to decrease PPRibP; and (4) dehydroepiandrosterone, at the concentration used expected to lower PPRibP concentrations by inhibiting glucose 6-phosphate dehydrogenase. For comparison, transport and PPRibP values for cells maintained in fructose or xylose are also tabulated (Table 1). All of the inhibitors stimulated glucose transport, and except for dehydroepiandrosterone, sharply decreased the PPRibP content.

Time course of transport and PPRibP responses

The time course of changes in glucose transport (Fig. 4a) and PPRibP (Fig. 4b) in cultures newly transferred to media containing fresh 3% dialysed calf serum and 5.5 mM-glucose, -fructose or -xylose revealed rapid responses to the new conditions regardless of the sugar supplied. This initial rise is dependent on the addition of fresh serum and is probably a response to insulin (Kletzien & Perdue, 1974; Musliner *et al.*, 1977). At 6 h the rates of transport reflect the sugar present. Cells responding to glucose exhibit increased transport for 3 h, but decrease their rate of uptake sharply between 6 and 9 h; it remains relatively constant for the next 15 h. In contrast, the rate of transport in cultures exposed to fructose or xylose continues to rise for 9 h, reaching plateau values which remain relatively constant for the remainder of the experiment. Taking the final steady-state transport value for xylose as 100%, the relative rates for cultures incubated in fructose and glucose are 52% and 16% respectively,

Table 1. Effects of metabolic inhibitors on 3-O-methylglucose transport and PPRibP concentrations

Cells grown in Eagle's minimal essential medium + glucose (5.5 mM) + 3% dialysed calf serum for 2 or 3 days were washed with 3×2 ml of balanced salt solution and incubated for 1 day further in the Eagle's medium + 3% dialysed calf serum and fructose, xylose or glucose (5.5 mM) and the indicated concentration of inhibitor. PPRibP concentrations and 3-O-methylglucose transport were determined as described in the Materials and methods section. Data (transport or PPRibP measurements converted into percentages for each experiment) are expressed as means $\pm$ S.E.M. for the numbers of separate experiments shown in parentheses. The value for 100% 3-O-methylglucose transport is that for xylose-grown cells; the average absolute value for eight experiments was 97 pmol of 3-O-methylglucose/min per 10^6 cells. The 100% value for PPRibP is that for glucose-grown cells in the absence of inhibitor; for 11 experiments the average was 1110 pmol of PPRibP/ 10^6 cells.

Sugar	Additions	3-O-Methylglucose transport	PPRibP
Glucose	None	14 ± 3 (10)	100 ± 18 (11)
Glucose	Dinitrophenol (0.2 mM)	87 ± 17 (6)	4 ± 1 (5)
Glucose	Methylene Blue (0.01 mM)	83 ± 20 (6)	10 ± 5 (4)
Glucose	Rotenone (0.01 mM)	69 ± 31 (5)	39 ± 10 (3)
Glucose	Dehydroepiandrosterone (0.1 mM)	85 ± 18 (7)	97 ± 21 (6)
Fructose	None	45 ± 9 (9)	41 ± 17 (9)
Xylose	None	100 ± 10 (8)	11 ± 5 (8)

Fig. 4. Time course of changes in glucose transport and PPRibP contents in cells incubated in glucose, fructose or xylose. Primary chick-embryo fibroblast cultures were grown for 2 days in standard growth medium (Eagle's minimal essential medium + 3% dialysed calf serum + 5.5 mM-glucose). At zero time, cultures were washed with 3 × 2 ml of balanced salt solution and incubated in the Eagle's medium + 3% dialysed calf serum with 5.5 mM-glucose (○), -fructose (▲), or -xylose (■), or no wash or change of medium (●). Rates of 3-O-methylglucose transport (a) and contents of PPRibP (b) were determined at various times after the change of medium. Transport is expressed as means ± S.D. for measurements performed on three vials; PPRibP-concentration assays were performed on sample extracts pooled from two vials.

similar to values obtained in the 1-day pre-incubation experiments shown in Fig. 3 and Table 1.

Initial increases in PPRibP were also observed in these cultures irrespective of the sugar supplied. This too is probably a serum-induced response and has been reported in cultured fibroblasts (Smith & Buchanan, 1979). Divergence in the concentrations of PPRibP becomes apparent within 2 or 3 h. PPRibP pools in cultures provided with glucose continued to rise throughout the time course of this experiment; cultures maintained in fructose increased PPRibP for 12 h. Once again the PPRibP values at 24 h were inversely related to the glucose-transport rates at the same time point.

Unexpected results in this series of experiments raise two questions that must be addressed. First, cells maintained for 2 days in glucose without change of medium had the expected repressed rate of transport. Compared with the PPRibP concentrations observed in cells switched to fresh medium containing glucose (Fig. 4b, 24 h), cultures remaining for 2 days in the same medium exhibited relatively low concentrations of PPRibP (Fig. 4b, 0–24 h). The lower PPRibP content was maintained despite glucose concentrations sufficient to sustain high steady-state PPRibP contents (R. J. Gay & H. Amos, unpublished work). The second question concerns the timing of the transport increase in cells newly provided with fructose in lieu of glucose. In

these cells the major increase in transport took place concurrently with increasing accumulation of PPRibP. Both observations are at variance with the hypothesis that PPRibP is a negative effector of transport.

Evidence that conditioned medium enhances PPRibP utilization

The aforementioned discrepancies draw attention to the experimental conditions used in these experiments. In essence, three changes were introduced: (1) old medium was removed; (2) cells were rinsed and new medium containing fresh dialysed calf serum was added; and (3) in the new medium glucose was replaced by other sugars, with or without serum. The influence of these modifications on the observed changes in glucose transport and PPRibP content were assessed. In Table 2 transport, PPRibP and cell number were measured in cells to which glucose and/or dialysed calf serum were added directly to the 2-day-old culture medium and in cells with fresh media with and without glucose and serum. No significant differences in hexose transport or PPRibP content were evident in cultures unless the culture medium was replaced by fresh medium. The addition of dialysed calf serum to existing medium did, however, induce growth in these cultures.

In fresh medium, sugar deprivation induced

Table 2. *Effects of change of medium, glucose and dialysed calf serum on glucose transport, PPRibP concentrations and cell number*

All cells were grown for 2 days in Eagle's minimal essential medium (MEM) + glucose (5.5 mM) + 3% dialysed calf serum (DCS). At this time additional 5.5 mM-glucose with or without 3% DCS was added directly to the medium (no wash or change of medium),* or cells were washed with 3 × 2 ml of balanced salt solution and incubated in MEM with the indicated additions. Then 1 day after these interventions cultures were assayed for glucose transport, PPRibP concentrations and cell number. Values for transport and cell number are the means of determinations performed on three vials; S.D. did not exceed 15% of the value shown. PPRibP was determined on extracts pooled from three vials. Values in parentheses were obtained from a separate experiment.

Wash and change of medium	Additions	3-O-Methylglucose transported (pmol/min per 10 ⁶ cells)	PPRibP (pmol/10 ⁶ cells)	10 ⁻³ × Cell number
No	None	12 (17)	244 (266)	298 (296)
No	Glucose	4	184	283
No	DCS	18	103	414
No	Glucose + DCS	10	132	372
Yes	MEM	26 (24)	36 (77)	192 (340)
Yes	MEM + glucose	3 (3)	113 (108)	212 (312)
Yes	MEM + DCS	92 (55)	95 (53)	274 (308)
Yes	MEM + glucose + DCS	21 (14)	644 (1310)	388 (372)

* Results from preliminary experiments indicated that washing before return of old medium did not significantly alter transport or PPRibP values.

increases in transport with or without serum, but rates were relatively higher in the presence of serum. PPRibP contents were higher in the presence of glucose regardless of the serum component. In the absence of serum some cell loss occurred; optimal growth was observed only in medium containing both glucose and serum.

Of primary interest is the dramatic increase in PPRibP pools observed in cultures transferred to fresh medium containing dialysed calf serum and glucose (Figs. 3 and 4). No comparable increase in PPRibP was elicited by the direct addition of these components to conditioned medium. The tentative conclusions drawn from these results are: (1) an unknown component(s) present in the conditioned medium (probably a metabolite released from the cells) is responsible for the low PPRibP concentrations in cells maintained in medium for several days; (2) this same component(s) may block the increase in PPRibP in cultures to which fresh glucose and serum are added without change of medium; and (3) this component may also be involved in suppressing glucose transport. The restriction on PPRibP could result from impaired synthesis or enhanced utilization of PPRibP.

Hyper-repression of glucose transport; guanine and hypoxanthine block the PPRibP surge

It is well known that the purine-salvage enzyme hypoxanthine/guanine phosphoribosyltransferase (EC 2.4.2.8), rather than the amidophosphoribosyltransferase (EC 2.4.2.14) of the 'de novo' purine pathway will preferentially utilize PPRibP provided that purine bases are available in the medium (Gordon *et al.*, 1979). In addition, cultured cells

have been reported to excrete purine bases into the culture medium (Willis & Seegmiller, 1979). This information led us to test hypoxanthine and guanine as potential inhibitors of the PPRibP surge observed in the previous experiment. Under the conditions found to produce the PPRibP 'surge' (Table 2), namely replacement of old medium with fresh medium containing dialysed calf serum and glucose, the addition of the purine bases, guanine and hypoxanthine prevented the increase in PPRibP (Table 3). Concentrations of guanine and hypoxanthine as low as 10 μ M and 100 μ M, respectively, decreased the detectable PPRibP at 24 h to approx. 6% of the value attained in cells growing on glucose alone. In the same cells 3-O-methylglucose entry was lowered to values barely above those attributable to passive diffusion. A change in the principal carbon source from glucose to xylose rendered transport refractory to the presence of guanine or hypoxanthine. This result may reflect a requirement for nucleotide synthesis from the exogenous purine base to effect the transport repression observed in cells supplied with glucose. One might expect little nucleotide formation in cells maintained in xylose, since the decreased PPRibP of these cells may be limiting for the purine-base-salvage reaction. If this line of reasoning is correct, purine nucleosides, which provide a source of both ribose 5-phosphate (for PPRibP synthesis) and a purine base, should prove more effective than purine bases alone in blocking transport derepression of xylose-grown cells. Such proved to be the case (Table 4). Guanosine and its immediate precursor inosine (1 mM) significantly decreased transport in cells incubated in media supplemented with xylose or

fructose. These additions also decreased transport in cells grown in glucose for 1 day. Guanosine was a more potent blocker than inosine, inhibiting transport by 89%, 74% and 51% compared with the rates observed in cells grown in media containing glucose, fructose and xylose respectively, in the absence of any additions. The other ribonucleosides tested (adenosine, cytidine, uridine and thymidine) were

ineffective in preventing transport enhancement. The effects observed with guanosine and inosine cannot be attributed to general cytotoxicity, since in the presence of xylose cell growth is enhanced by the addition of these nucleosides (results not shown). In fact, under these conditions none of the nucleosides significantly decreased cell numbers. Regardless of the sugar present, the inclusion of any of the listed ribonucleosides dramatically decreased *PPRibP* to values comparable with those seen in xylose-maintained cultures (results not shown).

Table 3. *Repression of 3-O-methylglucose transport and inhibition of PPRibP surge by guanine or hypoxanthine*

Cells grown for 2 days on Eagle's minimal essential medium + glucose (5.5 mM) + 3% dialysed calf serum were washed and incubated in fresh medium containing 3% dialysed calf serum and either glucose or xylose (5.5 mM) and the indicated additions for 1 day. At this time 3-O-methylglucose transport and *PPRibP* concentrations were determined; the former values are means of determinations performed on three vials (s.d. less than 10%). *PPRibP* was determined from extracts pooled from three vials (s.d. less than 15%). This experiment has been repeated several times with selected concentrations of guanine or hypoxanthine, with essentially identical results.

Sugar present	Addition	3-O-Methylglucose transport (pmol/min per 10 ⁶ cells)	<i>PPRibP</i> (pmol/10 ⁶ cells)
Glucose	None	17	1616
	Guanine		
	(10 μ M)	<1	90
	(100 μ M)	1	83
	(1000 μ M)	2	30
	Hypoxanthine		
	(10 μ M)	2	577
Xylose	(100 μ M)	<1	75
	(100 μ M)	<1	38
	None	54	56
	Guanine		
	(1000 μ M)	50	17
	Hypoxanthine		
	(1000 μ M)	62	17

Purine analogues as repressors of glucose transport

As the results thus far obtained were compatible with a role for guanosine nucleotides in the 'hyper-repression', guanosine and selected purine analogues were tested as potential blockers of transport derepression (Fig. 5). As shown, the inclusion of 6-thioguanosine at 3.3 μ M concentration inhibited the transport stimulation that results on shifting from glucose to fructose by approx. 50%; a concentration of 100 μ M-guanosine was required to achieve the same effect. In addition, 6-thioguanine, 6-methylmercaptapurine riboside and 8-azaguanine blocked transport enhancement. General toxicity can be ruled out, since the presence of the analogues did not significantly decrease the cell numbers in these and other experiments. In addition, transport repression by guanosine and 6-thioguanosine is reversible, i.e. washing cultures after exposure to these compounds and reincubating in medium containing fructose restored high glucose transport within 12–24 h (results not shown). Like guanosine, all of these compounds dramatically lowered *PPRibP* concentrations (results not shown), either by utilizing *PPRibP* directly via the salvage pathway (Stutts & Brockman, 1963; Henderson & Khoo, 1965*b*) or indirectly by inhibiting ribose phosphate pyrophosphokinase (EC 2.7.6.1) (Planet & Fox, 1976; Yen & Becker, 1979). These analogues are reported to have similar metabolic fates and effects, since all are raised at least to the 5'-monophosphate (Elion,

Table 4. *Influence of nucleosides on 3-O-methylglucose transport*

Cells grown for 2 days in standard medium were washed and incubated in Eagle's minimal essential medium + 3% dialysed calf serum and glucose, fructose or xylose (5.5 mM) with or without the indicated ribonucleoside for 1 day. At this time rates of glucose transport (for 0.1 mM-3-O-methylglucose) were measured. For purposes of comparison, the transport by cultures maintained in xylose is assigned a value of 100% (actual value 108 pmol of 3-O-methylglucose/min per 10⁶ cells). The mean values $\pm$ s.e.m. are computed from percentages calculated for each of four to seven separate experiments. In each experiment assays were done in triplicate or quintuplicate.

Sugar	Addition (1 mM)	...	Transport						
			None	Adenosine	Guanosine	Inosine	Cytidine	Uridine	Thymidine
Glucose			14 ± 2	6 ± 1	3 ± 1	7 ± 3	29 ± 8	27 ± 8	14 ± 6
Fructose			45 ± 4	44 ± 12	12 ± 4	24 ± 6	55 ± 11	61 ± 8	53 ± 9
Xylose			100 ± 10	91 ± 14	49 ± 10	55 ± 8	87 ± 11	87 ± 8	75 ± 2

Fig. 5. Inhibition of transport derepression by guanosine and purine analogues

For this, 2-day-old cultures were washed and incubated in fresh media containing 3% dialysed calf serum and 5.5 mM-glucose (control) or 5.5 mM-fructose and the indicated concentrations of 6-thioguanosine (concns., left to right: 0, 0.33 μ M, 3.3 μ M, 33 μ M, 333 μ M), guanosine (concns., left to right: 10 μ M, 100 μ M, 500 μ M, 1.0 mM, 5.0 mM), 6-methylmercaptapurine riboside (175 μ M), 8-azaguanine (300 μ M), or 6-thioguanine (left, 60 μ M; right, 300 μ M) for 1 day. Results are means $\pm$ S.D. for transport measurements performed on three vials.

1967; Lee & Sartorelli, 1981) and inhibit purine synthesis *de novo* (McCollister *et al.*, 1964; Miech *et al.*, 1967; Nelson *et al.*, 1975).

Discussion

A major contribution of this study is the finding that the rate of 3-O-methylglucose transport, in chick-embryo fibroblasts growing on glucose, and thus repressed for transport, can be decreased further to barely detectable values by guanine, hypoxanthine or analogues of guanine. The limit of sensitivity under these conditions has not been determined, but a concentration of 10 μ M-guanine added to the culture medium proved maximally effective. The effectiveness of the purine bases in restricting transport appears to correlate directly with the ability of the carbon source to generate *PPRibP* in the chick fibroblasts. This is taken to mean that nucleotide synthesis from the base is a requirement for action. The availability of *PPRibP* is well known to play a critical role in both purine and pyrimidine nucleotide synthesis and could be an important influence in the results reported here (Henderson, 1972).

This work was prompted by transport studies using intact cells (Amos *et al.*, 1977; Christopher, 1977; Gay & Hilf, 1980), which predicted that an intermediate of carbohydrate metabolism was a 'repressor' of glucose transport. Additional support for this hypothesis has subsequently been offered in

a report showing that enhanced rates of transport are demonstrable in membrane vesicles prepared from glucose-starved fibroblasts, compared with rates of transport observed in vesicles derived from cells incubated in the presence of glucose (Decker & Lipmann, 1981). In addition, Pessin *et al.* (1982), using a novel photoaffinity-labelling technique employing [³H]cytochalasin B, have shown that chick fibroblasts starved for glucose contain 4 times as much putative carrier as do fed cells. These data strongly support the notion that the starvation-induced increase in glucose transport observed in intact cells reflects an increase in the number of glucose carriers present in the plasma membrane. Studies with a phosphoglucose isomerase-defective mutant have implicated the pentose cycle in the repressive regulation of hexose transport (Pouyssegur *et al.*, 1980; Ullrey *et al.*, 1982; Ullrey & Kalckar, 1982).

With different experimental objectives, other investigators have documented the effects of altered culture conditions on *PPRibP* metabolism. The magnitude of *PPRibP* synthesis as a function of exogenous sugar has been reported in Ehrlich ascites-tumour cells, with the order of decreasing effectiveness being: glucose or mannose > fructose > galactose > ribose or no sugar (Henderson & Khoo, 1965a). Previous studies using cultured chick fibroblasts have shown the approximately reverse order for hexose-transport activity as a function of the carbon source in the medium (Martineau *et al.*,

1972; Musliner *et al.*, 1977; Bader *et al.*, 1981). In the present paper the data obtained with chick fibroblast cultures preincubated in media containing various sugars and assayed for both *PPRibP* and glucose transport were in general agreement with the results cited. Chick-embryo fibroblasts grown on xylose as the sole carbon source (Demetrakopoulos & Amos, 1976; Demetrakopoulos *et al.*, 1977) were previously found to be derepressed for transport. In fact, in the experiments reported here, medium containing xylose induced higher rates of transport than were observed in cells maintained in the total absence of sugar. Under the same conditions, xylose is a poor carbon source for *PPRibP* synthesis.

Decreased 3-*O*-methylglucose transport (Gay & Hilf, 1980) and increased *PPRibP* concentration (Henderson & Khoo, 1965a) as a function of increasing glucose concentrations in the medium have been reported, but not previously linked. In the present work an inverse relationship between these two parameters was observed over a range of glucose concentrations.

Anaerobiosis or treatment of Ehrlich ascites-tumour cells with 2,4-dinitrophenol inhibited *PPRibP* synthesis from glucose (Henderson & Khoo, 1965a). Anoxia (Reeves, 1975; Gould, 1979; Ullrey & Kalckar, 1981) and uncouplers of oxidative phosphorylation (Kalckar *et al.*, 1979, 1980; Bader *et al.*, 1981) have also been shown to enhance glucose transport. Our findings are confirmatory in that 2,4-dinitrophenol decreased *PPRibP* concentrations, probably by decreasing ATP, which would in turn limit synthesis of glucose 6-phosphate and *PPRibP*. This reagent also increased glucose transport in the presence of glucose.

Rotenone has been reported to decrease indirectly pentose phosphate-cycle activity in fat-cells (Kather *et al.*, 1972b) and to induce a carbohydrate metabolic profile similar to that observed in cells obtained from starved animals (Kather *et al.*, 1972a). In our hands the addition of rotenone to glucose-fed chick-embryo fibroblasts resulted in the expected decrease in *PPRibP*, with an increase in hexose transport.

The artificial hydrogen acceptor Methylene Blue rapidly oxidizes NADPH to NADP⁺ and has been shown to stimulate the oxidative branch of the pentose phosphate cycle and thus increase concentrations of ribose 5-phosphate and *PPRibP* in human erythrocytes (Sciaki *et al.*, 1973; Hershko *et al.*, 1969), Ehrlich ascites-tumour cells (Henderson & Khoo, 1965a) and human fibroblasts (Raivio *et al.*, 1979). This reagent can also act as an uncoupler of oxidative phosphorylation (Racker, 1956) and decrease intracellular *PPRibP* in 3T6 fibroblasts (Smith & Buchanan, 1979). In the experiments reported here, Methylene Blue appeared to act as a uncoupler and decreased *PPRibP*, with concomitant

increase in glucose transport. Rapid (15 min) serum-induced increases in the rates of *PPRibP* synthesis and in purine synthesis *de novo* have been observed in resting 3T6 fibroblasts (Smith & Buchanan, 1979). These authors concluded that serum stimulated the cycling of the nicotinamide nucleotide coenzymes NADP⁺ and NADPH, which led to enhanced oxidative shunt activity and this increased the formation of ribose 5-phosphate and *PPRibP*; this in turn accelerated the rate of purine nucleotide synthesis. In that study, as in our experiments, fresh dialysed serum and glucose-containing medium were employed.

The requirement for the removal of conditioned medium (Table 2) to assure the *PPRibP* surge led us to hypothesize that a component in the 2-day-old medium may be responsible for maintaining the relatively low *PPRibP* concentrations observed. Significant amounts of purine base, 3–5% (Willis & Seegmiller, 1979) and 4–7% (Gordon *et al.*, 1979) of the total purine synthesized *de novo*, are excreted into the medium of cultured human lymphocytes and fibroblasts respectively. It is also known that in cultured cells the phosphoribosyltransferase salvage enzymes rather than the amidophosphoribosyltransferase of the '*de novo*' pathway preferentially utilize *PPRibP* provided that purine bases are available in the medium (Hershfield & Seegmiller, 1977; Bagnara *et al.*, 1974; Gordon *et al.*, 1979). These authors demonstrated that the addition of micromolar hypoxanthine concentrations depleted intracellular *PPRibP* and inhibited purine synthesis *de novo*. In our hands the *PPRibP* surge was totally prevented by the inclusion of 10 μ M-guanine or 100 μ M-hypoxanthine in the culture medium (Table 3). This result is consistent with a rapid rate of *PPRibP* utilization via hypoxanthine/guanine phosphoribosyltransferase.

Since guanine and guanosine both depleted *PPRibP*, whereas only guanosine was capable of preventing transport derepression in the absence of glucose, we speculated that (1) the nucleoside provides sufficient ribose 5-phosphate for synthesis of *PPRibP*, and hence of 5'-GMP, and (2) a nucleotide of guanine may be important in repression of glucose transport.

The metabolic fate and effect of 8-azaguanine and 6-thioguanine are thought to be similar. Both are primarily converted into the nucleotide monophosphate by hypoxanthine/guanine phosphoribosyltransferase, deplete *PPRibP* and inhibit purine synthesis *de novo* (Sartorelli *et al.*, 1958; Stutts & Brockman, 1963; McCollister *et al.*, 1964; Henderson & Khoo, 1965b; Elion, 1967; Miech *et al.*, 1967; Hill & Bennett, 1969; Tay *et al.*, 1969; Parks *et al.*, 1973; Lee & Sartorelli, 1981). Upon entry into the cell and subsequent cleavage, the conversion of 6-thioguanosine parallels that of 6-thioguanine

(LePage & Junga, 1963; Paterson, 1960; Lee & Sartorelli, 1981).

The adenosine analogue, 6-methylmercaptapurine riboside, is phosphorylated to the monophosphate level via adenosine kinase (Bennett *et al.*, 1965; Henderson, 1972). The resulting phosphorylated derivative is a potent inhibitor of amidophosphoribosyltransferase (Henderson & Khoo, 1965b; Hill & Bennett, 1969; Shantz *et al.*, 1971) and can lead to increased *PPRibP* concentrations owing to decreased utilization (Paterson & Wang, 1970; Bagnara *et al.*, 1974). However, 6-methylmercaptapurine riboside 5'-monophosphate has also been reported to inhibit ribose phosphate pyrophosphokinase (Yen & Becker, 1979; Roth & Deuel, 1974) and to decrease *PPRibP* (Planet & Fox, 1976).

In the experiments reported here, these analogues proved to be potent inhibitors of the derepression of glucose transport in chick fibroblasts transferred from glucose to fructose-supplemented medium. These compounds decreased *PPRibP* concentrations to the same degree as that observed with naturally occurring ribonucleosides and bases. In the light of the reported high concentrations of monophosphate derivatives attained in cells exposed to 6-thioguanine, 6-thioguanosine or 6-methylmercaptapurine riboside (Miech *et al.*, 1967; Nelson & Parks, 1972; Parks *et al.*, 1973; Nelson *et al.*, 1975; Lee & Sartorelli, 1981), the potential role of guanine nucleotides, in particular 5'-GMP, in the physiological regulation of glucose transport should be an object of future studies.

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