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Effect of glutamine on fructose 2,6-bisphosphate and on glucose metabolism in HeLa cells and in chick-embryo fibroblasts

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Glutamine caused a dose-dependent decrease in fructose 2,6-bisphosphate concentration in both HeLa cells and chick-embryo fibroblasts. The effect was complete within 15 min in HeLa cells, but required more than 9 h in the fibroblasts. Half-maximal effects were obtained with 0.1–0.3 mM-glutamine. In chick-embryo fibroblasts, but not in HeLa cells, glutamine induced a time-dependent decrease in the activity of phosphofructokinase-2, which correlated with the decrease in fructose 2,6-bisphosphate. Glutamine decreased the glycolytic flux by about 25% only in chick-embryo fibroblasts. The difference in glycolytic response between the two types of cells might correspond to a difference in the sensitivity of phosphofructokinase-1 for fructose 2,6-bisphosphate. In HeLa cells, glutamine caused a 2–3-fold stimulation of the synthesis of glycogen, a 50% decrease in the concentration of fructose 1,6-bisphosphate and a more than 80% decrease in the concentration of 5-phosphoribosyl pyrophosphate; the concentrations of hexose 6-phosphates and ATP were not affected.

INTRODUCTION

Glutamine is a major respiratory fuel for certain cells, such as enterocytes (Neptune, 1965; Windmueller & Spaeth, 1974; Watford *et al.*, 1979), reticulocytes (Rapoport *et al.*, 1971), stimulated lymphocytes (Ardawi & Newsholme, 1982), fibroblasts in culture (Donnelly & Scheffer, 1976; Zielke *et al.*, 1984) and malignant cells (Kovacevic & Morris, 1972; Reitzer *et al.*, 1979). These cells have in common a relatively rapid growth rate, and therefore require active synthesis *de novo* of purines and pyrimidines, for which glutamine is an essential precursor. Thus the ready oxidation of the glutamine carbon skeleton could be related, at least in part, to the requirement for nitrogen atoms for the synthesis of purine and pyrimidine rings (Krebs, 1980). In enterocytes and HeLa cells, the main products of glutamine carbon are CO_2 and lactate (Windmueller & Spaeth, 1974; Watford *et al.*, 1979; Reitzer *et al.*, 1979; Windmueller, 1982). Another common feature of the cells that metabolize glutamine is their relatively poor capacity for glucose oxidation and their high glycolytic rate. In HeLa cells, glutamine is consumed to about the same extent as glucose; however, the CO_2 yield from glutamine is at least 4 times that from glucose (Reitzer *et al.*, 1979). The mitochondrial metabolism of glutamine (reviewed by Kovacevic & McGivan, 1983) has been re-investigated in Ehrlich tumour cells (Moreadith & Lehninger, 1984).

Our project was to study the influence of glutamine on glucose metabolism in such cells. More specifically we have examined whether glutamine could influence the glycolytic flux and the concentration of fructose 2,6-bisphosphate (Fru-2,6-P_2), which is a powerful stimulator of phosphofructokinase (PFK-1) (Hers & Hue, 1983). HeLa cells were chosen as a model for malignant cells because glutamine metabolism in these cells is well documented. Chick-embryo fibroblasts were chosen because the glycolytic rate and the concentration of Fru-2,6-P_2 in these cells are known to be increased under various conditions, including treatment with tumour promoters, mitogenic concentrations of insulin and transformation by Rous sarcoma virus (O'Brien, 1982; Farnararo *et al.*, 1984; Singh *et al.*, 1974; L. Bosca, G. Rousseau & L. Hue, unpublished work).

MATERIALS AND METHODS

Materials

All the biochemicals were purchased from Boehringer or Sigma. ^{14}C-labelled glucoses were from NEN, and [3-^3H]glucose was from Amersham International. Sephadex G-25 (medium grade) and Blue Sepharose were purchased from Pharmacia. Chemicals and poly(ethylene glycol) 6000 were from Merck.

Cell cultures

Primary cultures of chick-embryo fibroblasts prepared (Hunter, 1979) from 8–9-day embryos (c/o White Leghorn; Lohman, Cuxhaven, Germany) were maintained at 38 °C for 4 days in an atmosphere of air containing 6% CO_2. The cells were then collected by treatment with 0.05% (w/v) trypsin, followed by centrifugation (130 *g* for 5 min), and resuspended in growth medium (F10 medium, from Flow Laboratories) enriched with 5% (v/v) newborn-calf serum, 10% (w/v) tryptose/phosphate broth and 7 mM-$NaHCO_3$. This growth medium contained 1 mM-glutamine. Plastic Petri dishes (60 mm diameter) were seeded with 1×10^6 cells. After 4 days of secondary culture, the growth medium was removed and replaced by 5 ml of incubation medium (126 mM-NaCl/14 mM-$NaHCO_3$/3.8 mM-KCl/0.9 mM-Na_2HPO_4/0.6 mM-KH_2PO_4/0.6 mM-$MgSO_4$/0.3 mM-$CaCl_2$/6 mM-glucose/20 mM-Hepes, pH 7.2). The fibroblasts

Abbreviations used: Fru-2,6-P_2, fructose 2,6-bisphosphate; PFK-1, 6-phosphofructo-1-kinase (EC 2.7.1.11); PFK-2, 6-phosphofructo-2-kinase (EC 2.7.1.105).

were incubated in that medium for the indicated periods of time with or without glutamine.

HeLa cells were grown in suspension at 37 °C in an atmospheric incubator. The suspensions were maintained at 1×10^6 cells/ml in F10 medium supplemented with 3% newborn-calf serum with a daily change of the medium. The cells were harvested by centrifugation (130 *g* for 5 min), washed in incubation medium (the same medium as for fibroblasts, but supplemented with 1 mg of defatted bovine serum albumin/ml) and incubated at 37 °C for 45 min. After centrifugation (130 *g* for 5 min), the cells were resuspended in the incubation medium (2×10^6–3×10^6 cells/ml), and 5 ml samples were incubated in 20 ml plastic scintillation vials at 37 °C in a rotatory incubator for the indicated periods of time with or without glutamine.

Metabolism of glucose

The glycolytic flux was evaluated by the amount of glucose consumed and lactate released, and by the release of labelled anions (primarily lactate and pyruvate) from [U-^{14}C]glucose into the incubation medium. At the end of incubation in the presence of 6 mM-[U-^{14}C] glucose (0.5 μCi/ml), samples of the cell suspensions were centrifuged (1700 *g* for 30 s) and the supernatants were deproteinized in 0.5 M-$HClO_4$. After neutralization, 0.5 ml samples were applied on Dowex AG 1X8 (Cl^- form; 0.5 cm × 4 cm) columns; the anions were separated from glucose, which was washed out with 4 ml of water, and eluted with 1.5 ml of 1 M-NaCl; their radioactivity was measured. The flux through phosphofructokinase can be evaluated by the amount of ^3H_2O released from [3-^{3}H]glucose (Katz & Rognstad, 1976; Hue, 1981). After incubation with 6 mM-[3-^{3}H]glucose (1 μCi/ml), the incubation medium was deproteinized as above, and ^3H_2O was separated from [3-^{3}H]glucose as described by Bontemps *et al.* (1978). The oxidation of glucose was measured by the amount of $^{14}CO_2$ released from 6 mM-[1-^{14}C]- or -[6-^{14}C]-glucose (0.5 μCi/ml); after deproteinization, the radioactive CO_2 was collected in 0.5 ml of Lipoluma (Lumac Systems, Basle, Switzerland) added to a small plastic cup attached to the rubber stopper. The incorporation of [U-^{14}C]glucose into glycogen was measured as described by Hue *et al.* (1975).

Measurement of metabolites

For the measurements of hexose 6-phosphates and fructose 1,6-bisphosphate in HeLa cells, deproteinized extracts were prepared as follows: 3 ml portions of the cell suspension were centrifuged (700 *g* for 30 s at 0 °C) and the cell pellets were frozen in liquid N_2; the pellets were then homogenized (Potter) in 1 ml of 0.5 M-$HClO_4$ and, after centrifugation (5000 *g* for 3 min), the supernatants were neutralized with saturated K_2CO_3. Glucose 6-phosphate, fructose 6-phosphate and fructose 1,6-bisphosphate were measured as previously reported (Minatogawa & Hue, 1984). Fru-2,6-P_2 was measured in alkali extracts (Van Schaftingen *et al.*, 1982), and 5-phosphoribosyl pyrophosphate was measured in trichloracetic acid extracts, which were rapidly neutralized (Vincent *et al.*, 1984). Lactate (Hohorst, 1963) and glucose (Kunst *et al.*, 1984) were measured enzymically.

Measurement of enzyme activities

To measure enzyme activities, HeLa-cell extracts were prepared by homogenizing (Dounce; 15 strokes) pellets of frozen cells in 4 vol. of ice-cold buffer A (100 mM-KCl/5 mM-$MgCl_2$/1 mM-EGTA/1 mM-dithiothreitol/50 mM-Hepes, pH 7.2). For fibroblasts, the cell layers were scraped with a plastic spatula and the cells were collected in 1 ml of ice-cold buffer A and homogenized as above. After centrifugation (100000 *g* for 60 min), the supernatant was fractionated with 5% (w/v) and the resulting supernatant with 12% (w/v) poly(ethylene glycol) 6000; the pellet [5–12% (w/v) fraction] was washed in 12% poly(ethylene glycol) and, after centrifugation (2800 *g* for 15 min), the pellet was resuspended in buffer A, and PFK-2 activity was measured.

The enzyme was preincubated for 5 min at 30 °C and then incubated for up to 20 min at the same temperature with 1 vol. of buffer A containing 8 mM-ATPMg and fructose 6-phosphate/glucose 6-phosphate (1:3) at the concentrations indicated. Samples were taken at 0, 5, 10 and 20 min and were immediately treated with 1 vol. of 100 mM-NaOH at 80 °C for 10 min. The amount of Fru-2,6-P_2 formed was measured as indicated above. The formation of Fru-2,6-P_2 was linear for at least 20 min under these conditions.

The sensitivity of PFK-1 to Fru-2,6-P_2 was studied with partially purified enzymes. PFK-1 from fibroblasts was partially purified by ATP–agarose chromatography (Bosca *et al.*, 1982). PFK-1 from HeLa cells was purified by poly(ethylene glycol) fractionation (5–12% fraction), followed by chromatography on Blue Sepharose. A linear gradient of potassium phosphate (50–500 mM) and of ATP (0–7 mM) in 2 mM-dithiothreitol/30% (v/v) glycerol, pH 8.0, was applied to the column. PFK-1 activity was eluted at 100 mM-potassium phosphate/0.7 mM-ATP and was separated from PFK-2, which remained bound to the column under these conditions. PFK-1 activity was measured spectrophotometrically (Van Schaftingen *et al.*, 1980) at 25 °C and pH 7.0 in the presence of 3 mM-ATPMg, 5 mM-potassium phosphate, 1 mM-NH_4Cl, 0.05 mM-AMP, and the indicated concentrations of fructose 6-phosphate and Fru-2,6-P_2.

One unit of enzyme activity is the amount of enzyme that catalyses the formation of 1 μmol of product/min under the assay conditions.

RESULTS

Time-course and dose–response curves for the effect of glutamine on Fru-2,6-P_2 concentration

Under basal conditions in incubation medium, the concentration of Fru-2,6-P_2 was 8.0 ± 0.9 and 4.0 ± 0.4 (means ± S.E.M. for 11 observations) nmol/g of cells in HeLa cells and fibroblasts respectively. This content is similar to that in livers of fed rats, and about one order of magnitude greater than in rat skeletal muscle (Hue *et al.*, 1982). The addition of 2 mM-glutamine to the incubation medium caused a 70–80% decrease in the concentration of Fru-2,6-P_2 in both types of cells (Fig. 1). The effect was, however, much more rapid in HeLa cells (half-maximal effect obtained at 7 min) than in fibroblasts (9 h required for maximal effect). When fibroblasts were first preincubated for 10 h in the absence of glutamine, the concentration of Fru-2,6-P_2 remained stable for the next 15 h, indicating that the increase in Fru-2,6-P_2 observed under control conditions in Fig. 1(*b*) might result from the removal of glutamine that was present in the growth medium (see also below). Incubations of HeLa

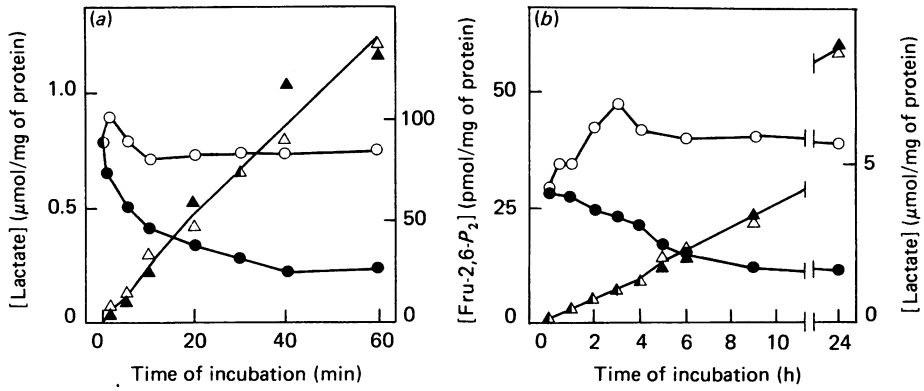

Fig. 1. Time course of the effect of glutamine on the concentration of fructose 2,6-bisphosphate (○, ●) and the production of lactate (△, ▲) in (*a*) HeLa cells and (*b*) chick-embryo fibroblasts

○, △, Control conditions; ●, ▲, with 2 mM-glutamine. Results shown are the means of two experiments.

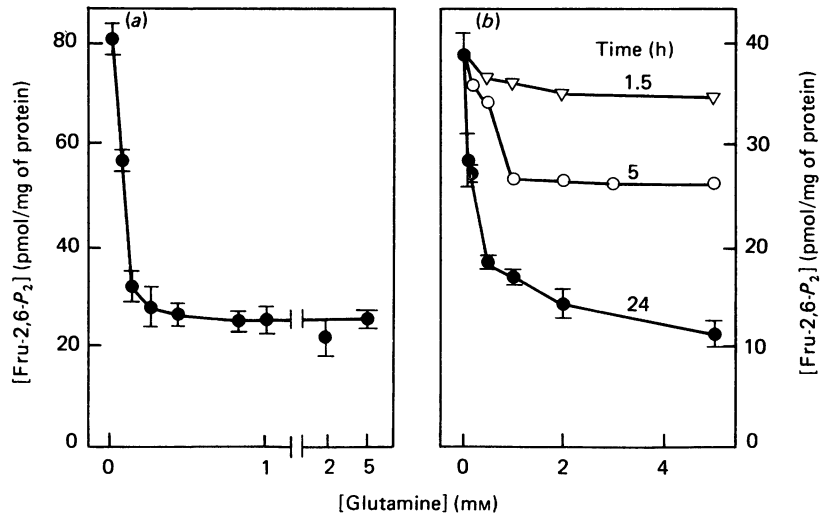

Fig. 2. Dose-dependency of the effect of glutamine on the concentration of fructose 2,6-bisphosphate in (*a*) HeLa cells and (*b*) chick-embryo fibroblasts

HeLa cells were incubated for 30 min in the presence of glutamine, whereas chick-embryo fibroblasts were incubated for the indicated periods of time. Values are means ± S.D. for two or three different experiments.

cells or fibroblasts with 10 mM- instead of 6 mM-glucose did not change the effect of glutamine on Fru-2,6-P_2 concentration (results not shown). Despite this large effect on Fru-2,6-P_2, the release of lactate (1.1 and 0.4 μmol/h per mg of protein in HeLa cells and in fibroblasts respectively) remained largely unaffected in both cases. However, since lactate might originate not only from glucose but also from glutamine, it is not excluded that a decreased lactate production from glucose was exactly compensated for by the lactate derived from glutamine.

When cells first incubated with 2 mM-glutamine were then incubated in a glutamine-free medium, 1 h (HeLa cells) or 10 h (fibroblasts) was respectively required to restore concentrations of Fru-2,6-P_2 similar to those found under basal conditions (results not shown).

The concentrations of glutamine required for half-maximal effect were below the millimolar range (about 90 μM in HeLa cells and about 300 μM in fibroblasts) (Fig. 2). These values are similar to the plasma concentrations of glutamine (0.2–0.6 mM, according to Windmueller, 1982) and at least one order of magnitude lower than the glutamine concentration present in the culture medium used (1 mM).

NH_4Cl, alanine and glutamate (each 2 mM), which all are products of glutamine metabolism, were without effect in HeLa cells (Table 1). However, in fibroblasts, alanine was as effective as glutamine in decreasing Fru-2,6-P_2 concentration, glutamate had no significant effect, and NH_4Cl increased Fru-2,6-P_2 concentration. Under the latter condition, lactate release was also increased by 50% (results not shown). All the other amino acids (1 mM) were without effect. In both types of cells, amino-oxyacetate (5 mM; an inhibitor of transaminases) was unable to prevent the effect of glutamine; however, it was able to prevent the effect of alanine in fibroblasts,

Table 1. Effect of several substances on Fru-2,6-P_2 concentration in HeLa cells and fibroblasts

Cells were incubated for 30 min (HeLa) or 24 h (fibroblasts). Values are means ± S.D. for at least three experiments.

Addition	Fru-2,6-P_2 (pmol/mg of protein)	
	HeLa cells	Fibroblasts
None	81 ± 11	44 ± 2
Glutamine (2 mM)	26 ± 2	22 ± 3
NH_4Cl (2 mM)	70 ± 8	80 ± 6
Alanine (2 mM)	78 ± 3	25 ± 4
Glutamate (2 mM)	73 ± 6	35 ± 2
Amino-oxyacetate (5 mM)	72 ± 5	39 ± 2
Amino-oxyacetate (5 mM) + glutamine (2 mM)	34 ± 3	25 ± 2
Amino-oxyacetate (5 mM) + alanine (2 mM)	—	42 ± 3
Glutamine (2 mM) + alanine (2 mM) + glutamate (2 mM)	28 ± 2	16 ± 1

Table 2. Effect of glutamine on the metabolism of glucose in fibroblasts and HeLa cells

Cells were incubated for 24 h (fibroblasts) or 30 min (HeLa cells) with or without 2 mM-glutamine. Values are means ± S.D. for three experiments.

Cell type	Addition...	Flux (nmol/min per mg of protein)	
		None	2 mM-Glutamine
Fibroblasts	Lactate released	6.0 ± 0.5	6.1 ± 0.6
	[^{14}C]Glucose converted into labelled anions	3.3 ± 0.5	2.4 ± 0.2
HeLa	Glucose consumed	18 ± 1	19 ± 1
	Lactate released	32 ± 3	33 ± 3
	[^{14}C]Glucose converted into labelled anions	16 ± 1	19 ± 1
	[U-^{14}C]Glucose incorporated into glycogen	0.25 ± 0.01	0.62 ± 0.05
	[1-^{14}C]Glucose into CO_2	1.1 ± 0.1	1.4 ± 0.3
	[6-^{14}C]Glucose into CO_2	0.2 ± 0.05	0.15 ± 0.04

Table 3. Effect of glutamine on the concentrations of hexose phosphates, ATP and phosphoribosyl pyrophosphate in HeLa cells

Cells were incubated for 30 min. Values are means ± S.D. for the numbers of experiments shown in parentheses.

Addition...	Metabolite concn. (nmol/mg of protein)	
	None	2 mM-Glutamine
Glucose 6-phosphate + fructose 6-phosphate	6.2 ± 0.8	5.8 ± 0.8 (5)
Fructose 1,6-bisphosphate	4.0 ± 0.5	1.8 ± 0.6 (6)
Fru-2,6-P_2	0.081 ± 0.011	0.026 ± 0.002 (3)
ATP	18 ± 2	20 ± 1 (3)
Phosphoribosyl pyrophosphate	1.7 ± 0.3	0.24 ± 0.05 (3)

suggesting that alanine could stimulate the formation of glutamine.

Metabolic fluxes and concentrations of metabolites

This series of experiments was performed to document the influence of glutamine on the glycolytic flux. In order to distinguish the lactate produced by glucose from that originating from glutamine, the amount of ^{14}C-labelled anions (mainly lactate and pyruvate) released from [^{14}C]glucose was measured in the incubation medium of fibroblasts and compared with the total lactate release. The results (Table 2) show that a 27% decrease in the production of labelled anions from glucose was observed in the presence of 2 mM-glutamine. Therefore glycolysis was partially inhibited. The situation was different in HeLa cells. In these cells, glucose consumption, lactate release and the release of labelled anions from [^{14}C]glucose were measured as well as glucose oxidation, which was evaluated by the production of $^{14}CO_2$ from [1-^{14}C]- and [6-^{14}C]-glucose. Table 2 shows that 2 mM-glutamine did not affect any of these parameters, demonstrating that

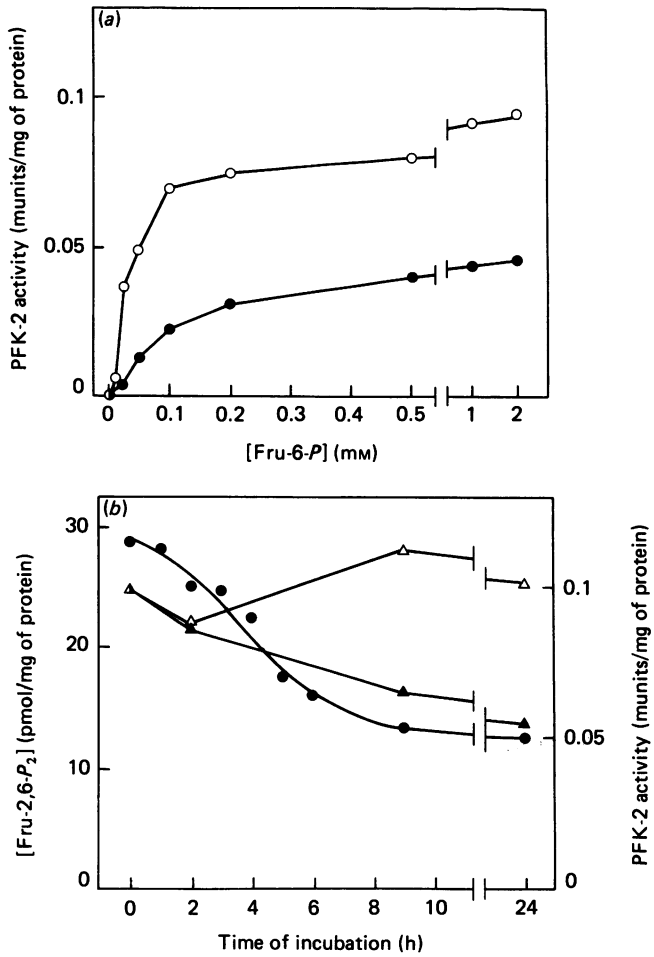

Fig. 3. Effect of 2 mM-glutamine on the concentration of fructose 2,6-bisphosphate and the activity of phosphofructokinase-2 in chick-embryo fibroblasts

(*a*) Effect of the concentration of fructose 6-phosphate (Fru-6-*P*) on the activity of PFK-2 from control cells (○) or cells treated with glutamine for 24 h (●). (*b*) Time course of the change in Fru-2,6-P_2 concentration (●) and PFK-2 activity in control (△) or glutamine-treated (▲) cells.

glycolysis was not influenced by glutamine in these cells. The proportions of glucose transformed into labelled anions (85%) and into total CO_2 (10% maximally) are in good agreement with the published data (Reitzer *et al.*, 1979). Table 2 also shows that the small proportion of glucose (1.4%) that was incorporated into glycogen was increased 2.5-fold by glutamine. This effect is reminiscent of that obtained in hepatocytes (Katz *et al.*, 1976).

When HeLa cells were incubated in the presence of glucose at concentrations equal to or less than 0.05 mM, the release of labelled anions from [U-^{14}C]glucose, and of ^3H_2O from [3-^{3}H]glucose, was, however, consistently ($P < 0.001$) decreased by 18% (results not shown).

The influence of glutamine on the concentrations of several metabolites was also measured in HeLa cells (Table 3). Whereas glutamine had no effect on the concentration of hexose 6-phosphates and ATP, it did, however, lower the concentration of fructose 1,6-bisphosphate by 50% and caused an impressive 7-fold decrease in the concentration of 5-phosphoribosyl pyrophosphate.

Effect of glutamine on the activity of 6-phosphofructo-2-kinase (PFK-2)

To investigate the mechanism of the glutamine-induced fall in the concentration of Fru-2,6-P_2, we measured the activity of PFK-2, the enzyme which catalyses the synthesis of Fru-2,6-P_2. PFK-2 was measured after precipitation of the enzyme by poly(ethylene glycol) in extracts of cells treated or not with 2 mM-glutamine (30 min for HeLa cells, 24 h for fibroblasts). Whereas no change was observed in HeLa cells (results not shown), a 50% decrease in $V_{max.}$ was obtained in fibroblasts (Fig. 3*a*). Moreover, the time course of the decrease in PFK-2 activity in fibroblasts was similar to that of Fru-2,6-P_2 concentration after addition of 2 mM-glutamine (Fig. 3*b*).

Stimulation of partially purified PFK-1 by fructose 2,6-bisphosphate

Since the fall in Fru-2,6-P_2 concentration resulted in different effects on the glycolytic flux in HeLa cells and in fibroblasts, we compared the kinetic properties of PFK-1 from these cells. Fig. 4 shows that, at physiological concentrations of fructose 6-phosphate (0.3–0.5 mM), PFK-1 from HeLa cells was indeed poorly sensitive to a change in the concentration of Fru-2,6-P_2 and was certainly less sensitive than the fibroblast enzyme. This difference in sensitivity was confirmed in the presence of 10% poly(ethylene glycol) (results not shown), which is believed to mimic the protein–protein interactions (the 'crowding') supposed to occur in the cell (Bosca *et al.*, 1985). From the data in Table 3, one can calculate (assuming that 1 g of HeLa cells contains 110 mg of protein and that the intracellular water space represents 60% of the cell volume) that the concentration of Fru-2,6-P_2 goes from 12.5 to 4.0 μM after glutamine. Fig. 4 shows that, at 0.3 mM-fructose 6-phosphate, this change in Fru-2,6-P_2 concentration has little, if any, influence on PFK-1 activity. The same poor sensitivity was observed when PFK-1 was studied in a less purified preparation, such as the 5–12%-poly(ethylene glycol) fraction (results not shown).

DISCUSSION

Glutamine and fructose 2,6-bisphosphate

Although glutamine induced a similar fall in Fru-2,6-P_2 in both fibroblasts and HeLa cells, the mechanism of action was clearly different. In fibroblasts, the effect was relatively slow and may be explained by the decreased activity of PFK-2, perhaps as a result of an effect on the turnover of the enzyme. In HeLa cells, a much more rapid glutamine effect was observed, which could not be explained by a stable change in the activity of PFK-2. We speculate that, in HeLa cells, glutamine caused the appearance of inhibitors or the disappearance of stimulators of PFK-2. These putative small-M_r modulators would have been lost or diluted in preparing the extracts.

Glutamine and glycolysis

A similar fall in Fru-2,6-P_2 produced by glutamine resulted in quite different effects on glycolysis in the two types of cells. This may be explained by the difference in sensitivity of HeLa-cell and fibroblast PFK-1 to Fru-2,6-P_2. Alternatively, and/or concomitantly, the fact

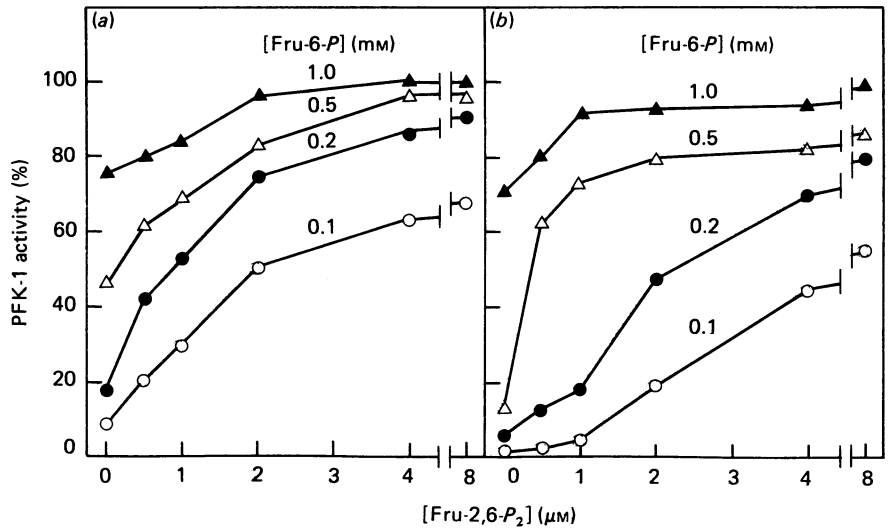

Fig. 4. Effect of Fru-2,6-P_2 on the activity of partially purified PFK-1 from HeLa cells (*a*) and chick-embryo fibroblasts (*b*)

The reaction was started by the addition of fructose 6-phosphate (Fru-6-*P*) at the indicated concentrations.

that fructose 1,6-bisphosphate, which is a stimulator of PFK-1, is 10 times as concentrated in HeLa cells as in fibroblasts (400 and 40 nmol/g respectively; L. Bosca, unpublished work) is probably relevant. Thus the actual concentration of fructose 1,6-bisphosphate in HeLa cells is much larger than the known K_a of PFK-1 for fructose 1,6-bisphosphate [well below 0.1 mM, according to Van Schaftingen *et al.* (1981), Foe *et al.* (1983) and Kitajima & Uyeda (1983)], and fructose 1,6-bisphosphate could indeed participate in the control of PFK-1 in these cells. Finally, it is not excluded that some other metabolites could interfere.

The 2-fold decrease in fructose 1,6-bisphosphate concentration caused by glutamine in HeLa cells may result either from an inhibition of its synthesis (by PFK-1), or a stimulation of its disposal (through aldolase and beyond), or a combination of both. Since the flux through PFK-1 did not seem to be affected by glutamine (Table 2), it appears that glutamine stimulated a step further down the pathway.

The dramatic fall in the concentration of 5-phosphoribosyl pyrophosphate caused by glutamine in HeLa cells probably indicates that this metabolite is effectively utilized as, e.g., in the synthesis *de novo* of purines. Glutamine is indeed an amide donor for 5-phosphoribosyl pyrophosphate in the reaction catalysed by 5-phosphoribosylamine synthase. Whether this has some bearing on the synthesis of glycogen, which is the only pathway to be changed by glutamine in these cells, is an attractive speculation and requires further experimentation. The influence of 5-phosphoribosyl pyrophosphate on the activity of PFK-2 is also worth considering. However, our preliminary results do not support this proposal.

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