Glucagon and ammonia influence the long-term regulation of phosphate-dependent glutaminase activity in primary cultures of rat hepatocytes

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1. Glutaminase activity was measured in primary cultures of hepatocytes. 2. Enzyme activity decreased markedly after 24–40 h in culture, and this loss of activity was accompanied by loss of enzyme protein. 3. The loss of activity was delayed by high concentrations of glutamine, and was abolished by the continuous presence of NH_4Cl in the culture medium. 4. In cells from rats fed on high-carbohydrate protein-free diet, glutaminase activity was increased by glucagon, but not by dexamethasone. This induction was observed only in the continuous presence of NH_3 or high concentrations of glutamine. 5. It is concluded that NH_3 and glutamine are essential for the stabilization and induction of glutaminase activity in hepatocytes. The inactivation of glutaminase in hepatocytes and *in vivo* under certain conditions may be due to lack of NH_3 in the extracellular medium.

INTRODUCTION

The mitochondrial enzyme phosphate-dependent glutaminase (EC 3.5.1.2) in rat liver is of key importance in the regulation of liver nitrogen metabolism and may also play a role in acid/base homeostasis [for reviews see McGivan *et al.* (1984), McGivan (1988) and Haussinger (1990)]. Liver glutaminase has different kinetic properties from the phosphate-dependent glutaminases which exist in kidney, brain and intestine and in ascites-tumour cells. The liver enzyme has been isolated (Heini *et al.*, 1987; Smith & Watford, 1988), and polyclonal antibodies to the enzyme protein have been prepared (Smith & Watford, 1988). These antibodies react only weakly with glutaminases from kidney and brain. Antibodies prepared to the kidney enzyme do not cross-react with liver glutaminase (Curthoys *et al.*, 1976). It has been concluded that liver glutaminase is a different protein from glutaminases which occur in other tissues, but has some epitopes in common.

Glutaminase activity is subject to both short-term and long-term control. The short-term regulation of liver glutaminase by pH and NH_3 concentration is well understood [see McGivan (1988) and Haussinger (1990) for reviews]. The enzyme has an absolute requirement for both phosphate and NH_3 as obligatory activators. Activation by NH_3 is highly pH-dependent, and it has been concluded that NH_3 rather than the NH_4^+ ion is the activating species. In isolated hepatocytes or perfused liver, glutaminase activity is stimulated by cyclic AMP, glucagon and Ca^{2+} -dependent hormones such as vasopressin. This activation is thought to be a consequence of hormone-induced mitochondrial swelling via an increase in cytosolic Ca^{2+} concentration. It has been concluded that a reversible change in association of the enzyme with the inner surface of the mitochondrial membrane on mitochondrial swelling is responsible for the changes in kinetic properties observed (McGivan *et al.*, 1985).

The long-term regulation of liver glutaminase has been less studied. The enzyme is induced *in vivo* in diabetes or after starvation (Watford *et al.*, 1984) or high-protein feeding (Watford *et al.*, 1985), and is partially repressed in low-protein feeding, but, unlike the kidney enzyme, is not induced in acidosis. Quesada *et al.* (1988) have shown that the maximum activity of glutaminase in the liver undergoes a remarkable decrease to less than half of its initial value within 24 h of implantation of ascites tumours in mice.

In order to elucidate some of the factors influencing the long-term activity of glutaminase in the liver, the regulation of glutaminase activity in primary cultures of rat hepatocytes over periods of up to 70 h has been studied. The regulation of glutaminase activity is compared with the well-characterized regulation of ornithine aminotransferase (Merrill & Pitot, 1985), another inducible mitochondrial-matrix enzyme involved in liver nitrogen metabolism.

MATERIALS AND METHODS

Materials

Collagenase was purchased from Boehringer. Cell-culture media, antibiotics and newborn-calf serum were from Gibco. Materials for Western blotting were purchased from Bio-Rad. Hormones and other biochemicals were from Sigma.

Animals and diets

Male Wistar rats (250 g) were used. Control animals had unrestricted access to a standard laboratory diet. 'Glucose-fed' rats were denied access to solid food, and the drinking water was replaced with a 20 % (w/v) solution of glucose for 3 days before use. This regime has been shown to produce large decreases in the levels of certain enzymes involved in liver nitrogen metabolism (Krebs, 1972; McGivan *et al.*, 1974).

Isolation and primary culture of hepatocytes

Hepatocytes were isolated by collagenase perfusion essentially as described by Berry & Friend (1969), except that collagenase digestion was preceded by 5 min of Ca^{2+} -free perfusion with Krebs–Henseleit bicarbonate-buffered medium plus 0.2 mM-EGTA. After this period, 1 mM- Ca^{2+} was added to the perfusate. The cells were washed and resuspended in sterile Eagle's Minimum Essential Medium containing 2 mM-glutamine, 10 % (v/v) newborn-calf serum, gentamycin (0.05 mg/ml), penicillin (50 units/ml), streptomycin (0.05 mg/ml) and amphotericin B (0.12 $\mu\text{g}/\text{ml}$). The cells were plated on Falcon Primaria tissue-culture dishes (60 mm diam.; Becton Dickinson Labware, Cowley, Oxford, U.K.) at a density of 5×10^6 cells per dish in 3 ml of medium, and allowed to adhere to the dishes for 2–4 h. The medium was then removed and replaced with 3 ml of the same medium except that the serum was omitted and 0.2 % BSA was

added together with glutamine as indicated in the legends to the Figures. The cells were incubated at 37 °C under a gas phase containing 5% CO₂. In experiments performed in bicarbonate-free medium the 25 mM-NaHCO₃ in the medium was replaced with 25 mM-sodium Hepes, pH 7.4, and the cells were incubated in air. The culture medium was changed every 24 h for fresh medium of the same initial composition as the original.

Glutaminase is severely inhibited by many detergents, which therefore could not be used in the extraction of enzyme activity. Extensive preliminary experiments (results not shown) established the following optimum extraction procedure. The culture medium was removed and the culture dishes were washed with 2 × 1 ml of cold 0.1 M-potassium phosphate, pH 8.0, to remove BSA. The cells were scraped off the dish in another 1 ml of cold phosphate, and the suspension was centrifuged at 10000 *g* for 20 s in a micro-centrifuge (MSE). The pellet was suspended in 0.1 ml of phosphate by repeated passage through a fine pipette tip and was immediately frozen in liquid N₂ until assayed. Before assay, the cell pellet was thawed, frozen and re-thawed. The protein was then resuspended as above. This suspension was used for enzyme and protein assays. For the assay of metabolites in the culture medium, a sample of the medium was frozen in liquid N₂ until required. It was then deproteinized with HClO₄ (final concn. 3%, w/v) and neutralized with a small volume of 3 M-KOH. The contents of three to five dishes were used to measure activity at each time point.

Assay of enzyme activity

Glutaminase activity was assayed essentially as described by Heini *et al.* (1987). A 0.025 ml portion of frozen and thawed cells in 0.1 M-potassium phosphate, pH 8.0, was added to 0.035 ml of a medium which contained 0.1 M-potassium phosphate, pH 8.0, 171 mM-glutamine plus 1.5 mM-NH₄Cl. These conditions are appropriate for the measurement of V_{max} for the enzyme. After incubation at 37 °C for between 10 and 30 min, the reaction was terminated by addition of 0.01 ml of trichloroacetic acid. After 15 min on ice, the protein was removed by centrifugation and NH₃ was assayed by using the *o*-phthalaldehyde-mercaptoethanol reagent (see Heini *et al.*, 1987) and measuring the A_{410} . The assay was calibrated on each occasion with standard NH₄Cl. The production of NH₃ was linear with time and with protein concentration under the conditions used. For each sample, the NH₃ present at zero time was measured by adding the frozen and thawed cell suspension to the assay medium and immediately deproteinizing the mixture and assaying as above; this was subtracted from the NH₃ present in the corresponding sample after incubation. It is important to note that, when using this procedure, the rate obtained was independent of the amount of NH₃ derived from the culture medium present in the frozen cell pellet. Ornithine aminotransferase activity was measured by using the *o*-aminobenzaldehyde reagent as described by Peraino & Pitot (1964) and taking the absorption coefficient of the product to be 2.71 M⁻¹·cm⁻¹ at 440 nm.

For the measurement of cell protein, the frozen and thawed cell suspension was dissolved in an equal volume of 2.5% (v/v) Triton X-100. Protein was measured as described by Bradford (1976), with BSA as standard in the presence of the appropriate concentration of Triton.

NH₃ (Kirsten *et al.*, 1963) and glutamine (Lund, 1974) in the culture medium were assayed by standard enzymic methods.

Antibodies and Western blotting

Rat liver glutaminase was purified as described by Smith & Watford (1988), and polyclonal antiserum to this protein was raised in rabbits. Western blotting was performed essentially as described by Towbin *et al.* (1979).

RESULTS

Glutaminase activity in cultured hepatocytes from control and glucose-fed rats

The activity of glutaminase in freshly isolated hepatocytes from normally fed rats was 87.3 ± 2.3 nmol/min per mg (mean ± S.E.M. from 10 preparations). The activity measured in freshly isolated hepatocytes was fully recovered in cells taken from culture dishes immediately after the attachment period. Fig. 1 shows the variation of glutaminase activity with time in cultured hepatocytes over a 70 h period after the first change of medium. The activity remained stable for approx. 10 h, but then sharply decreased in the interval between 15 and 30 h, reaching a new steady value at 35–40% of the original activity. This rapid decrease in activity was not observed for the enzyme ornithine aminotransferase in the same cells (results not shown). The initial activity of ornithine aminotransferase was 10.1 ± 0.8 nmol/min per mg (mean ± S.E.M., 6 preparations), and this decreased gradually to 85% of its initial value after 60 h, in accordance with previous reports (Merrill & Pitot, 1985).

The polyclonal antibody raised against rat liver glutaminase reacted with a single polypeptide band of M_r 59000 in isolated mitochondria, but cross-reacted with an additional undefined peptide in isolated hepatocytes. Fig. 2(a) shows a Western blot using the glutaminase antibody with hepatocytes which had been cultured for 0, 24 and 40 h. The upper band corresponds to glutaminase, as judged by comparison with mitochondrial extracts, where only one band is seen (results not shown). A densitometric scan of this blot is shown in Fig. 2(b). There was a marked decrease in the intensity of the M_r -59000 band representing glutaminase as a function of culture time. The intensity of the other cross-reacting peptide decreased to a much lesser extent. These results suggest that the decrease in glutaminase activity was due to a decrease in the amount of the glutaminase protein in the hepatocytes.

Table 1 shows that, in hepatocytes from rats fed on glucose for 3 days, the activity of glutaminase was decreased. The mean initial activity in such cells was 35 ± 3.0 nmol/min per mg (6 cell preparations), and this did not decrease appreciably over a 70 h culture period.

Fig. 1. Glutaminase activity as a function of time in primary cultures of hepatocytes

Hepatocytes from normally fed rats were cultured in Minimum Essential medium containing 2 mM-glutamine as described in the Materials and methods section. Each point is the mean of measurements from four culture dishes; S.E.M. values are omitted for the sake of clarity, but were always < 10% of the mean. The results are taken from ten separate cell preparations, in which the initial glutaminase activity was 87.3 ± 2.2 nmol/min per mg.

Fig. 2. Immunoblot of glutaminase in cultured hepatocytes at various times

(a) Hepatocytes from normally fed rats were cultured in Minimum Essential Medium containing 2 mM-glutamine. Samples were taken after zero time, 24 h and 40 h. Portions (0.02 mg) of cell protein were separated by SDS/PAGE and the proteins transferred to nitrocellulose. Rabbit anti-(rat liver glutaminase) antiserum was added. After incubation and washing, the antibody was located by using goat anti-rabbit IgG (peroxidase-labelled). Biotin-labelled protein standards were run on the same gel and located with peroxidase-labelled avidin. Lanes: A, protein standards; B, hepatocytes, time zero; C, 24 h; D, 40 h. The activities of glutaminase in the hepatocyte samples were 92.3, 50.2 and 33 nmol/min per mg respectively. (b) Densitometric scan of blot in (a).

Effect of high glutamine concentrations

The decrease in glutaminase activity between 15 and 30 h in control hepatocytes was further investigated. This decrease in activity was not affected by changing the culture medium; a similar decrease in activity occurred in Waymouth's medium, Ham's F12 medium and RPMI 1640 medium, each containing approx. 2 mM-glutamine. The decrease in activity was postponed, but not abolished, by increasing the glutamine content of the medium to supra-physiological values (Fig. 3). In the presence of 10 mM-glutamine, the activity was 90 % of the initial at 24 h and 62 % of the initial at 40 h, compared with values of 58 % at 24 h and 40 % after 40 h when 2 mM-glutamine was present. At

Table 1. Time course of glutaminase activity in hepatocytes from normal and glucose-fed rats

Hepatocytes were isolated from normal and glucose-fed rats and cultured in Minimum Essential Medium containing 2 mM-glutamine. The medium was changed every 24 h. The results are from one typical cell preparation from each source. Each value is the mean $\pm$ S.E.M. of the activity measured for four separate culture dishes.

Time in culture (h)	Glutaminase activity (nmol/min per mg)	
	Control cells	Cells from glucose-fed rat
0	91.4 $\pm$ 4.8	31.7 $\pm$ 3.5
19	77.6 $\pm$ 3	27.7 $\pm$ 3.1
25	54.8 $\pm$ 2	25.5 $\pm$ 3.5
31	45.7 $\pm$ 4.1	29.8 $\pm$ 2.1
40	34.7 $\pm$ 3.7	27.7 $\pm$ 3.2
50	35.2 $\pm$ 2.1	35 $\pm$ 6.1

Fig. 3. Effect of increasing glutamine concentrations on the time course of changes in glutaminase activity

Hepatocytes from normally fed rats were cultured in Minimal Essential Medium containing the initial glutamine concentrations shown. The medium was changed every 24 h. Glutaminase activity was measured after 24 h (▲), 40 h (■) and 65 h (●).

30 mM-glutamine the initial activity was retained for 24 h, and > 70 % was retained after 40 h, but in each case the activity declined to less than 40 % of the initial value after 65 h. These results suggest that high concentrations of glutamine stabilize the enzyme in some way and delay the onset of its degradation. However, the decrease in activity is not due to a lack of glutamine itself, since, under the conditions used, the hepatocytes decreased the glutamine concentrations in the medium by only 2 mM in 24 h when the initial concentration was 5 mM.

Effect of omission of bicarbonate

In order to assess the effect of pH changes on glutaminase activity in hepatocyte cultures, NaHCO_3 in the culture medium was replaced with an equal concentration of sodium Hepes in the absence of glutamine. At pH 7.4 under these conditions, glutaminase activity decreased over the first 20 h, but then stabilized at approx. 60 % of the initial value (Fig. 4). At pH 7.2, the decrease in activity was slower, and the activity stabilized at a higher value (80 % of the initial). The addition of 5 mM-

Fig. 4. Effect of bicarbonate-free media on glutaminase activity over 40 h

Hepatocytes from normally fed rats were cultured in Minimum Essential Medium in which the bicarbonate was replaced by 25 mM sodium Hepes. The medium was changed every 24 h. Glutaminase activity was measured at the times shown. All points are the mean of cells from four culture dishes. (a) pH 7.4; (b) pH 7.2. ■, No glutamine; ▲, + 5 mM-glutamine.

Table 2. Stabilization of glutaminase activity in hepatocytes cultured in the absence of bicarbonate at pH 7.4

Hepatocytes were cultured in Minimum Essential Medium in which the NaHCO_3 was replaced by an equal concentration of sodium Hepes. Glutamine was present initially at the concentrations shown. The medium was changed after 24 h. The results show the glutaminase activity after 40 h of culture. The values shown are means $\pm$ S.E.M. of activities measured in four culture dishes for each point. The initial glutaminase activity in the cell preparation was 88.4 ± 2.4 nmol/min per mg.

[Glutamine] (mM)	Glutaminase activity after 40 h (nmol/min per mg)
0	52.0 ± 3
0.2	55.5 ± 6
0.5	67.3 ± 8.7
1	77.7 ± 2.5
2	74.8 ± 2.3
5	78.1 ± 5.4

glutamine at either pH value virtually abolished the decrease in glutaminase activity. In four separate cell preparations, the activity of glutaminase in the absence of HCO_3^- but in the presence of 5 mM-glutamine was $95 \pm 4\%$ of the initial value. Under these conditions, glutamine consistently exerted a half-maximum effect at less than 1 mM; Table 2 shows a representative concentration-dependence.

In the absence of added bicarbonate, the synthesis of urea in the hepatocyte culture was much decreased, and NH_3 accumulated. Fig. 5 shows the production of NH_3 in a representative experiment. In the presence of bicarbonate, the NH_3 concentration in the medium was kept below 0.1 mM while the urea concentration as a result of metabolism of the amino acids present in the medium reached 3 mM in 24 h. At pH 7.4 in the absence of bicarbonate, NH_3 accumulated in the medium. The accumulation of NH_3 was faster at pH 7.2 as the rate of urea synthesis was further decreased. When 5 mM-glutamine was added, the rate of NH_3 production was further increased. These

Fig. 5. Accumulation of NH_3 in cell cultures

Hepatocytes were cultured in media containing: Minimal Essential medium with 5 mM-glutamine, pH 7.4 (Δ); bicarbonate-free medium with no glutamine, pH 7.4 ($\blacktriangle$); bicarbonate-free medium with no glutamine pH 7.2 ($\blacksquare$); bicarbonate-free medium with 5 mM-glutamine, pH 7.4 ($\bullet$). Samples of the culture media were deproteinized at the times shown and the neutralized extracts assayed for NH_3 as described in the Materials and methods section.

results suggest that, in the absence of bicarbonate, the accumulation of NH_3 in the medium may be responsible for the stabilization of glutaminase activity. The accumulation of NH_3 at pH 7.4 was slower than at pH 7.2, and glutaminase activity was stabilized earlier at pH 7.2. In the presence of glutamine, the NH_3 concentration increased rapidly and glutaminase activity was stable. If this is the correct interpretation of these results, it appears that a continuous concentration of NH_3 of between 0.5 and 1 mM in the culture medium may be sufficient to prevent the decrease in glutaminase activity.

It was not possible to test this hypothesis in the presence of bicarbonate, since NH_3 added to the culture medium under these conditions was rapidly converted into urea. NH_3 added at 5 mM was completely metabolized, leaving an NH_3 concentration of less than 0.1 mM after 15 h. High concentrations of NH_3 (> 10 mM) were found to impair hepatocyte viability. Addition of urease to the culture medium led to rapid production of NH_3 , but also to a drastic loss of cell viability and glutaminase activity over a 24 h period.

Hormonal induction of glutaminase activity

Several liver enzymes involved in amino acid metabolism are induced over a period of hours in isolated hepatocytes by glucagon, dexamethasone or a mixture of these hormones. Preliminary work showed that the presence of either or both of these hormones in cultures of hepatocytes from control rats had no effect on glutaminase activity over a period of 40 h in experiments similar to that in Fig. 1. Similarly, hormones added at 40 h, i.e. after the decrease in activity had occurred, did not increase glutaminase activity over the following 30 h (results not shown).

In hepatocytes from glucose-fed rats, glutaminase activity is repressed. It seemed possible that, in order to demonstrate hormonal induction, stabilization of this enzyme by NH_3 or high glutamine concentrations might be required. Table 3 shows the effect of addition of glucagon to cells cultured in Minimal Essential Medium containing 2 mM-glutamine, 30 mM-glutamine, or 5 mM-glutamine plus 2.5 mM- NH_4Cl . In normal Minimal Essential Medium containing 2 mM-glutamine, glucagon added after 16 h did not increase cell glutaminase activity measured after 40 h, even though in these same cell cultures it was found

Table 3. Conditions for the induction of glutaminase activity by glucagon in hepatocytes from glucose-fed rats

Hepatocytes from glucose-fed rats were cultured in Minimum Essential Medium containing 2 mM-glutamine, 30 mM-glutamine, or 5 mM-glutamine plus 2.5 mM-NH₄Cl. Glucagon (0.1 μ M) was added after 16 h and glutaminase activity was determined after 40 h. The activities (nmol/min per mg) shown are means $\pm$ S.E.M. of values obtained from four separate cell preparations. The activity for each cell preparation was taken as the mean of that from four separate culture dishes. Significance was assessed by a paired Student's *t* test: **P* < 0.05 versus appropriate control without glucagon. The mean activity of glutaminase at zero time was 37 ± 3.2 for the four cell preparations.

Medium	Glutaminase activity after 40 h	
	Control	+ Glucagon
2 mM-Glutamine	31.8 ± 3.6	35.5 ± 4.7
30 mM-Glutamine	28.3 ± 3.8	$42.1 \pm 1.1^*$
5 mM-Glutamine plus 2.5 mM-NH ₄ Cl	28.8 ± 6.0	$45.9 \pm 7.0^*$

that the activity of ornithine aminotransferase increased from 3.4 ± 0.4 to 8.2 ± 0.6 nmol/min per mg under these conditions. When cells were cultured in the presence of 30 mM-glutamine or of 5 mM-glutamine plus 2.5 mM-NH₄Cl, glucagon caused a significant increase in glutaminase activity after 40 h. The presence of 30 mM-glutamine or of 2.5 mM-NH₄Cl did not itself increase glutaminase activity. Hepatocytes from glucose-fed rats are known to metabolize NH₃ more slowly than hepatocytes from control rats, owing to a decreased concentration of *N*-acetylglutamate, the obligatory activator of carbamoyl phosphate synthase in these cells (McGivan *et al.*, 1976). In these experiments 0.4 mM-NH₃ was still present in the culture medium after 24 h; the NH₃ concentration in the culture medium was therefore at least as high as this throughout. Further experiments (results not shown) established that the increase in glutaminase activity reached a maximum within 16 h after the administration of glucagon.

Table 4. Induction of glutaminase and ornithine aminotransferase in the presence of NH₄⁺ ions

Hepatocytes from glucose-fed rats were cultured in a medium containing 2.5 mM-NH₄Cl plus 5 mM-glutamine. Glucagon (0.1 μ M), dexamethasone (10 μ M) or both hormones were added after 16 h and enzyme activity was measured after 40 h. The values shown are means $\pm$ S.E.M. of values from three separate cell preparations. For each preparation the activity was determined as the mean of the values from four culture dishes. Enzyme activities at zero time were: glutaminase, 40.2 ± 3.1 nmol/min per mg; ornithine aminotransferase, 2.8 ± 0.2 nmol/min per mg. Significance was assessed by a paired Student's *t* test: **P* < 0.05, ***P* < 0.01 versus control without hormones.

Additions	Activities after 40 h of:	
	Glutaminase	Ornithine aminotransferase
None	30.6 ± 4.7	2.9 ± 0.7
Dexamethasone	33.5 ± 3.6	3.4 ± 0.9
Glucagon	$44.4 \pm 3.5^{**}$	$6.47 \pm 1^*$
Glucagon + dexamethasone	$46.2 \pm 3.4^*$	$7.2 \pm 1.4^*$

Table 4 shows the effects of glucagon and dexamethasone added separately and together on the activity of glutaminase measured in 40 h cell cultures in a medium containing 2.5 mM-NH₄Cl. In accordance with data in the literature, ornithine aminotransferase activity was induced by glucagon, but not by dexamethasone, and dexamethasone did not potentiate the effect of glucagon. A similar pattern was observed with glutaminase. In this case a 50 % increase in activity over the control was produced by glucagon, but not by dexamethasone. Western-blotting experiments (results not shown) did not show a convincing increase in the cell content of glutaminase protein corresponding to the increase of activity after 40 h caused by glucagon. However, it is unlikely that the immunoblotting system is sensitive enough to detect relatively small increases in glutaminase protein in cell extracts.

DISCUSSION

The results in this paper show that, in primary cultures of hepatocytes, liver glutaminase activity increased in response to glucagon. In this respect, glutaminase closely resembles the mitochondrial-matrix enzyme ornithine aminotransferase, which undergoes induction and repression in similar conditions and with a similar time course. However, the long-term regulation of glutaminase differs from that of glutamine synthetase, which is induced in culture by growth hormone plus tri-iodothyronine rather than by glucagon (Gebhardt & Mecke, 1979). A number of other liver amino-acid-metabolizing enzymes, such as serine dehydratase, tyrosine aminotransferase, tryptophan oxygenase and phosphoenolpyruvate carboxykinase, are also induced by glucagon, and repressed by high-carbohydrate feeding (see Merrill & Pitot, 1985). However, glutaminase and ornithine aminotransferase are unusual in that induction by glucagon is not potentiated by glucocorticoids in culture. This may point to different mechanisms of induction for mitochondrial-matrix and cytosolic enzymes.

The present results indicate that the decrease in glutaminase activity in primary cultures of hepatocytes results from decreases in the amount of glutaminase protein, as detected by glutaminase-specific antiserum. In previous work, the amount of antibody-detectable glutaminase protein was found to increase in the liver of diabetic rats (Smith & Watford, 1988). It has been shown that injection of cyclic AMP into rats causes an increase in glutaminase mRNA in the liver within 5 h (Watford & Smith, 1989), and the glutaminase mRNA increases in diabetic-rat liver (Smith & Watford, 1990). These results strongly suggest that the increased activity of glutaminase observed in cell cultures in the presence of glucagon in the present investigation is due to enzyme induction rather than activation. The recent availability of a partial cDNA to the glutaminase gene (Smith & Watford, 1990; Watford & Smith, 1990) will now allow the study of the regulation of glutaminase synthesis in cell cultures at the transcriptional and translational level.

A novel and important feature of the present investigation is the finding that glutaminase is unstable in cultured hepatocytes unless NH₃ is continuously present. Under normal conditions of cell culture, the NH₃ concentration in the culture medium is kept low, owing to urea synthesis; under these conditions there was a marked decrease in glutaminase activity with time. Activity could be stabilized only by culturing cells under artificial conditions where NH₃ concentration was continuously above approx. 0.4 mM. This was done by decreasing the rate of urea synthesis either by omitting bicarbonate or by using hepatocytes from glucose-fed rats. Further, it was impossible to demonstrate glutaminase induction unless NH₃ or high levels of glutamine were present in the culture medium, although glucagon, in

addition to NH_3 , was required for glutaminase induction. Stabilization of enzyme activity by glutamine is not likely to be of physiological importance, since the glutamine concentrations required were very high.

Stabilization of glutaminase by ammonia in cell cultures probably mimics the situation *in vivo*. Glutaminase is located mainly or exclusively in the periportal cell population (Watford & Smith, 1990). These cells are exposed to the portal blood flow, which contains levels of NH_3 of the order of 1 mM as an end product of intestinal glutamine metabolism. It is likely that on low-protein/high-carbohydrate feeding less glutamine is metabolized in the intestine, and consequently there is less NH_3 in the portal blood. Under these conditions, glutaminase activity in the liver has been shown to decrease.

Although no experimental data are available, it may be speculated that the decrease in liver glutaminase activity within 24 h of transplantation of ascites tumours in mice (Quesada *et al.*, 1988) also results from decreased portal-blood NH_3 concentrations. Tumours are known to metabolize glutamine rapidly. Decreased availability of glutamine to the intestine as a result of rapid glutaminolysis by the tumour would again be expected to lead to a lower concentration of NH_3 in the portal vein and consequent repression of liver glutaminase.

Finally, the mechanism by which glutaminase activity is decreased to 40 % of its initial value in a few hours in cell culture requires investigation. Glutaminase is a mitochondrial-matrix enzyme, and degradation via a lysosomal or cytoplasmic pathway would require either the specific exit of the enzyme from the mitochondria, or intramitochondrial degradation. It is possible that the liver mitochondrial matrix contains an enzyme activity which specifically degrades glutaminase. Characterization of such an activity is presented in the accompanying paper (McGivan *et al.*, 1991).

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