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Effect of Glutamine on the Degradation of Glutamine Synthetase in Hepatoma Tissue-Culture Cells

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In certain lines of hepatoma tissue-culture cells, the extracellular glutamine concentration regulates the specific activity of glutamine synthetase. By quantifying the radioactivity in immunoprecipitated glutamine synthetase on polyacrylamide gels, we found that the rate of degradation, but not of synthesis, of glutamine synthetase is a sensitive function of extracellular glutamine. The activity that degrades this enzyme appears to be labile.

Glutamine synthetase [L-glutamate–ammonia ligase (ADP), EC 6.3.1.2] specific activity has been reported to be regulated by the extracellular concentration of glutamine in a variety of animal cell lines (DeMars, 1958; Paul & Fottrell, 1963; Kirk & Moscona, 1963; Kulka *et al.*, 1972; Tiemeier & Milman, 1972). In certain clones of hepatoma tissue-culture cells, a decrease in the glutamine concentration in the medium from 0.2mM to 2mM results in a 6–8-fold increase in the specific activity of intracellular glutamine synthetase over a 20h period (Kulka & Cohen, 1972). This induction occurs in the absence of RNA synthesis, but requires protein synthesis. Depression of the induced activity, which results from adding 2mM-glutamine to cells grown in 0.2mM-glutamine, occurs with a half-time of 3–4h. It is independent of concomitant RNA synthesis, but partially dependent on concomitant protein synthesis. Immunological evidence suggests that glutamine affects the degradation of glutamine synthetase in hepatoma tissue culture cells (Arad *et al.*, 1976). There is also immunological evidence that glutamine regulates the amount of glutamine synthetase in Chinese hamster lung cells (Milman *et al.*, 1975).

We have examined the phenomenon in hepatoma tissue culture cells by using an antibody raised against purified rat liver glutamine synthetase (Crook *et al.*, 1978). Polyacrylamide gels of anti-(glutamine synthetase) immunoprecipitates from cells incubated in medium containing [^{35}S]methionine show the presence of a variety of proteins. We have identified one band as glutamine synthetase by four criteria. (1) Only one band specifically requires anti-(glutamine synthetase); all other bands are present in an identical immunoprecipitate made without anti-(glutamine synthetase); (2) this band co-electrophoreses with pure rat liver glutamine synthetase; (3) it is the only protein that is induced by exposure of cells to glucocorticoids; and (4) it is greatly decreased in immunoprecipitates from a glutamine synthetase-deficient line of hepatoma tissue-culture cells.

By using the amount of radioactivity in the glutamine synthetase band on gels as an assay, we have studied the effect of glutamine concentration on the rates of synthesis and degradation of glutamine synthetase in hepatoma tissue-culture cells.

Materials and Methods

Chemicals

All chemicals were from Sigma (St. Louis, MO 63178, U.S.A.) or Mallinckrodt (St. Louis, MO 63160, U.S.A.) and were of the highest quality available unless otherwise specified. [^{35}S]Methionine (320Ci/mmol) was obtained from New England Nuclear (Boston, MA 02118, U.S.A.).

Media and growth of cells

The cells used in the present study are from a subclone of a hepatoma tissue-culture line derived from Morris Hepatoma 7288C. Subclone GM22 is a glutamine prototrophic subclone selected by growth in soft agar without glutamine (Kulka *et al.*, 1972). Cells were grown in spinner culture in Swim's S-77 medium as described by Peterkofsky & Tomkins (1967), except that glutamine was not included in the powder, but was added to the concentration required.

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Other procedures

The glutamine synthetase enzyme assay, immunological techniques, radioactive labelling of cellular protein and preparation of cell extracts for reaction with an antibody raised against purified rat liver glutamine synthetase, the electrophoresis of immunoprecipitates on sodium dodecyl sulphate/polyacrylamide slab gels, and the analysis of the amount of radioactivity in glutamine synthetase in the gel pattern have all been described in detail elsewhere (Crook *et al.*, 1978).

Results

Effect of glutamine on specific activity

Fig. 1 shows the dependence of glutamine synthetase specific activity on the extracellular concentration of glutamine.

The effects of several concentrations of actinomycin D on the induction of glutamine synthetase by glutamine stepdown are shown in Fig. 2(*a*). These concentrations, which inhibit total RNA synthesis by 90–97% (Peterkofsky & Tomkins, 1967), have no effect on the induction of glutamine synthetase activity. Cycloheximide (0.2mM), which inhibits protein synthesis in hepatoma tissue-culture cells by 98% (Peterkofsky & Tomkins, 1968), blocks the increase in glutamine synthetase activity almost totally. These data suggest that protein synthesis, but not RNA synthesis, is required for the induction of glutamine synthetase by glutamine stepdown.

Fig. 2(*b*) shows the effects of actinomycin D and cycloheximide on the decrease of glutamine synthetase activity that occurs when 2mM-glutamine is

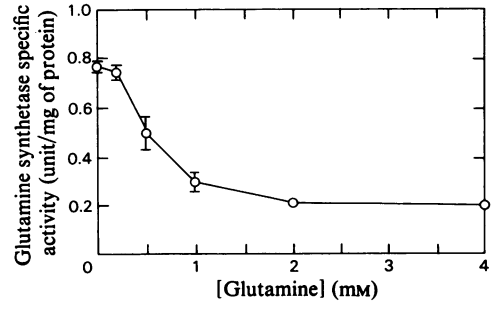

Fig. 1. *Effect of the extracellular glutamine concentration on glutamine synthetase activity*

Cells were grown in growth medium (Swim's S-77; Gibco, Grand Island, NY 14072, U.S.A.) without glutamine, supplemented with 10% (v/v) calf serum (Gibco), containing various concentrations of glutamine, for 20h. The specific activities of glutamine synthetase were then determined by the γ-glutamyltransferase assay (Crook *et al.*, 1978). One unit is defined as the amount of enzyme that produces 1μmol of γ-glutamylhydroxamate/h. Glutamine synthetase specific activity is plotted versus the glutamine concentration in the culture medium. Bars indicate the spread of duplicate samples; values without bars represent identical duplicates.

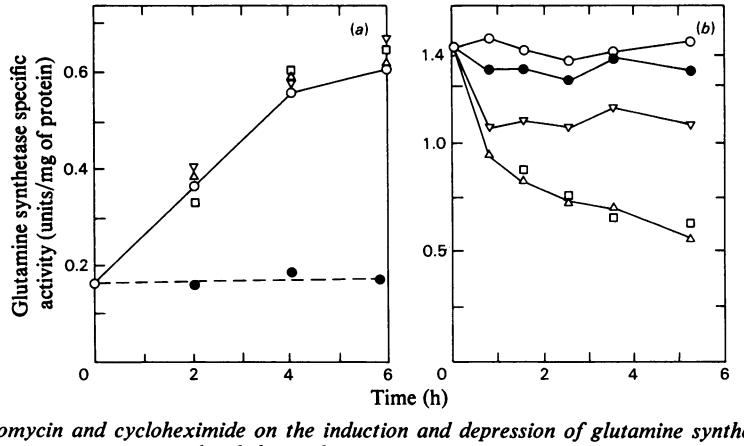

Fig. 2. *Effect of actinomycin and cycloheximide on the induction and depression of glutamine synthetase specific activity by shifts in glutamine concentration*

(*a*) Cells grown in growth medium containing 2mM-glutamine were centrifuged at 160*g* for 5min at 25°C, the supernatants decanted, and the cells pellets were resuspended in growth medium containing 0.2mM-glutamine plus: ○, no additions; △, 0.1μg of actinomycin D/ml; ▽, 0.5μg of actinomycin D/ml; □, 5μg of actinomycin D/ml; ●, 0.2mM-cycloheximide. Cells were incubated for various lengths of time, and then enzyme activities were determined. (*b*) Cells grown 2 days in 0.2mM-glutamine plus 0.1μM-dexamethasone were given: ○, no additions; ●, 0.2mM-cycloheximide; ▽, 0.2mM-cycloheximide plus 2mM-glutamine; △, 2mM-glutamine; □, 0.5μg of actinomycin D/ml plus 2mM-glutamine. After various times enzyme activities were determined.

re-added to cells grown for 20h in medium containing 0.2mM-glutamine. Again actinomycin D has no effect, whereas there is a partial inhibition of glutamine synthetase repression by cyclohexamide. These results suggest that there is no requirement for RNA synthesis, and a partial requirement for protein synthesis for the complete repression of glutamine synthetase by glutamine, and confirm previous data (Arad *et al.*, 1976).

Effect of glutamine on glutamine synthetase synthesis

The rate of synthesis of glutamine synthetase was studied in cells grown in either 0.2mM- or 2mM-glutamine. Cells were grown for 20h in the required glutamine concentrations and then incubated in medium containing 170μCi of [^{35}S]methionine/ml for various periods of time. Immunoprecipitates were made from the cell extracts, electrophoresed on sodium dodecyl sulphate/polyacrylamide gels, and the amount of radioactivity in glutamine synthetase was quantified by weighing the peak of material that corresponded to glutamine synthetase on a tracing of an X-ray film of the gel. This methodology has been described in detail elsewhere (Crook *et al.*, 1978). The results are shown in Fig. 3. The rate of synthesis of glutamine synthetase in cells grown in 0.2mM-glutamine is about 80% of that from cells grown in 2mM-glutamine. The rate of general protein synthesis in 0.2mM-glutamine-grown cells is decreased by 60% relative to those grown in 2mM-glutamine; however, glutamine synthetase synthesis appears to be partially independent of this general effect.

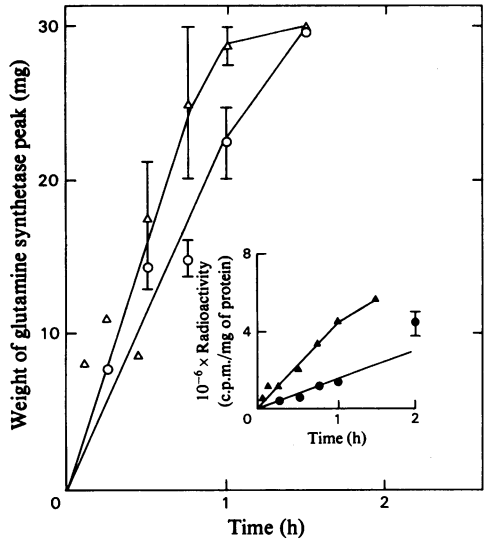

Fig. 3. *Rate of synthesis of glutamine synthetase in cells grown in* 0.2*mM- and* 2*mM-glutamine*

Cells were grown for 20h in growth medium containing 0.1μM-dexamethasone and either (○, ●) 0.2mM-glutamine or (△, ▲) 2mM-glutamine. They were then labelled with 170μCi of [^{35}S]methionine/ml for various lengths of time. Immunoprecipitates of the labelled cell extracts were prepared and analysed on polyacrylamide gels as previously reported (Crook *et al.*, 1978). The amount of radioactivity in glutamine synthetase is expressed as the weight of the glutamine synthetase peak on a tracing of X-ray film exposed to the gel, normalized to a standard amount of extract protein added to the gels (25μg). Values without bars are those of identical duplicates. Inset: the rate of total protein synthesis in these cultures. Samples (2μl) were taken from the extracts and analysed for incorporation of radioactivity into trichloroacetic acid-precipitable material as previously described (Crook *et al.*, 1978).

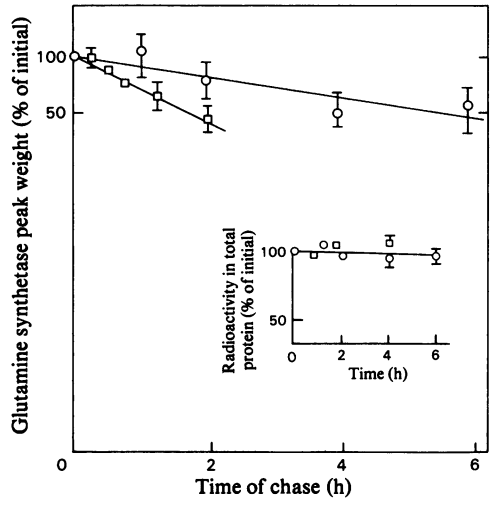

Fig. 4. *Rate of glutamine synthetase degradation in cells grown in* 2*mM- and* 0.2*mM-glutamine*

Cells grown in growth medium containing 0.1μM-dexamethasone, and either 0.2mM-(○) or 2mM-(□) glutamine, were incubated in 170μCi of [^{35}S]-methionine/ml for 30min, then washed and resuspended in growth medium containing either 2mM- or 0.2mM-glutamine, for the indicated lengths of time. Immunoprecipitates were prepared and analysed as in Fig. 3. Inset: samples (2μl) were analysed for the rate of radioactivity incorporation into total protein (symbols as in Fig. 3). Glutamine synthetase specific activity from unlabelled samples with 0.2mM-glutamine is 21 ± 2units/mg of protein and with 2mM-glutamine is 5 ± 1units/mg of protein.

Effect of glutamine on glutamine synthetase degradation

The rate of degradation of glutamine synthetase was measured as a function of the concentration of glutamine in the culture medium. Cells grown in monolayers in 0.2mM- or 2mM-glutamine were incubated in medium containing [^{35}S]methionine for 35min. Then they were washed in unlabelled medium, which was removed by suction, resuspended in this medium and incubated for various lengths of time before harvesting. Immunoprecipitates were prepared and analysed as above. Fig. 4 shows the rate of degradation of glutamine synthetase from cells grown in 0.2mM-glutamine. Glutamine synthetase from cells grown in 0.2mM-glutamine has a half-life of 6–8h, whereas glutamine synthetase from cells grown in 2mM-glutamine decays with a half-life of 1.75h. The ratio of these half-lives is roughly that of the specific activities of glutamine synthetase from the two sets of cultures (see the legend to Fig. 4).

To characterize further the dependence of the rate of glutamine synthetase degradation on the glutamine concentration, cultures of cells grown for 20h at different concentrations of glutamine were pulsed with 170μCi of [^{35}S]methionine/ml for 35min, and then washed and incubated in unlabelled medium containing the same glutamine concentrations, for 3h. Fig. 5 shows that the radioactivity in glutamine synthetase disappears as a function of the glutamine concentration in the cell medium. Also glutamine synthetase specific activity varies as the rate of glutamine synthetase degradation. The inset shows the rate of synthesis of glutamine synthetase at 35min incubation as a function of glutamine concentration. Again the decrease in rate of synthesis at lower glutamine concentrations is apparent.

Kinetics of the shift in degradation rate

Our data show that glutamine synthetase turns over with a half-time of 6–8h in cells grown in 0.2mM-glutamine, and with a half-time of 1.75h in cells grown in 2mM-glutamine. We next studied the kinetics of the shift of degradation rates that occurs when cells grown in medium containing 0.2mM-glutamine are transferred to 2mM-glutamine. Cells grown in 0.2mM-glutamine were incubated in medium containing [^{35}S]methionine for 30min, then washed and transferred to unlabelled medium containing 2mM-glutamine. The radioactivity in the glutamine synthetase population was then followed as a function of time. Fig. 6 shows that a degradation rate characteristic of cells grown at the high concentration of glutamine ($t_{\frac{1}{2}} = 1.5$h) is established after about 40min. Glutamine synthetase specific activity, shown on the upper part of Fig. 6, falls with a slightly longer half-time (1.9h), as would be expected if the specific activity measured at any time is a function of the rate of synthesis of glutamine

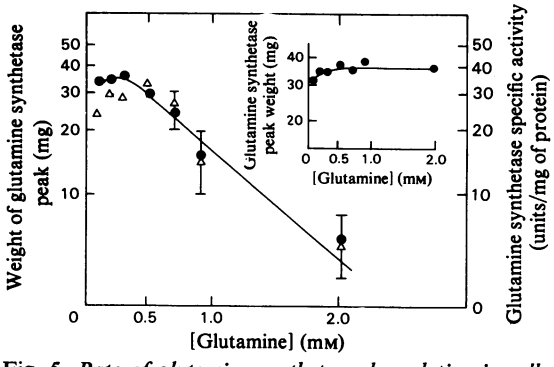

Fig. 5. *Rate of glutamine synthetase degradation in cells grown in various concentrations of glutamine*
Cells were grown in growth medium containing 0.1μM-dexamethasone and various concentrations of glutamine for 12h. They were then incubated in [^{35}S]methionine for 35min and chased for 3h as in Fig. 4, or not 'chased' (see the inset). Immunoprecipitates were prepared and analysed as in Fig. 3. ●, Weight of glutamine synthetase peak; △, glutamine synthetase specific activity. Inset: rate of glutamine synthetase synthesis at various concentrations of glutamine.

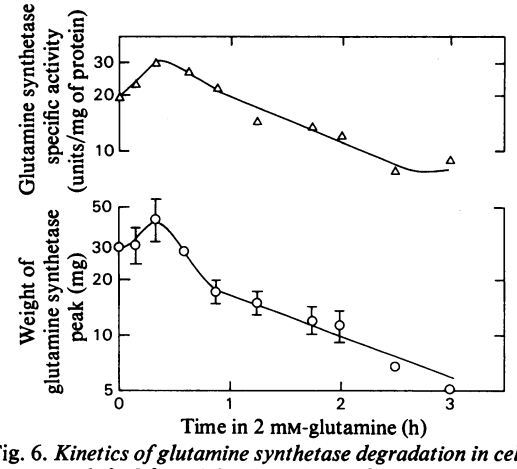

Fig. 6. *Kinetics of glutamine synthetase degradation in cells shifted from* 0.2*mM- to* 2*mM-glutamine*
Cells grown for 20h in growth medium containing 0.1μM-dexamethasone and 0.2mM-glutamine were incubated in [^{35}S]methionine for 10min. They were then washed and resuspended for the indicated times in growth medium containing 0.1μM-dexamethasone and 2mM-glutamine. Immunoprecipitates were prepared from cell extracts and analysed as in Fig. 3, and glutamine synthetase activity was determined on the same extracts. ○, Glutamine synthetase peak weight; △, glutamine synthetase specific activity.

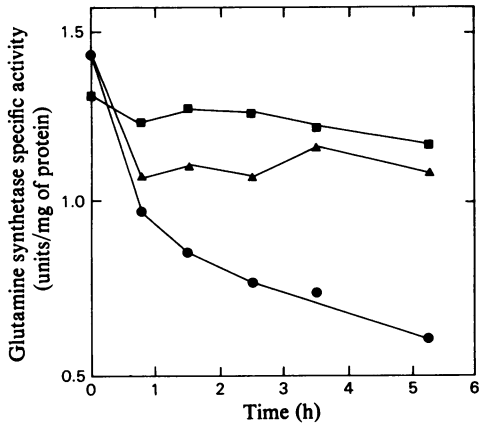

Fig. 7. *Effect of cycloheximide preincubation on repression of glutamine synthetase by glutamine*

Cells grown in growth medium containing 0.2mM-glutamine were split into two sets of cultures. To the first was added 0.2mM-cycloheximide; no additions were made to the other. At 2h, the set of cultures without cycloheximide was split. To one set was added 2mM-glutamine (●) and 0.2mM-cycloheximide plus 2mM-glutamine (▲). To the preincubated set of cultures was added 2mM-glutamine (■). Enzyme activity was determined after various times.

synthetase molecules as well as of the rate of degradation.

Lability of glutamine synthetase-repressing activity

Glutamine appears to influence the rate of glutamine synthetase degradation, but does not affect the rate of glutamine synthetase synthesis. We further characterized this process. The effect of cycloheximide on the repression of glutamine synthetase by glutamine (Fig. 2*b*) raised the possibility that an activity, which brings about the degradation of glutamine synthetase, itself requires concomitant protein synthesis for the maintenance of its activity. This suggested that the preincubation of induced cells with cycloheximide for several hours before addition of 2mM-glutamine might eliminate the glutamine synthetase-repressing activity. Accordingly, cells that had been grown in medium containing 0.2mM-glutamine were exposed to 0.2mM-cycloheximide for 2h; they were then exposed to 2mM-glutamine. Cultures not preincubated with cycloheximide were also given combinations of cycloheximide and 2mM-glutamine as controls. The results (Fig. 7) indicate that glutamine synthetase repression in preincubated cultures is completely eliminated for 2.5h, then glutamine synthetase activity does decrease. This result supports the view that glutamine synthetase is degraded by an activity that requires concomitant protein synthesis for its maintenance, and that, in the absence of protein synthesis, the degrading activity disappears in 1h (Fig. 2*b*).

Discussion

The present results suggest that the effect of glutamine on the specific activity of glutamine synthetase is achieved through modulation of the rate of degradation of glutamine synthetase rather than by synthesis. This effect is not reflected in the degradation of general cellular protein, which proceeds with an average half-time of greater than 10h in cells grown in either 0.2mM- or 2mM-glutamine. These data are consistent with those of Hershko & Tomkins (1971), who found that general protein turnover proceeds at a rate of 3% in cells grown in complete medium, and 5% in cells grown in nutritionally deprived media.

Data of Arad *et al.* (1976) indicate more than one antigenic form of glutamine synthetase in hepatoma tissue-culture cells. We found this only when glutamine synthetase is precipitated from cell extracts with anti-(glutamine synthetase) in the absence of anti-(immunoglobulin G). The inclusion of anti-(immunoglobulin G) in the reaction mixture results in equivalence curves for glutamine synthetase from cells grown in 0.2mM- or 2mM-glutamine, with or without dexamethasone, which are identical (R. B. Crook, unpublished work; Crook *et al.*, 1978).

The mechanism of degradation of glutamine synthetase alters both the catalytic activity and antigenic activity of glutamine synthetase (Crook *et al.*, 1978, and Fig. 6). The identity of the process, however, is unknown. It could involve a covalent modification of glutamine synthetase, such as occurs in prokaryotes (Stadtman & Ginsburg, 1974). However, the fact that the reaction of anti-(glutamine synthetase) with glutamine synthetase does not alter glutamine synthetase catalytic activity (Crook *et al.*, 1978) suggests that the primary antigenic determinants of this enzyme are not located at the active site. Thus such a proposed covalent modification would have to affect two separate sites on the enzyme.

An alternative mechanism, which to us seems more compatible with the data, is proteolytic cleavage of glutamine synthetase, followed by hydrolysis to amino acids. An analogous activity has been ascribed to a proteinase in liver, which specifically degrades pyridoxal-requiring enzymes when they are not bound by pyriodoxal (Kominami *et al.*, 1972).

At present, the mechanism of glutamine synthetase degradation, and the molecular role of glutamine in the process, are unknown. For the purpose of discussion, we shall refer to the degrading

activity as glutamine synthetase-inactivating factor.

The obliteration of glutamine synthetase-inactivating faction by cycloheximide (Fig. 6) suggests that it turns over rapidly. However, other models could explain the data. For example, an activator of the inactivating factor may be turning over rapidly. Such cascade effects are involved in the control of bacterial glutamine synthetase (Stadtman & Ginsburg, 1974). Alternatively, the pool of a molecule that modifies the inactivator, such as aminoacyl-tRNA, might change with the blockage of protein synthesis by cycloheximide, thus modifying the rate of glutamine synthetase degradation. Such a tRNA has been implicated in the regulation of the histidine operon in *Salmonella* (Singer *et al.*, 1972).

The control of glutamine synthetase would appear to function as follows. Under conditions of glutamine deprivation, the cell's glutamine concentration is raised by increasing specific activity of glutamine synthetase. This would be difficult via the mechanism of increasing synthesis of the enzyme polypeptide, since the effect of glutamine starvation is to slow cellular protein synthesis (Fig. 3, inset). Decreasing the rate of degradation of glutamine synthetase, however, requires no additional protein synthesis. Thus the concentration of glutamine synthetase molecules is increased, which promotes an increase in the concentration of intracellular glutamine.

Although G. M. T. died before this work was completed, his ideas and encouragement contributed overwhelmingly to the direction and development of this project. I thank Keith Yamamoto, Bob Ivarie, Mike Stallcup, David Martin and Jim Spudich for careful readings of the manuscript. This work was done while R. B. C. was a student of G. M. T. It was supported by Grant no. GM 17239 from the National Institute of General Medical Sciences.

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