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Regulation of glutamine synthetase by glutamine and steroids in rat hepatoma cells

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REGULATION OF GLUTAMINE SYNTHETASE BY GLUTAMINE AND STEROIDS IN RAT HEPATOMA CELLS

by

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DISSERTATION

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for my parents, Valerie and Richard Crook

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It was my honor and priviledge to be a student of Gordon Tomkins'. Gordon died last year, a profound loss on many levels to many people, but the greatest loss, next to that of those who loved him, undoubtedly belongs to those who never got the chance to meet him. Gordon was the most brilliant scientist I have ever met. I think he was a genius; a student can hardly ask more. But he was blessed with a profusion of other talents, foremost among them being the ability to make other people feel that their own gifts, which they were perhaps not aware of, had in fact been lying just below the surface, and now were coming, glinting and shimmering, to light. Gordon was a splendid human being; I will never forget him.

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ABSTRACT

Glutamine synthetase (GS) was studied in a line of Hepatoma Tissue Culture cells called GM22. GS specific activity is induced three-fold by 10^{-7} M dexamethasone (dex). This induction requires RNA and protein synthesis. Deinduction caused by dex removal requires RNA synthesis. Immunological studies, using an antibody prepared against purified rat liver GS, indicate that dex causes a three-fold increase in the rate of GS synthesis, as measured by analyzing SDS acrylimide gels of immunoprecipitates from cells incubated with $[^{35}S]$ methionine; while dex has no effect on the rate of GS degradation. The dex-induced rate of GS synthesis is established with a half-time of two to three hours. The decrease in the rate of synthesis, which occurs when dex is washed out of induced cells, is prevented by addition of 0.5 µg/ml actinomycin D.

Reduction of the extracellular glutamine concentration from 2 mM to 0.2 mM causes a six to eight-fold increase in GS specific activity. This increase requires protein synthesis but not RNA synthesis. Re-addition of 2 mM glutamine to cells induced for GS by growth in 0.2 mM glutamine causes a fall in GS activity with a half-time of three hours. This fall does not require RNA synthesis but has a partial requirement for protein synthesis. Immunological experiments of the kind described above reveal that the glutamine concentration determines the rate of GS degradation, which is inversely proportional to the GS specific activity, while having a minor effect on the rate of GS synthesis.

Immunological evidence is presented which suggests the existence of a second form of GS. A variety of experiments fail to support this finding, however.

The frequency of appearance of the gln^+ phenotype from a cloned line of HTC cells, called ST3, which are glutamine auxotrophs, is shown to be 3×10^{-5}. This frequency is not enhanced by treatment of ST3 with nitrosoguanidine or ethyl methane sulfonate, whereas the frequency of 6-thioguanine resistant clones is.

INTRODUCTION

In 1971, work in this laboratory was largely focussed on the biology of the steroid induction of tyrosine aminotransferase in hepatoma tissue culture cells. As a side light, it appeared to be an interesting puzzle that the cells were glutamine auxotrophs. Rat liver, from which these cells were derived, is an organ rich in the enzyme responsible for glutamine production, glutamine synthetase. Hepatoma tissue culture cells, on the other hand, possess barely detectable amounts of this activity. The question arose as to why they had lost glutamine synthetase activity. Had the loss occurred irreversibly, for example by chromosomal exclusion, or was the genetic information for glutamine synthetase present but unexpressed? In light of the fact that most hepatomas contain only trace amounts of glutamine synthetase activity, we thought the phenomenon might be related to the process of malignant transformation.

Plating cells in soft agar containing growth medium without glutamine resulted in roughly 1% of the cells surviving. Of those survivors which were checked, all had clearly detectable glutamine synthetase activity. Therefore, it seemed that in the process of becoming a neoplastic line, hepatoma tissue culture cells had in some reversible way suppressed the expression of this enzyme.

At this juncture, the finding took on a new significance when it was realized that in several eukaryotic systems glutamine synthetase had been reported to be inducible either by glutamine or steroids, or

both. The idea of having several functions under the control of steroids in the same cell line had seemed attractive for some time. Investgation of a second inducible function, surface factor, which promotes cell adhesion to glass, had allowed inferences to be drawn concerning the uniqueness of the steroid receptor in hepatoma tissue culture cells. Unfortunately, the usefulness of the function is limited by the fact that its chemical nature is not known.

It was further imagined that the existence of several inducible functions would be valuable in the eventual use of in vitro protein synthesizing systems and cell-cell hybridization experiments.

For these reasons, a study of the regulation of glutamine synthetase in hepatoma tissue culture cells by glutamine and steroids was undertaken. This thesis presents the results of the study in Chapters 2 through 6. In Chapter 7, some work directed at understanding the interesting puzzle of the glutamine auxotrophy to prototrophy transition in these cells is presented.

CHAPTER ONE

REVIEW OF THE LITERATURE

In 1933, H. A. Krebs noted that glutamic acid stimulated greater oxygen uptake into kidney slices than any other amino acid of the L-series (1). Yet unlike any other α-amino acid, it actually reduced ammonia formation. The observation prompted him to study the relationship between glutamate and ammonia and led to the first description of glutamine synthetase activity in 1935 (2). His work showed that this formation of amide nitrogen was energy dependent, and could be detected in retina, brain, and liver in a variety of animals.

Studies by Elliot (3) and Speck (4) demonstrated that ATP could be utilized as the energy source for the conversion. Speck first defined the Mg^{2+} dependent reaction: (5)

$$\text{L-glutamate} + NH_3 + \text{ATP} \xrightarrow{Mg^{2+}} \text{L-glutamine} + \text{ADP} + \text{Pi} \quad (1).$$

Elliot extended this observation to preparations from sheep brain, bacteria, and plants (6).

In 1954, Levintow and Meister demonstrated that the reaction catalysed by purified pea glutamine synthetase is reversible, with an equilibrium constant of 1.2×10^{-3} (pH 7.0, $37^{\circ}C$)(7). From this value it was possible to obtain the estimate of -8,000 calories for the free energy of ATP hydrolysis.

Today, glutamine synthetase is known to figure centrally in nitrogen metabolism. The amide nitrogen of glutamine is used in the synthe-

sis of tryptophan, histidine, and p-amino benzoate; carbamyl phosphate, purines and pyrimidines; and glucosamine-6-phosphate. Through transamination reactions, it is involved in the formation of alanine and glycine. Furthermore, the amide nitride nitrogen serves as a principal source of the α-amino group for the synthesis of all amino acids in ammonia restricted prokaryotes. When the concentration of ammonia in the medium is less than 1 mM, its incorporation in glutamate also requires glutamate synthase (8), as follows:

$$\text{glutamate} + NH_3 + ATP \longrightarrow \text{glutamine} + ADP + Pi \quad (1)$$

$$\text{glutamine} + \alpha\text{-ketoglutarate} + NADPH + H^+ \longrightarrow 2\ \text{glutamate} + NADP^+ \quad (2)$$

When the concentration of ammonia is greater than 1 mM, incorporation of ammonia into glutamate proceeds by the reaction catalyzed by glutamate dehydrogenase, without consumption of ATP:

$$\alpha\text{-ketoglutarate} + NH_3 + NADP + H^+ \longrightarrow \text{glutamate} + NADP^+ + H_2O.$$

The amide of glutamate is then transferred, by the appropriate transaminases, to the α-keto acid of the amino acids.

Clearly, the regulation of the ammonia assimilation into glutamine by glutamine synthetase bears directly on the many diverse and independent metabolic pathways utilizing glutamine. Because it links pathways of amino acid biosynthesis, nucleic acid biosynthesis, complex polysaccharide formation, and oxidative metabolism, glutamine synthetase has become a growing subject of cell regulation research.

Glutamine Synthetase Reaction Mechanism

The early work of Speck and Elliott established that free intermediates of the reaction could be detected (3-5, 9,10). In experiments

with N-acetyl-γ-glutamyl phosphate and ammonia, glutamine was produced. It was not clear whether this was enzymic. Further, the tendency of γ-glutamyl phosphates spontaneously to cyclize to pyrrolidone carboxylic acid limited the usefulness of this approach (11).

Meister and his colleagues have done essentially all the work from this point on the reaction mechanism of eukaryotic glutamine synthetase (GS). The enzyme purified from sheep brain has been demonstrated (12) to catalyse four other reactions besides the one described by Speck:

$$\text{glutamate} + \text{ATP} + \text{hydroxylamine} \rightarrow \gamma\text{-glutamyl-hydroxymate} + \text{ADP} + \text{Pi} \quad (2)$$

$$\text{glutamine} + \text{hydroxylamine} \xrightarrow[\text{Pi or As}]{\text{ADP or ATP}} \gamma\text{-glutamyl-hydroxymate} + NH_3 \quad (3)$$

$$\text{glutamine} + H_2O \xrightarrow[\text{As}]{\text{ADP}} \text{glutamate} + NH_3 \quad (4)$$

$$\text{glutamic acid} + \text{ATP} \rightarrow \text{pyrrolidone carboxylate} + \text{ADP} + \text{Pi} \quad (5)$$

Meister's work supports the idea of a phosphorylated intermediate bound to the enzyme. The evidence flows from essentially four kinds of experiments. (1) In pea GS, both optical isomers of glutamic acid are substrates. D and L γ-glutamyl-hydroxymate are made at the same rate, whereas D-glutamine is made at a much slower rate than L-glutamine. This suggests that an activation step of low optical specificity is followed by a more specific reaction with ammonia which becomes rate-limiting in the case of D-glutamate. Such a limitation does not occur with hydroxylamine, which is known to react non-emzymatically with acyl phosphates, thiol esters, and certain other compounds (13).

(2) If a small amount of isotopically labelled glutamate is allowed to react with GS in the presence of ATP and Mg^{++}, and then hydroxylamine and a large excess of unlabelled glutamic acid are added, one sees a preferential conversion to labelled γ-glutamyl-hydroxymate. This suggests that no equilibration occurs between labelled and unlabelled glutamate during the course of the reaction, implying that glutamic acid is bound to the enzyme (14).

(3) Sedimentation and ultrafiltration studies demonstrate that glutamate is bound to GS, and that both ATP and Pi are required, for this binding to occur (14).

(4) β -glutamate, which has the amide group on the β-carbon of the glutamyl carbon skeleton, is a substrate for GS. The acyl phosphate is far more stable than its α-counterpart, and had been shown to produce ATP when added to the enzyme (15).

The general scheme for the reaction, then, is thought to include (1) a binding site on the enzyme incomplete for glutamate, requiring ATP to complete the site; and (2) an enzyme-bound γ-glutamyl phosphate intermediate. Thus, ATP and Mg^{++} bind to the enzyme, followed by glutamate. An activated intermediate is formed which is then attacked by ammonia to give glutamine, ADP and Pi which are then released from the enzyme.

Further work with substrate analogues and model building by Meister and his co-workers have led to the postulate that L-glutamate is attached to the active site of glutamine synthetase in an extended conformation in which the carbonyl groups of L-glutamate are as far apart as possible, and the α-hydrogen atom is directed away from the enzyme (12). The failure of L-aspartate to attach to the enzyme indicates that the dis-

tance between the substrate carboxyl atoms must be substantially greater than about 4 Å.

Structural Comparison of Prokaryotic and Eukaryotic Glutamine Synthetases

Although all species are essentially identical in reaction mechanism and substrate specificity (16), the prokaryotic and eukaryotic enzymes are fundamentally different in subunit structure. Bacterial glutamine synthetase is approximately 600,000 MW consisting of twelve 50,000 subunits; whereas the eukaryotic enzyme is approximately 350,000 MW consisting of eight subunits (Table I).

In electron micrographs, the E. coli molecule is seen as a pair of facing hexagons (17). The molecule from Chinese Hamster (18), pig brain (19), and chick retina (20) appears as a pair of facing tetramers.

The enzyme's amino acid composition from representative species reinforces this difference and is presented in Table II. All mammalian enzymes look very similar. If it is true that there is a difference between the glutamate compositions of rat liver and rat brain, this suggests an organ specific variation in primary structure within species. Consistent with its evolutionary remoteness, the pea enzyme differs signigicantly from the mammalian enzymes. Specific aromatic differences tend to balance out, but it possesses nearly twice as much leucine, 50% more valine, and 40% less glycine, rendering it somewhat more hydrophobic. It contains 50% less methionine and only 17% the cysteine residues. In this last respect, pea GS is closer the E. coli enzyme. The prokayotic enzyme is also distinguished by a greater threonine and histidine content.

Regulation of Bacterial Glutamine Synthetase

In bacteria, GS is regulated in several ways: feedback inhibition and activation by small molecules; adenylylation and deadenylylation of the enzyme, resulting in modification of specific activity; and regulation of the transcription of GS.

1. Small Molecule Modification. In E coli, activity is inhibited in vivo and in vitro by CTP, AMP, glucosamine -6-P; histidine, tryptophan, carbamyl-phosphate, alanine, glycine, and serine (28,29). The amide nitrogen of glutamine is utilized directly in synthesizing all of these molecules except alanine, glycine and serine, and these are indirectly derived from glutamine via glutamate synthase and various transaminases.

These inhibitions constitute a feedback regulation system in which each end product of a highly branched metabolic pathway inhibits the first common enzyme in the pathway.

At physiological concentrations, these small molecules each partially inhibit GS (28). They appear to act independently of one another, as judged by the fact that the fractional activity obtained with any combination of two or more inhibitors is approximately equal to the product of the fractional inhibition of each inhibitor measured independently. Although the evidence is in some cases equivocal, it is felt that there are separate sites for at least most of the small molecules.

Much has been made of the "taut" and "relaxed" conformations of GS which are a function of the concentration of Mg^{2+} or Mn^{2+} (21,30,31). Essentially, with little or no Mg^{2+} present, GS is in one conformation and inactive, while with Mg^{2+} present GS is in another conformation and active. It therefore has been proposed that GS is "regulated" by Mg^{2+}.

However, it seems unremarkable that the components of the reaction as defined by Speck must be present in order for the reaction to occur.

2. Reversible Adenynylation of Glutamine Synthethase. Studies from the laboratories of Holzer (32,33) and Stadtman (34,35) have shown that the ability of glutamine synthetase to catalyze the formation of glutamine can be decreased by the covalent attachment of adenylyl groups to the enzyme, and increased by the removal of these groups. A single enzyme, adenylyltransferase (designated ATase; 130,000 MW), catalyzes both the addition of AMP using ATP as the substrate, and its removal (34,36,37). The site is a specific tyrosyl residue on each of the twelve subunits (38). The number of sites per oligomer adeylylated at one time can range from zero to twelve (34). It is a sensitive function of the metabolic environment and can be approached from either a fully adenylylated or a fully deadenylylated population.

The reaction which ATase catalyzed is governed by a regulatory protein, called P_{11} (50,000 MW dimer)(39,40). In its native state, P (designated P_{11A}) combines with Atase to adenylylate glutamine synthetase and reduce its activity. However, P_{11} is itself subject to modification by the covalent addition of UMP. In that form, P_{11} (designated P_{11D}) combines with ATase to catalyze the deadenylylation of glutamine synthetase (41,42,43).

The addition of UMP to P_{11} is catalyzed by uridylyltransferase (designated UR enzyme)(42,44). The two have not yet been separated and may constitute a multi-enzyme complex.

UTase is, in turn, activated by ATP, magnesium or manganese, and α-ketotoglutarate (45). The last accumulates when ammonia is limiting and subsequently stimulates the formation of the deadenylylated enzyme,

the ammonia scavenger the cell requires. UTase is inhibited by glutamine and inorganic phosphate, both enzyme products, which inhibit the formation of deadenylylated enzyme, and themselves. The UR enzyme has a specific requirement for manganese, which reflects the specific requirement of the adenylylated enzyme, are not formed.

Finally, to amplify these metabolic signals, the "cascade" system is sensitive to the same compounds at the next stage--adenylylation and deadenylylation (45). ATP and α-ketoglutarate inhibit the adenylylation reaction and stimulate the deadenylylation directly. In addition, UTP, the initial substrate in the "cascade" leading to deadenylylated enzyme and maximal glutamine synthetase activity, has been reported to inhibit the adenylylation reaction directly.

3. Repression and Derepression of Glutamine Synthetase Synthesis. The rate of synthesis of glutamine synthetase in bacteria is controlled by the nitrogen source of the growth medium: it is high in media whose nitrogen source is growth limiting and low in media containing an excess of ammonia (32,46,67).

This phenomenon has been investigated in Klebsiella aerogenes, where Magasanik and his colleagues have mapped by P·1 transduction four genes which influence the activity of GS (47-51). They and their functions are:

gln A - structural gene for GS
gln B - structural gene for P_{II}
gln D - structural gene for UTase
gln E - structural gene for ATase

Gln A is assumed to code for the GS subunit because (1) mutants in gln A display a loss of GS specific activity; some show cross-reacting material, whereas others do not; and (2) the concentration of GS molecules

(catalytically active or inactive) is proportional *in vivo* to the level of RNA specific for the gln A gene (51). The functions of gln B, D, and E were deduced by complementation of mutant extracts with G-200-separated wild type UTase, ATase, and P_{II} activities (52).

Mutants in gln B and gln D show constitutively low specific activities of glutamine synthetase due to an inability to deadenylylate the enzyme (52). Mutants in gln E, on the other hand, show constitutively high specific activities of glutamine synthetase, due to a modified ATase which cannot adelylylate the enzyme. Gln B and D mutants can be suppressed by a mutant in gln E. Further experiments have shown that:

(1) gln B or D mutants causing a constitutively low specific activity of GS, contain proportionately low levels of CRM as well (51).

(2) gln E mutants which display constitutively high specific activities of GS, contain proportionately high levels of CRM (51).

(3) gln A - specific RNA is reduced in gln B mutants (51,52).

(4) Some mutants in gln A, the structural gene for the GS, can suppress gln B or D mutations. That is, an alteration in the GS polypeptide results in the resumption of normal levels of gln A transcription. Gln A wild-type is dominant to this gln A mutant merodiploids (47,112).

These results have led to the hypothesis that the adenylylated form of glutamine synthetase acts as inhibitor of the transcription of GS.

As another mode of transcriptional regulation, glutamine synthetase has been suggested to be under cAMP control (53). In an adenyl cyclase mutant of *E. coli*, the addition of 5 mM cAMP results in a two-fold increase in the specific activity of GS in cells grown in either high or low levels of ammonia. The fact that chloramphenicol blocks this increase is taken to suggest that the effect is on synthesis of the GS polypeptide (53). In light of the complexity of the cascade control of

GS however, it seems possible that the block of protein synthesis might exert an effect at any of several other sites. In any case it seems likely that at some level the production of active glutamine synthetase is controlled by cAMP. The effect requires active cAMP binding protein, since this effect does not occur in a mutant lacking cAMP binding protein (53). An effect of cAMP also can be seen, in the same mutants on the other enzymes of glutamine metabolism: glutamate synthase, glutamate dehydrogenase, and glutaminase A. The effect is positive for glutamate dehydrogenase and negative for glutamate synthase and glutaminase A. Once again, the complexity of the GS control network, and the effect of GS on other enzyme systems to be discussed below make it difficult to determine whether the cAMP effects on these enzymes is direct or indirect.

Regulation of Other Enzymes by Glutamine Synthetase

1. Glutamate dehydrogenase. The gln C^- mutants are gln B^- mutants with a second mutation, thought to be in gln A, leading to constitutively high production of GS molecules (54). These were found to lack glutamic dehydrogenase (GDH) regardless of the nitrogen source. Examination of the wild type cell showed that GDH activity is high when its GS is repressed by high ammonia levels, and almost devoid of GDH when its GS is derepressed by growth in an ammonia-limiting medium. Repression of GDH does not require GS to be catalytically active: the GDH level is low in a glutamine requiring mutant derepressed for the synthesis of GS cross reacting material (54). Moreover, a high level of GDH is found in a strain which probably causes a deletion in gln A. Whether the adenylylated or the unadenylylated of glutamine synthetase is responsible for the repression of GDH is not known. Under conditions

where the repression is observed, the non-adenylylated form predominates.

2. Tryptophan Permease. Gln C^- cells, grown on glucose-ammonia, excrete indolepyruvate when tryptophan is added to the medium (47). The product polymerizes to give a brown product, and so can be visualized in agar. When gln A^- cells are grown on excess ammonia together with glucose and tryptophan, they fail to produce indolepyruvate, in spite of their enzymatic ability to transaminate tryptophan to α-ketoglutarate. This transaminating activity is present in gln A^+, gln A^- and gln C^- cells, irrespective of nitrogen or tryptophan concentration in the medium. On the other hand, gln C^- cells, and gln A^+ cells grown on a limiting nitrogen source both have the ability to produce indolepyruvate when exposed to glucose and tryptophan. From these data, the conclusion has been tentatively drawn that the production of indolepyruvate appears to require a transaminase, present in cells regardless of the level of GS, and a permease present in amount sufficient for rapid tryptophan entry, only in cells with a derepressed level of GS. The regulation of this permease appears not to require GS enzymatic activity, since indolepyruvate is produced in a mutant containing enzymatically inactive, immunologically reactive GS. In the wild-type gln A^+ strain, the activity of the permease depends entirely on the nitrogen source in the medium.

3. Histidine Degrading Enzymes. The histidine utilization pathway consists of four enzymes capable of sequentially degrading histidine to glutamate, ammonia, and formimide (55). Klebsiella aerogenes can produce histidase, the first enzyme in the pathway, when grown in glucose and histidine without ammonia, but not in glucose, histidine and ammonia.

Further experiments demonstrated that in glucose grown cells, the level of histidase is high whenever the level of GS is high. Mutants unable to produce GS have low levels of histidase even when grown in limiting amounts (56). Mutants constitutive for GS synthesis (eg. gln C^-) have high levels of histidase even when grown with an excess of ammonia. In the GS mutant which constitutively produces enzymatically inactive, immunologically active GS, the levels of histidase are high, even in cells grown in excess ammonia. In this same mutant containing an E. coli episome containing wild-type gln A, repression of GS CRM is normal, and histidase control is normal. Thus GS is thought to be involved in the regulation of histidase in a manner which does not involve its catalytic activity (56).

Further experiments were performed using phages λ and φ80, into which the hut-specific DNA of Salmonella typhimurium had been introduced (57,58). These λphut and φ81 dhut phages contain little bacterial DNA other than the hut genes; consequently RNA complementary to hut DNA could be measured. In an in vitro system, Smith and Magasanik (59) could show that φ81dhut RNA hybridizing to the correct (H) strand of φ81hut DNA is stimulated by highly purified non-adenylylated GS isolated from K. aerogenes. Highly adenylylated GS fails to stimulate. In these experiments, the concentration of GS and the molar ratio of GS to hut DNA did not exceed that found in intact cells. GS did not stimulate the transcription of λDNA, or gal DNA. Transcription of hut DNA from a mutant defective in hut P, the promoter on one of the two S. typhimurium hut operons, was only slightly stimulated by GS (59).

At this point, it is not known whether GS binds to DNA polymerase, or to DNA, although the latter would seem more likely, due to the specificity of the effect.

GS appears to play a major role as a regulatory protein in nitrogen metabolism. In an ammonia rich environment, GS in its adenylylated form appears to repress its own synthesis. In an ammonia-poor environment, it represses glutamate dehydrogenase. Furthermore, it then activates the synthesis of at least three systems of enzymes potentially capable of providing the cell with glutamate by the degradation of tryptophan, histidine, and proline. There is some evidence that in K. pneumoniae, GS also is involved in the regulation of nitrogenase synthesis (60).

Eukaryotic Glutamine Synthetase

In the study of GS of eukaryotes progress has been much slower. In eukaryotic organisms, particularly mammals, the function and possibly the regulation of an enzyme may vary from organ to organ. Besides its metabolic function of synthesizing glutamine, it has been suggested that GS is involved in pH regulation in the kidney, ammonia detoxification in the liver, and neurotransmitter metabolism through association with glutamate and γ-aminobutyrate metabolism in the brain. Progress in the study of chromosomal regulation of GS has been limited by the current level of knowledge of the structure and function of the eukaryotic chromosome. Finally, the lack of manipulatable segregation and recombination in most eukaryotic cells have so far prevented a genetic approach of the kind which has proved so powerful in prokaryotes.

Three major types of regulation of GS have been studied in eukaryotes: direct metabolite inhibition and activation, induction by glutamine, and induction by steroid hormones.

1. Metabolite Inhibition and Activation. Early reports of cumulative feedback inhibition of GS in prokaryotes prompted a search for such a phenomenon in eukaryotic systems. Nothing that elaborate has been observed, but it has been reported that GS is responsive to certain cellular metabolites in vitro (18,16). The effectors vary with the species and cell type or organ, but include α-ketoglutarate and citrate (stimulants) and glycine, alanine, serine, carbamyl phosphate, and AMP (inhibitors). Meister and his co-workers have suggested that the physiological function of the α-ketoglutarate activation, which is as great as two-fold, is to convert to glutamine excess α-ketoglutarate formed by the citric acid cycle and transamination of glutamate and glutamine. It is noteworthy, however, that the concentrations at which these metabolites were reported to be effective are high: α-ketoglutarate, citrate and glycine, serine and alanine all are 20 mM, and carbamyl phosphate is 10 mM (26). Similar concentrations were reported to be effective by Temeier and Milman (18). Moreover, it has been argued that the effects of α-ketoglutarate and citrate on the mammalian enzyme are due to their metal chelation rather than direct allosteric binding. At low metal ion concentration their effect is reversed and they act as inhibitors (61).

2. Regulation of Glutamine Synthetase by Glutamine. In 1958, Demars first reported that the specific activity of GS in Hela cells was 15-fold higher in cells grown without glutamine than cells grown in 2 mM glutamine (62). Paul and Fottrell (63) described the kinetics of the

same phenomenon in L cells, showing that the increase in specific activity which occurs upon deleting glutamine from the medium is achieved in about two days, whereas the reduction of activity which results from re-addition of glutamine was complete in about 16 hours. Since reducing nucleotides and a nitrogen gas atmosphere inhibit the decrease, they suggested inactivation of the enzyme by oxidation as the mechanism of decrease in activity.

Two other labs reported similar results with L cells. Barnes et al. found enzyme activity was depressed after 12 hours in glutamine although only a two-fold difference between induced and depressed levels was noted (64). Stamatiadou in 1972 reported a six-fold stimulation of GS activity by glutamine removal (65). The depression by glutamine, complete within two hours, required neither RNA nor protein synthesis. Induction was found to require protein synthesis but not RNA synthesis. The experiments reported in these papers generally contain only a few data points, taken at relatively long times, so the kinetics of the responses are unclear. However, the phenomenon of glutamine control GS activity appears to be common to Hela and L cells. In much more convincing detail, Temeier and Milman in 1972 reported that GS in Chinese Hamster lung cells is induced eight to ten fold in 48 hours following removal of glutamine (66). Repression is achieved in eight hours, with a half-life of twelve minutes. Once again, repression does not require RNA or protein synthesis, and induction does not require RNA synthesis. The decrease in specific activity is directly proportional to the glutamine concentration for concentrations less than 0.3 mM. On the basis of these experiments they suggest a glutamine-induced modification of GS leading to less activity when glutamine is present. Subsequent work

from Milman's lab has shown that the amount of immunologically reactive GS is a function of glutamine concentration, and that the specific activity of GS is a reflection of the amount of material reacting with antibody specific for pure Chinese Hamster liver GS (67). These results are taken to suggest that GS is degraded or altered to some non-cross-reacting form in the presence of glutamine.

Kulka and his co-workers have investigated GS control by glutamine in hepatoma tissue culture cells. Their results with inhibitors essentially parallel Milman's except that a partial requirement for protein synthesis in repression of GS is noted in this system (68).

In subsequent work from Kulka's lab, compounds structurally related to glutamine were investigated for their ability to mimic or antagonize the effect of glutamine (69). Of the compounds tested, 6-diazo-5-oxo-L-norleucine (DON), like glutamine, depressed the activity of GS. L-methionine sulfone, albizziin, L-methionine sulfoxide, L-glutmyl-γ-hydrozide, and γ-N-methyl-L-glutamine (in order of decreasing potency) were antagonists which prevented the effect of glutamine of GS activity. These antagonists had little effect on glutamine transport or protein synthesis in HTC cells, and their effects were reversible. When the effects of these compounds on GS activity in cell-free extracts of HTC cells were examined, it was found that albizziin inhibited neither the transferase nor the synthetase activity of GS. This observation was taken to mean that the glutamine binding site involved in the regulation of GS is not the active site of the enzyme.

3. Regulation of Glutamine Synthetase by Steroids. The neural retina of the chick embryo is the system most extensively characterized with respect to steroid regulation of GS. At one to two weeks of age,

the eye is a relatively prominent structure in the embryo. The retina itself is easily accessible within this structure and can be isolated by simple dissection. An avascular sheet of approximately 10^8 cells (3 mg protein), it is uncomplicated by the presence of blood, vascular cells, or connective tissue elements. The isolated retina can be cultured up to a week with little qualitative difference in RNA or protein synthesis (70).

GS follows a characteristic developmental pattern in the embryonic retina. During the initial two weeks of life, enzyme activity increases at a slow rate, at the end of which time cell proliferation ceases. From the 16th to the 21st day, GS activity increases one hundred fold to the high activity characteristic of the adult retina (70). At three weeks of age, the retina is functionally mature. This rapid enzyme activity increase corresponds temporally and topographically to the retina's final morphological differentiation (71).

Though enzyme induction does not occur until the sixteenth day, the embryo is competent for induction as early as the seventh day. Adult serum can trigger premature induction in vivo or in cultured retinas (73). Calf serum is most effective, although sera from chicken, horse, rat, and man also work.

In 1966, Reif and Amos (74) and Moscona and Piddington (75) demonstrated that 11-β-hydroxycorticosteroids at physiological concentrations are excellent inducers of GS in chick retina. Investigations of steroid structure indicate that the 11-hydroxyl and the keto α,β-unsaturated (Δ^4, 3 keto) grouping in the A ring are required for induction. Non-metabolized flourine-substituted steroids such a dexamethasone, flumethasone, 9-α-flourohydrocortisone, and triamcinilone, are active at con-

centrations one order of magnitude below hydrocortisone. The active dialyzable component of serum has been identified as corticosteroid (76). The similarity is kinetics and extent of precocious induction to the normal developmental pattern of GS increase is taken as strong evidence that the normal in vivo inducer is steroid: but the assumption remains unproven.

A glucocorticoid-specific receptor in the chick embryonic retina is detectable as early as the sixth day in ovo, which is consistent with the ability to precociously induce GS (71). The receptor binds natural (cortisol) and synthetic (dexamethasone) inducers as well as progesterone, which is not an inducer. Its molecular weight by sucrose density sedimentation is approximately 200,000. The receptor-steroid complex migrates to the nucleus, where binding occurs and presumably the activation of GS-specific sequences in the chromosome (77,78).

The steroid induction of GS in the retina is inhibited by actinomycin D, suggesting that RNA synthesis is required (79). However, actinomycin D can enhance steroid induction of GS under some condition (79,80). This is reminiscent of "superinduction" of steroid induced tyrosine aminotransferase by actinomycin D in HTC cells (81). The inhibition of GS induction by Cordycepin is taken to indicate the requirement of RNA transport (82). But in light of the nonspecific nature of the drug's effects, this conclusion seems premature.

Steroid induction is inhibited by cycloheximide or purmycin, suggesting new protein synthesis is required (79). That this synthesis is of new enzyme molecules is suggested by the increase of material cross-reacting with a GS-specific antibody that accompanies steroid induction (83). Moreover, Schwartz has reported the isolation of a RNA

fraction (450,000MW) coding for GS from a polysome profile, and he has demonstrated by enzyme assay and antibody precipitation in vitro GS synthesis relative to control retinal RNA. In the presence of steroid, the ability to translate GS molecules increases. Cultures incubated in steroid plus cycloheximide and switched to steroid plus actinomycin D are able to induce GS (84). However, the extent of incubation in cultures treated with actinomycin D plus steroid after hour hours of steroid alone depends upon the actinomycin D concentration. In the range .05 to 0.2 µg/ml actinomycin D blocks 70-90% of the control induction. However, 10 µg/ml actinomycin D has no inhibitory effect on GS induction. Removal of steroid after four hours ultimately has the same effect as continued incubation in steroid plus low actinomycin D concentrations: enzyme activity levels off at a fraction of the activity in control cultures.

The model proposed to account for these data include a "suppressor" and a "desuppressor" of enzyme synthesis (85,92). Both would be transcriptionally controlled but the production of the latter is assumed to be more sensitive to inhibition by actinomycin D and depend on the presence of steroid. At a high enough antibiotic concentration, neither would be made and the increase in translation of GS which occurs with addition of steroid would occur in its absence. Upon removal of steroid, the rapidly turned over "desuppressor" would be lost and further enzyme synthesis would be blocked. The "suppressor" might be an mRNA nuclease or a regulatory RNA species blocking processing or translation of the message.

The model of Moscona is essentially identical to the model proposed by Tomkins et al. for steroid control of tyrosine aminotransferase in HTC cells (81).

Two other reports of regulation by steroids of GS have been made, but they are, on the whole, preliminary. Barnes et al. reported a three-fold increase in GS activity in L cells grown in either the presence or absence of glutamine when 10^{-5}M cortisol was added to the growth medium (64). Other steroids were tested with corticosterone being the most effective: a ten-fold induction of GS was observed using this steroid. In developing rat liver, Wu has presented evidence for the precocious induction of GS by the injection of hydrocortisone prior to the normally scheduled developmental rise in GS activity which occurs on the 14th day of post natal development (86).

Table 1. Comparison of oligomer and subunit molecular weight and subunit number of glutamine synthetases from prokaryotes and eukaryotes.

Table 1

	Molecular Weight	Number of Subunits	Subunit Molecular Weight
Prokaryotes			
E. coli (21)	592,000	12	49,000
B. subtilis (22)	600,000	12	50,000
Eukaryotes			
Yeast (23)	394,000	8	45,500
Pea (24)	360,000	8	45,500
Sheep Brain (25)	392,000	8	49,000
Rat Liver (26)	352,000	8	44,000
Chinese Hamster Liver (20)	336,000	8	42,000
Chicken Retina (20)	340,000	8	42,000

Table 2. Amino acid composition of several eukaryotic and prokaryotic glutamine synthetases. (27).

Table 2

Amino Acid Composition of Mammalian Liver and Brain and of Pea and *E. coli* Glutamine Synthetase (27).

Amino Acid	Moles of Amino Acid/45,000 g Enzyme						
	Rat Liver	Rat Brain	Ovine Brain	Human Brain	Pig Brain	Pea	50,000 g *E. coli*
Lye	25	26	25	22	25	26	24
His	13	13	11	12	11	8	24
Arg	21	23	25	26	23	19	18
Asp	48	47	41	44	44	42	43
Thr	20	20	20	20	20	23	37
Ser	24	21	25	25	20	26	26
Glu	33	41	44	46	47	41	38
Pro	23	29	25	22	24	19	23
Gly	42	41	41	43	39	26	35
Ala	28	28	29	30	30	29	42
½ Cys	12		12			2	4
Val	20	18	18	22	18	27	28
Met	12	13	11	11	13	6	15
Ile	23	22	21	21	19	23	24
Leu	25	25	22	27	19	40	31
Tyr	17	16	16	16	16	14	15
Phe	21	23	21	20	20	28	21
Thy	6		7			6	4

CHAPTER TWO

MATERIALS AND METHODS

Chemicals. All chemicals were from Sigma or Mallinckrodt unless otherwise specified. $[^{3}H]$ leucine (53 Ci/mmol) and $[^{35}S]$ methionine (320 Ci/mmol) were from New England Nuclear.

Media and Growth of cells. The cells used in this study are subclones of a hepatoma tissue culture line derived from Morris Hepatoma 7288C. GM22 is a glutamine prototrophic subclone selected by growth in soft agar without glutamine (87). ST3 is a glutamine auxotrophic subclone, picked under non-selective conditions from soft agar. Cells were grown in spinner and monolayer as previously described (88,89), except that Growth Medium was also made lacking L-glutamine. Glutamine was then added to the concentration desired.

Preparation of Cell Extracts. 20 mls of 5×10^{5} cells per ml were centrifuged at 160 xg for 5 minutes, the supernatant decanted and the pellet resuspended by gentle shaking. The cells were then washed once in .15 M NaCl and resuspended in 1 ml of the same. The cells were sonicated with a Bronwill Biosonik Sonicator set at 20% output, for two 10 second bursts at $4^{\circ}C$. The sonicated cells were then centrifuged at 160 xg for 5 minutes at $4^{\circ}C$, and the supernatant saved. This solution is used for enzyme assays. More concentrated extracts, if required, were made by resuspending more cells in 1 ml of .15 M NaCl.

Glutamine Synthetase Activity Assay. Glutamine synthetase was measured by the γ-glutamyl transferase assay (87). A one ml reaction mix contained 120 mM L-glutamine, 40 mM imidazole-HCl, 10 mM Na_2AsO_4, 50 mM ATP, 0.5 mM $MnCl_2$, 30 mM NH_2OH, final pH 6.0 and up to 500 µg of the extract. The reaction ingredients were made up in two solutions: I contained glutamine, imidazole-HCl, $NaAsO_4$, and ATP; and II contained $MnCl_2$ and NH_2OH. These were stored at 4°C, and were stable for up to six months. The enzyme reaction was allowed to proceed for up to 75 min., when it was stopped with .75 mls of Stop Mix. Stop Mix contains .31 M trichloroacetic acid, .8 M HCl, and .063 M $FeCl_3$. Denatured protein was removed by centrifugation at 550 xg for 2 minutes in a Sorvall CW-1 centrifuge. The absorbance at 500 nm of the supernatant was then measured in a Zeiss spectrophotometer and compared to a γ-glutamyl hydroxamate curve. One unit of GS activity is defined as the amount of enzyme which produces 1 µmol of γ-glutamyl hydroxamate per hour. GS specific catalytic activity is expressed as units per milligram of protein. Protein was determined according to the method of Lowry et al. (90) using bovine serum albumin (Sigma) as a standard.

Immunological Techniques. Rabbit antiserum raised against purified rat liver GS was prepared by Dr. Thomas Deuel. This antiserum was purified by ammonium sulfate precipitaiton and DEAE cellulose chromatography.

Sheep antiserum to rabbit IgG was a gift from Dr. Patricia Jones. This antiserum was purified by a 30-40% ammonium sulfate fractionation followed by dialysis overnight against several liters of 10 mM NaH_2PO_4/ Na_2PO_4 and 50 mM NaCl pH 7.5.

Antibody-Antigen Reaction. Precipitin tests were performed by adding increasing volumes of the sheep anti-rabbit IgG to a fixed concentration of rat IgG in a 1 ml reaction mixture at .15 M NaCl. The solution was allowed to incubate overnight. The next day the precipitate formed was washed three times in .15 M NaCl, dissolved in .5 ml 0.1 M NaOH, and a sample read at 280 nm in a Zeiss spectrophotometer.

Equivalence Curves. Extracts from 1 liter of cells were prepared as described above, with a subsequent centrifugation at 30,000 xg for 10 minutes. The supernatant was retained and used in the assays described here. Equivalence titrations were performed in two ways. (1) To a fixed volume of .15 M NaCl containing a fixed concentration of GS antiserum, increasing amounts of cell extract were added. The final protein concentration was adjusted to a value between 5 and 8 mg with bovine serum albumin. The reaction was allowed to incubate for 2 hours, at which time a fixed amount of IgG antiserum, usually two-fold in excess of the equivalence point of the GS antiserum previously, was added. The total volume was 1 ml. The reaction was then allowed to incubate overnight at $4^{o}C$. The next day the pellet was sedimented by centrifugation at 30,000 xg for 10 minutes, and the supernatant saved and assayed for GS activity. (2) The same protocol was followed, except that an increasing amount of cell extract was added to a fixed amount of GS antiserum.

DEAE Cellulose Chromotography. 4 ml DEAE cellulose columns were prepared in 6 ml disposable plastic syringes. Extracts equilibrated in .01 M tris (hydroxymethyl) aminomethane, pH 8.0 (at $4^{o}C$). Extracts taken through the 30,000 xg sedimentation step were layered on the column, no

more than 1 mg of protein per ml of bed volume. After being run into the column and allowed to sit for ten minutes, 1 ml fractions were collected with an Isco fraction collector as the resin was washed with a 0-.5 M NaCl gradient, which was linear and 50 mls.

Isoelectric Focussing. A 10-60% glycerol gradient containing .08% ampholines (LKB) distributed envenly throughout, was poured into a 1.9 x 10 cm (internal diameter) glass column with a dialysis membrane bound by rubber bands at the lower end. 1-2 mg of extract (purified through the 30,000 xg centrifugation step) was mixed into the chamber of the gradient maker containing the 60% glycerol (Mallinckrodt) when the gradient was about to be poured. This initial distribution of the extract minimizes the denaturation which occurs at the upper end of the column, which is initially acidic. The upper electrode (anode) solution is 1% (w/v) H_3PO_4, and the lower (cathode) solution is 2% ethanolamine (Mallinckrodt). The gradient was exposed to 250 volts from an Isco power supply for 20 hours. Under these conditions, the pH gradient takes about 10 hours to form. Focussing of various standards as well as GS was complete by 20 hours. Fractions were collected from the lower end of the column by puncturing the dialysis membrane with a syringe needle fixed in a small cap of rubber tubing which covers the end of the column.

Sucrose Gradient Density Sedimentation. 5-20% sucrose gradients were made according to Yamamoto (91) in .01 M tris buffer, pH 7.5. Alkaline phosphatase (Sigma, 6.2S), and catalase (Sigma, 11.3S) were routinely included as internal markers. 1 mg of extract was layered on top of the gradient, and then the gradients were centrifuged for 5 hours in

a Beckman Model L2-65B preparative ultracentrifege, using a SW 50.1 rotor at 50,000 rpm. Drops were collected from the lower end of the tube by a Buchler Piercing Unit, with positive pressure applied to the top of the gradient with a Gilson Minipuls II motor. Fractions were assayed for GS activity. Alkaline phosphatase activity was measured by the method of Yamamoto (91); hemoglobin was measured by absorbance at 540 nm; and catalase was measured by absorbance at 600 nm.

Thermal Inactivation of GS. Extracts were prepared through the 30,000 xg step. All concentrations were equalized by dilution with .15 M NaCl. Then to each sample was added 5 mM ATP, 50 mM glutamic acid, 5 mM $MnCl_2$, and 50 mM NH_4Cl. These compounds stabilize GS to heat inactivation (data not shown). The samples were then placed in a water bath of the desired temperature, for the desired time, then placed in an ice bath to cool. The denatured protein was sedimented by a 30,000 xf centrifugation for 10 minutes, and the supernatant was assayed for GS activity. In a reconstruction-type experiment in which the precipitate was not removed but resuspended, no additional GS activity could be detected compared to the control with the precipitate removed.

General Labelling of Cell Proteins. 10 μCi of [^{3}H-] leucine was placed in a 50 ml plastic centrifuge tube. 10 mls of suspended cells were added (final isotope concentraiton = 1 μCi/ml) and allowed to shake in a 37°C water bath for 15 minutes. Incorporation was linear during this time (data not shown). The incubation was stopped by adding cold PBS to 50 mls, then the cells were centrifuged at 160 xg for 5 minutes at 4°C, in an International Centrifuge model PR-J. decanted, and the washing repeated. The supernatant was discarded, and cells were lysed

with 1 ml of 0.5 % NP40 (Shell) in PBS. Protein was precipitated with 1 ml of 10% TCA, then the suspension was poured over a 2.4 cm glass fiber filter (Whatman), washed 3 times with 5% TCA, dried, dissolved in "½ strength NCS", and counted in a Beckman Model Ls-233 scintillation counter. Counting efficiency was 40%. "½ strength NCS" is: 100 ml NCS, 15.2 gm omniflour, 3790 ml toluene, and 16 ml distilled H_2O.

Labelling of Protein For SDS Gels. 5×10^6 cells were plated on a 60 x 15 mm tissue culture dish (Corning). After an hour the medium was removed and fresh medium was added and the cells were allowed to grow overnight. The next morning the cells were again refed. 1-2 hours later, the medium was removed and 1.3 mls of Labelling Medium with glutamine added as desired was added to each dish. Labelling Medium is Growth Medium without glutamine or methionine, made up from a 10x concentrated stock without serum (Pacific Biological). 1.3 mls was just enough to cover the bottom of the dish. After 5 minutes, 0.2 mls of 1.3 mCi/ ml $[^{35}S]$ -L-methionine, made in labelling medium, was added to the dish, and the dish was returned to a 37°C warm room. The incubation was stopped by adding 5 mls of ice cold PBS. Then the PBS was siphoned off and 5 mls of STV (saline, trypsin, and versine) added to remove the cells from the dish bottom. After 5-10 minutes the unattached cells were poured into a 50 ml plastic disposable centrifuge tube, diluted to 50 mls with .15 M NaCl, and centrifuged for 50 minutes at 160 xg. This wash was repeated once, and then the cell pellet was transferred to a 100 µl plastic conical tube (Analytical Aids), centrifuged again, and all the supernatant except approximately 50 µl was removed. The cells were disrupted by two rounds of freezing in liquid N_2, followed by thawing in a 37°C water bath. After the first freeze, pellets may be

stored at -60°C for up to two weeks without loss of GS activity. Extracts were then centrifuged at 30,000 xg for 10 minutes to remove disrupted membranes, nuclei, and other large structures, and the supernatant removed with a Clinac Micropipetter holding a 50 µl glass tip and transferred to a disposable 10 x 75 mm glass test tube. Extracts were all adjusted to 50 µl with .15 M NaCl.

To the extracts were added 42.2 µl of a solution containing equal parts of 0.1 M ATP, pH 7.0, 0.1 M $MnCl_2$, 1 M glutamate, and 1 M NH_4Cl. Final concentration to 50 µl is necessary because GS stability at 64°C required the addition of the four reagents just mentioned. However, they also prevent the formation of the GS-anti-GS complex. This inhibition is reversed with a 20-fold dilution. The extracts are brought to room temperature, then sealed with parafilm and heated in a water bath set at 64°C for 5 minutes. This step results in a 3-4 fold decrease in protein concentration with no loss of GS activity. The tubes were placed in an ice bath to cool; then the samples were transferred to 12 x 75 mm polystyrene test tubes and centrifuged at 30,000 xg for 10 minutes to remove denatured protein. The supernatant was recovered, transferred to 5 ml Pyrex conical glass tubes, and diluted with cold .15 M NaCl to .9 mls. A 50 µl sample was taken for protein determination, and the GS activity of nonlabelled samples prepared at the same time was determined. From these values, the amount of GS antiserum required to be 1.5-2.0 x in excess of the GS activity was determined and added to all labelled samples.

Preparation of [^{35}S] methionine-labelled Samples for SDS Gels. Immunoprecipitates formed during the overnight incubation at 4°C were washed

in .15 M NaCl and centrifuged 3 times at 990 xg for 20 minutes, and finally washed in glass distilled water by centrifugation at 2,100 xg for 10 minutes. The pellet was then resuspended in 30-50 µl of sample buffer (10% (w/v) glycerol, 5% (v/v) B-mercaptoethanol, 2.3% (w/v) SDS, and .0625 M tris-HCL, pH 6.8). This solution was either stored immediately at -60°C, or heated in boiling water for 1-2 minutes to dissaggregate proteins. 20-40 µl containing no more than 40 µg of protein were applied to each of the gel wells. Protein determinations of the extract in sample buffer were made using the method of Heil and Zilling (92).

SDS Acrylimide Slab Gels. Discontinuous SDS slab gels were made, run, and exposed to X-ray film according to the method of Laemmli (93), as adapted to a slab gel system by O'Farrell (94), except that the stacking gel was poured 1½ inches high. This was done so that the teeth of the plastic comb used to form the wells in the stacking gel, and which were ½ inch high, would be 1 inch from the top of the running gel. Gels were destained and dried under vacuum according to O'Farrell (94).

Quantitation of Gel Bands. The optical density of the image on X-ray film increases linearly with respect to radioactivity from .5 to 3.2 (94). All data reported here were either on the linear portion of the curve, or were corrected to linearity using the curve.

The film developed after exposure to the dried gel were cut into 2-3 sections between gel columns on the film in order to fit onto the sample tray of a Joyce-Loebel Microdensitometer, Films were scanned using the appropriate wedge. The peak of a tracing on paper corresponding to the band in question was then cut out and weighed.

Samples were standardized to the protein concentration of the sample before adding the antisera. Cpm in total protein was not used as a standard because many of the experiments involve varying pulse or chase times, in which case radioactivity in general protein is a variable. Unequal incorporation of label into samples therefore is a possibility. It was corrected for by doing each point in duplicate or triplicate, and each experiment at least two or three times.

CHAPTER THREE

RESULTS

In GM22 cells, GS specific activity is increased by addition of 10^{-7} M dexamethasone (dex) or reduction of the extracellular glutamine concentration from 2 mM to 0.2 mM (Fig. 1). The dex induction of GS takes about 10-15 hours to achieve a 2.5-3 fold increase. The glutamine induction requires 15-20 hours to reach a 6-8 fold increase in specific activity. The two effects are multiplicative; together the effect is a 15-20 fold increase in GS activity.

INDUCTION BY DEXAMETHASONE

The effect of inhibitors of RNA and protein synthesis on the dex induction of GS was investigated. 2×10^{-5} M cycloheximide, a concentration which inhibits protein synthesis in HTC cells by greater than 99% (95,68), totally prevents the increase in GS mediated by dex (Fig. 2). This result suggests that protein synthesis is required for the increase in GS activity.

0.1 µg/ml actinomycin D (AMD) inhibits the dex-dependent rise in GS activity as well (Fig. 2), suggesting that RNA synthesis is also required for the dex induction of GS.

Moscona and his colleagues (79,80,96) reported that at high concentrations of AMD, GS is induced in chick embryo neural retina in the presence or absence of cortisol. With this result in mind, several concentrations of AMD were tested. Table 3 shows that concentrations

from 0.1 to 5.0 µg/ml had no significantly enhancing effects on the dex induction.

The dose-response curve for dex induction of GS is shown in Figure 3. The half-maximal concentration of dex is about 5×10^{-8}M. This curve is identical to that of TAT in these cells, suggesting that the steroid receptor implicated in the induction of TAT is the same molecule as that involved in GS induction.

When dex is washed out of GM22 cells, GS activity falls to the basal level (Fig. 4a). The kinetics of this phenomenon are somewhat variable, but the half-time is approximately 4-6 hours. Such variability in the deinduction kinetics of TAT is thought to be due to an effect of serum (97).

The dependence on RNA synthesis of dex deinduction was next investigated. At the time of removal of dex, one set of cultures was given 0.1 µg/ml AMD, while the other received no additions. The results, shown in Figure 4b, indicate that GS activity falls in the absence of AMD but remains at a higher level in the presence of AMD. This suggests that RNA synthesis is required for the full deinduction of GS activity by removal of dex. These observations parallel those made on TAT in HTC cells (98).

INDUCTION BY GLUTAMINE

The activity of GS is a function of the extracellular glutamine concentration (Figure 5).

Actinomycin D at a variety of concentrations has no effect on the glutamine induction of GS (Figure 6). This suggests that, unlike dex, glutamine does not require RNA synthesis to bring about an increase in

GS activity. In some experiments of GS induction by glutamine stepdown, a 10-29% increase in GS activity is seen in the presence of AMD.

2×10^{-5}M cycloheximide completely inhibits the rise in GS activity (Fig. 6), suggesting that protein synthesis is required for a rise in GS activity due to glutamine to occur.

If 2 mM glutamine is added to cells grown for 12 hours on 0.2 mM glutamine, the GS activity falls with a half-time of 3 hours (Fig. 7). This suggested the possibility that an activity responsible for GS inactivation disappeared after an hour in cycloheximide. To test this idea, cells grown in 0.2 mM glutamine were exposed to cycloheximide two hours before addition of 2 mM glutamine. Figure 8b shows that glutamine inactivation of GS activity is inhibited 90% by this procedure, suggesting that the activity involved in decreasing the activity of GS has been largely removed by this treatment.

Since the inhibition of protein synthesis by cycloheximide results in a partial inhibition of the repression of GS activity by glutamine, and since glutamine stepdown likely causes a decrease in protein synthesis, the rate of protein synthesis in cells grown at various concentrations of glutamine was investigated. Figure 9 shows that the rate of protein synthesis is a sensitive function of glutamine concentration, and that GS specific activity varies inversely as the rate of protein synthesis.

This result raised the possibility that GS repression is a function of the rate of protein synthesis rather than specifically the concentration of glutamine. Accordingly, the following experiment was done. Cells grown in 2 mM glutamine were washed and resuspended in Growth Medium containing either 0.25 mM leucine and 2 mM glutamine, no leucine

and 2 mM glutamine, or 0.5 mM leucine and 0.2 mM glutamine. To avoid the contribution of the leucine in serum, this experiment was performed in serum-free medium. The results are shown in Figure 10.

The inhibitor experiments suggest that RNA synthesis is not required for the inactivation of GS by glutamine. Using enucleated GM22 cells, it was possible to ask whether processing and transport of previously synthesized RNA is required. Cells induced for GS by growth in 0.2 mM glutamine were enucleated by Dr. Warner Fan as reported (99). After enucleation, one set of cultures was given 2 mM glutamine, while the control culture was not. Specific activities of GS were followed over 6 hours. The results (Fig. 11) show that repression by 2 mM glutamine proceeds for about four hours with kinetics slower than in nucleated cells, followed by a cessation of repression. Given the complexity of the phenomenon of enucleation, it is difficult to interpret the plateau after four hours in 2 mM glutamine. However, it is clear that for four hours repression can proceed in the absence of RNA synthesis, processing and transport.

DISCUSSION

The results with inhibitors suggest that both RNA and protein synthesis are required for the increase in GS specific activity due to dex addition. The experiments do not bear on the identity of the RNAs and proteins which are required. While the results are consistent with a model in which the only protein synthesis necessary is that of the enzyme polypeptide, which is apparently the case for TAT induction in HTC cells (81), it cannot be ruled out that other RNA and protein species are required, as in the case of steroid induction of alkaline phosphatase in Hela cells (100).

The dependence of GS deinduction on RNA synthesis bears close resemblance to the dependence of TAT deinduction on RNA synthesis (98). It is not possible at this point to ascribe the maintenance of GS activity to a maintenance of GS (or some other protein's) synthesis, or to a stabilization of existing molecules.

Glutamine appears to influence GS activity by a mechanism different from that of dex. No RNA synthesis is required for either induction or repression of GS activity. Control is therefore at a post-transcriptional level. That in the case of repression of GS activity it is beyond processing and transport of RNA is suggested by the repression of GS by glutamine in enucleates. We also investigated the requirement for a nucleus for induction of GS by glutamine removal, but were not able to see induction. This result is of questionable significance, since the absence of an effect could be ascribed to damage done to cells by the process of enucleation. General protein synthesis, as measured by incorporation of $[^3H]$ leucine into TCA-precipitable material, was about 50% of nucleated controls at 3 hours post-enucleation. Total protein synthesis in cells in 2 mM and 0.4 mM glutamine were roughly the same (data not shown).

The rapid decrease in GS activity caused by glutamine could be due either to a decrease in the rate of synthesis of GS or a GS-activating protein, which together with a fast inactivation time would result in loss of activity (as in the case of TAT in the presence of cycloheximide (89)); or it could be due to an increase in the rate of inactivation of GS with a no change in the rate of synthesis; or elements of both. The kinetics of activity increase and decrease do not conclusively favor either alternative. However, the fact that in the absence of protein

synthesis modulation alone does not account for the depression of GS by glutamine.

If, then, the inactivation of the enzyme is being controlled by some function whose activity is in some way modulated by glutamine, the results of preincubating cells grown in low glutamine with cycloheximide 2 hours before adding 2 mM glutamine suggests that this activity itself is labile in the absence of protein synthesis. The simplest model would be of a protease which is synthesized and degraded rapidly (with a stable message, however--Figure 8a), which is activated by glutamine (either binding to GS or the protease). Precedent for other, more complicated "cascade" models exists in prokaryotes (see Review of the Literature).

Fig 1. Induction of glutamine synthetase activity by dexamethasone and glutamine. Cells grown for 12 hours in 2 mM glutamine were centrifuged at 160 xg for 5 minutes, the supernatant decanted, and cells resuspended in Growth Medium containing: ▽ , 2 mM glutamine; ○ , 10^{-7}M dex and 2 mM glutamine; Δ ,0.2 mM glutamine; and □ , 0.2 mM glutamine and 10^{-7}M dex. Enzyme activity was determined at various times as descibed in Materials and Methods.

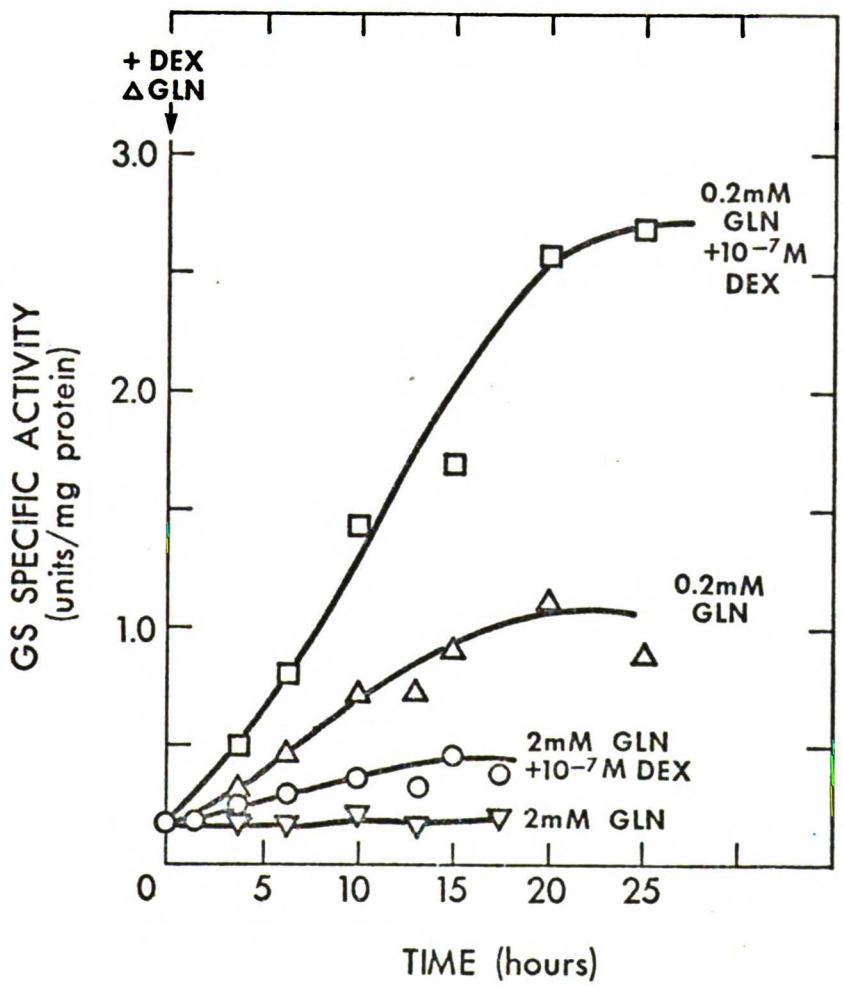

+ DEX
Δ GLN
3.0
2.0
1.0
0
5
10
15
20
25
GS SPECIFIC ACTIVITY
(units/mg protein)
TIME (hours)
0.2mM GLN +10⁻⁷M DEX
0.2mM GLN
2mM GLN +10⁻⁷M DEX
2mM GLN

Fig 2. Effect of actinomycin D and cycloheximide on the induction of glutamine synthetase by dexamethasone. Cells grown in 2 mM glutamine were given: ○, 10^{-7} M dex; △, 10^{-7} M dex and 0.1 µg/ml AMD; □, 10^{-7} M dex plus 2 x 10^{-7} M cycloheximide (CHX); and ● , no additions.

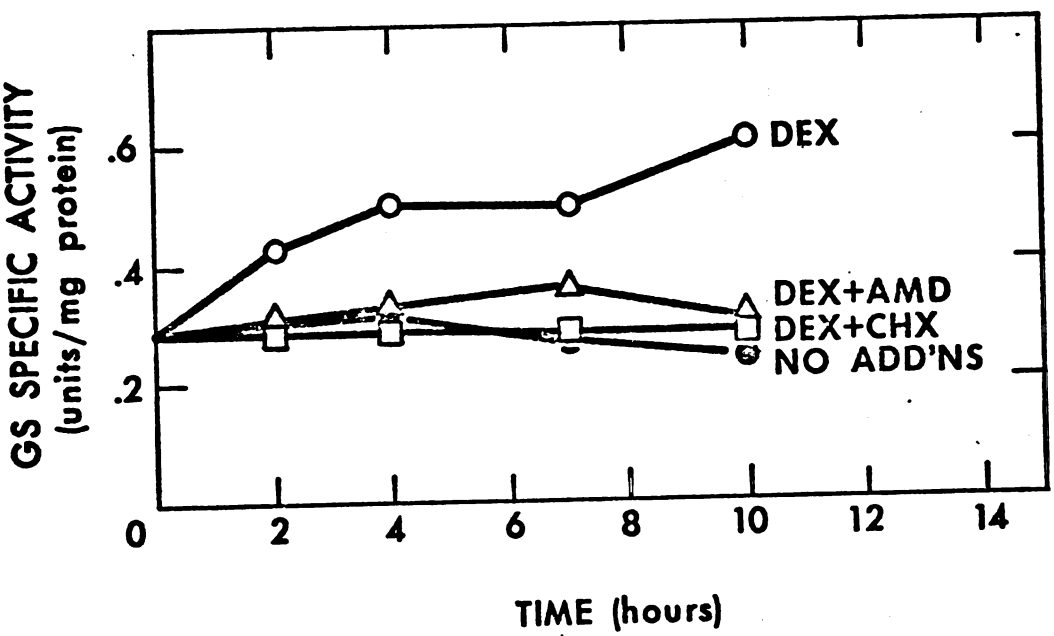

GS SPECIFIC ACTIVITY
(units/mg protein)
.6
.4
.2
0
2
4
6
8
10
12
14
TIME (hours)
DEX
DEX+AMD
DEX+CHX
NO ADD'NS

Table 3. Effect of various concentrations of actinomycin D on induction of Glutamine Synthetase by dexamethasone. Cells grown in Growth Medium containing 2 mM glutamine were given 10^{-7} M dex and various concentrations of AMD. GS specific activity was determined 6 hours later.

Actinomycin D (µg/ml)	Inhibition of Dexamethasone Induction (%)
0	0
0.1	96
0.5	95
1.0	97
5.0	96

Fig 3. Dose response curve of dexamethasone effect on glutamine synthetase and tyrosine aminotransferase in GM22 cells. Cells grown in Growth Medium containing 2 mM glutamine were given various concentrations of dex. 12 hours later, GS and TAT specific activities from each set of cultures were determined as described in Materials and Methods.

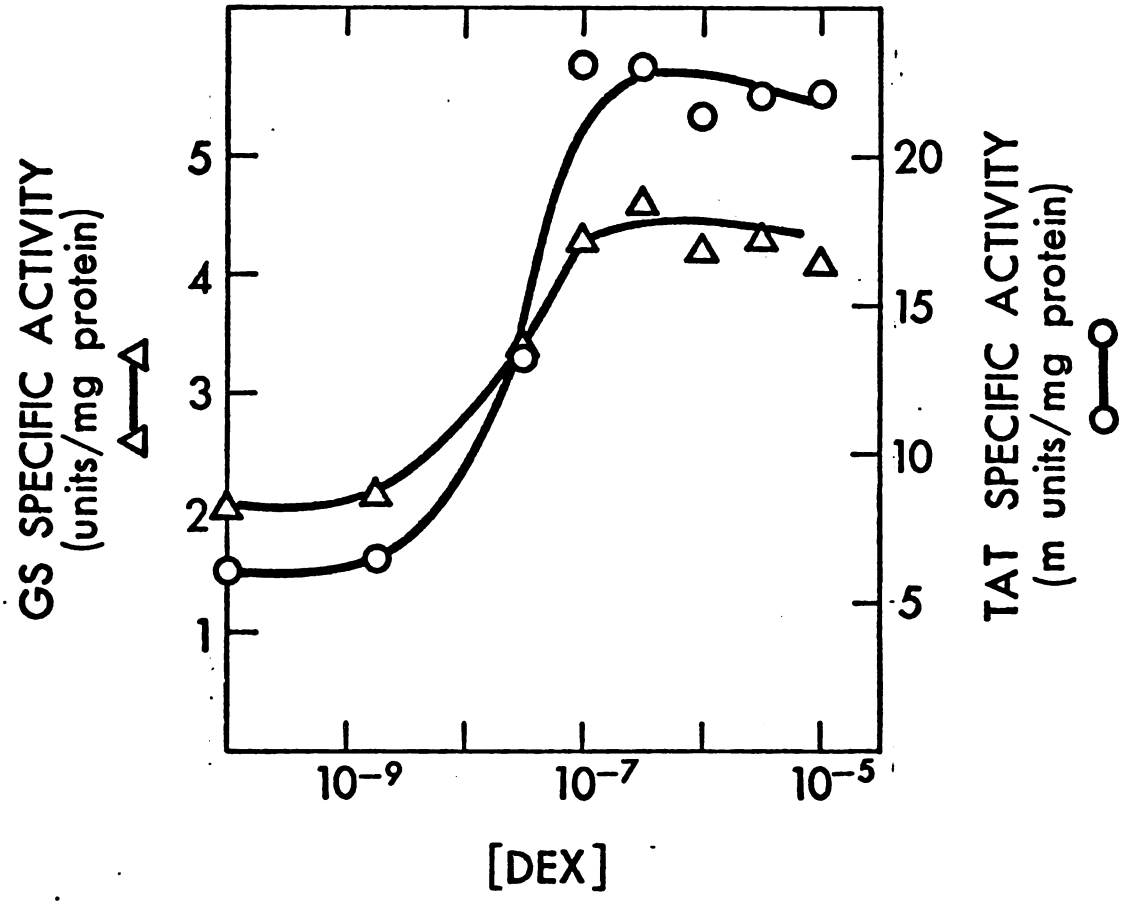
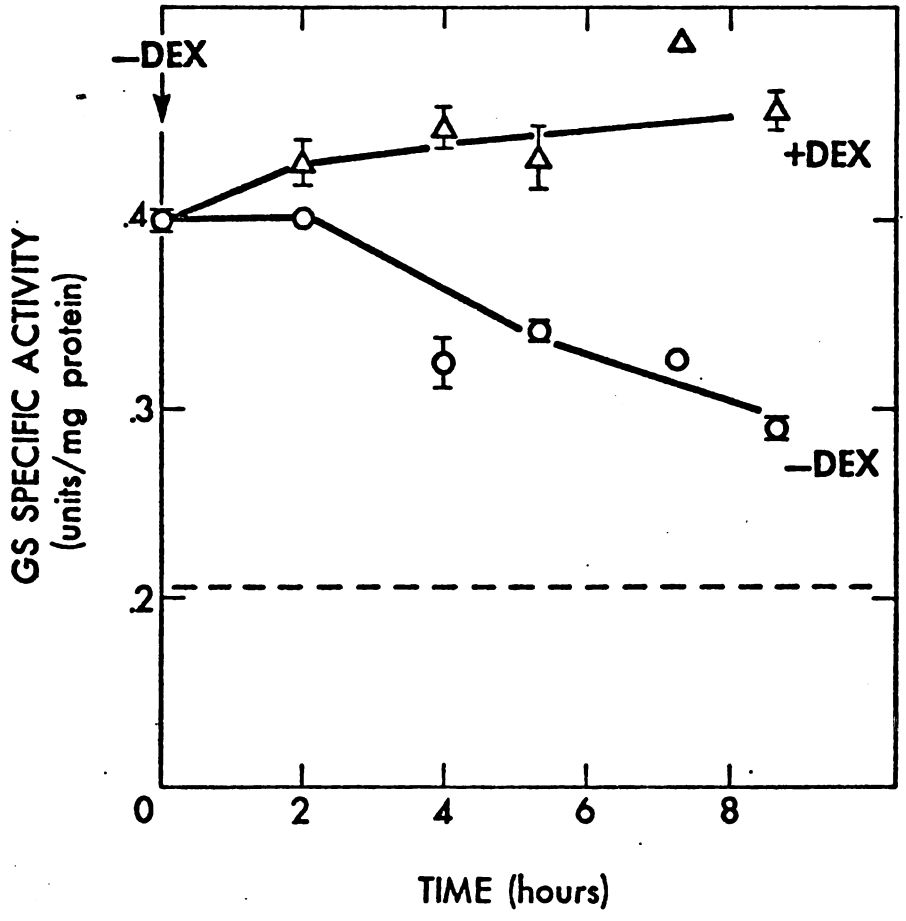

GS SPECIFIC ACTIVITY
(units/mg protein)
TAT SPECIFIC ACTIVITY
(m units/mg protein)
5
4
3
2
1
20
15
10
5
10^{-9}
10^{-7}
10^{-5}
[DEX]

Fig 4a. Effect of dexamethasone removal on glutamine synthetase activity. Cells grown 12 hours in Growth Medium containing 2 mM glutamine plus 10^{-7} M dex were centrifuged at 160 xg for 5 minutes at 25°C, the supernatant decanted and the cell pellet resuspended in Growth Medium 2 mM glutamine, either: △ , with dex; or ○ , without dex; enzyme activity was determined. b. Effect of 0.5 µg/ml AMD on GS activity following dex removal. The protocol in 4a was followed except that cells were resuspended in Growth Medium containing 2 mM glutamine and either: ○ , no additions; or △ , 0.5 µg/ml AMD.


–DEX
+DEX
–DEX
.4
.3
.2
0
2
4
6
8
GS SPECIFIC ACTIVITY
(units/mg protein)
TIME (hours)

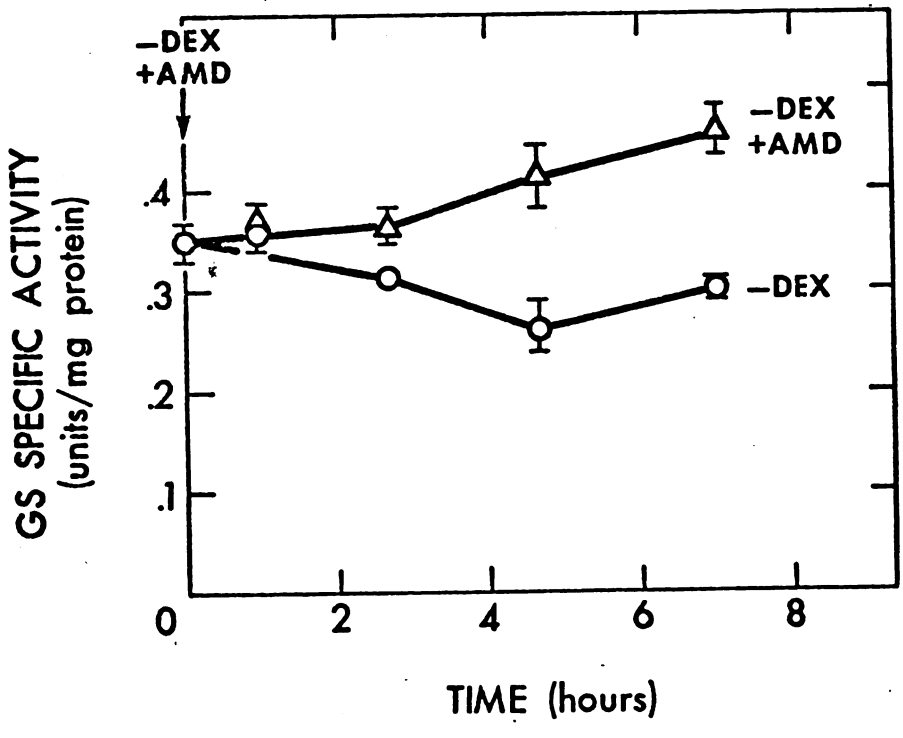

–DEX
+AMD
–DEX
+AMD
–DEX
GS SPECIFIC ACTIVITY
(units/mg protein)
.4
.3
.2
.1
0
2
4
6
8
TIME (hours)

Fig 5. Effect of the extracellular glutamine concentration of glutamine synthetase specific activity. Cells were grown in Growth Medium containing various concentrations of glutamine for 20 hours. The specific activities of GS were then determined.

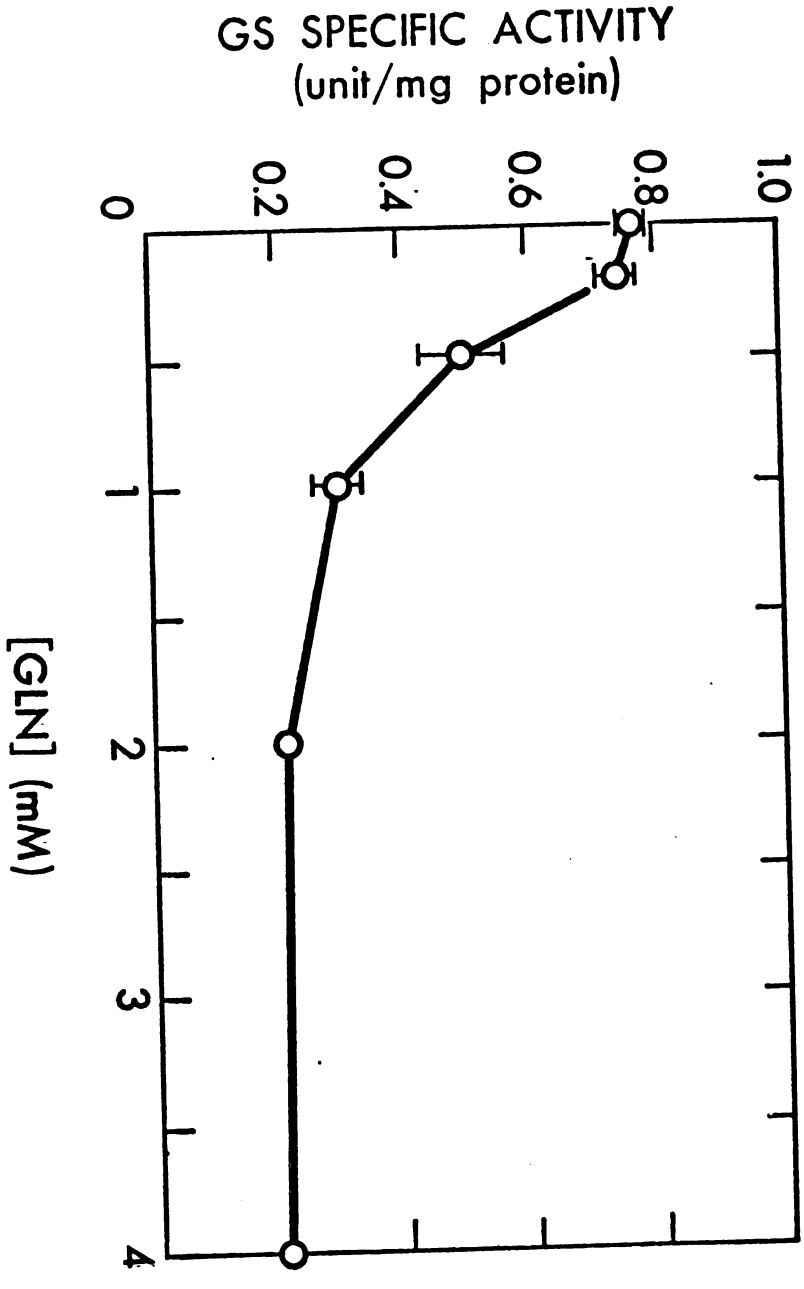

GS SPECIFIC ACTIVITY
(unit/mg protein)
0
0.2
0.4
0.6
0.8
1.0
[GLN] (mM)
1
2
3
4

Fig 6. Effect of actinomycin D and cycloheximide of the induction of glutamine synthetase by glutamine stepdown. Cells grown in Growth Medium containing 2 mM glutamine were centrifuged at 160 xg for 5 minutes at 25°C, the supernatant decatanted, and the cell pellets resuspended in Growth Medium containing 0.2 mM glutamine plus: ○ , no additions; △ , 0.1 µg/ml AMD; ▽ , 0.5 µg/ml AMD; and □ , 5 µg/ml AMD; and ● , 2 x 10^{-4} M cycloheximide. Cells were allowed to incubate various lengths of time, and then enzyme activity was determined.

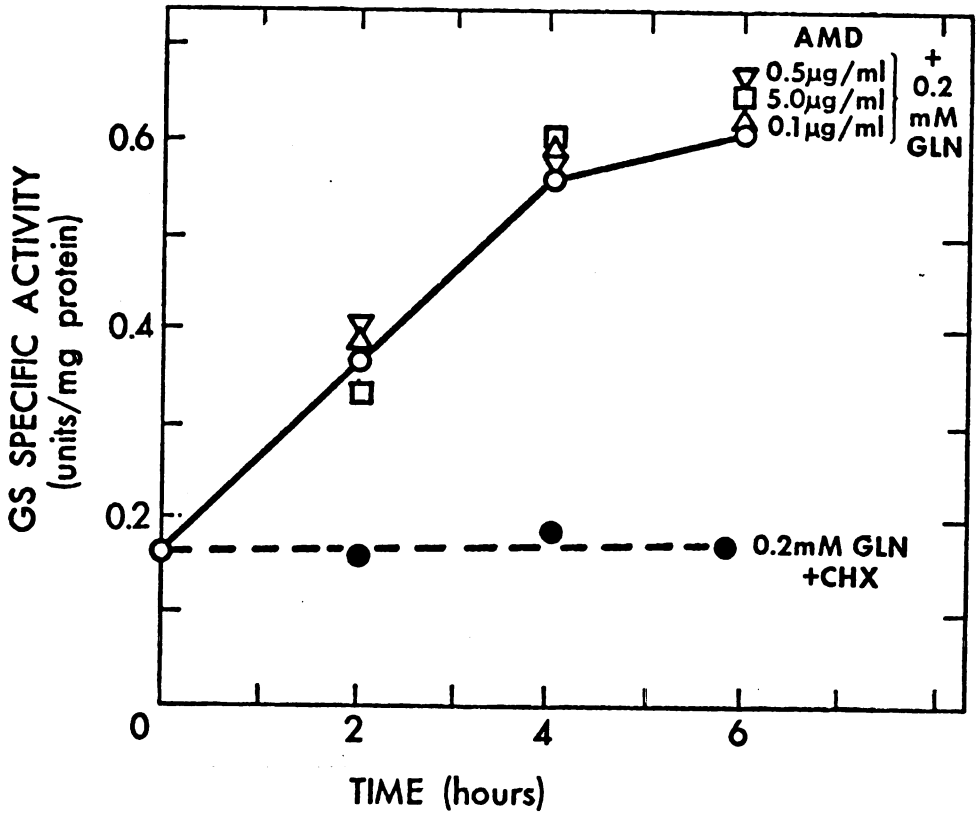

AMD
0.5µg/ml
5.0µg/ml
0.1µg/ml
+
0.2
mM
GLN
0.2mM GLN
+CHX
GS SPECIFIC ACTIVITY
(units/mg protein)
0.6
0.4
0.2
0
2
4
6
TIME (hours)

Fig 7. Effect of actinomycin D and cycloheximide on the depression of glutamine synthetase activity by 2 mM glutamine. Cells grown 2 days on 0.2 mM glutamine plus 10^{-7} M dex were given: ○, no additions; ●, 2×10^{-4} M cycloheximide; ▽, 2×10^{-4} M cycloheximide plus 2 mM glutamine; △, 2 mM glutamine; and □, 0.5 μg/ml AMD plus 2 mM glutamine. After various times, enzyme activities were determined.

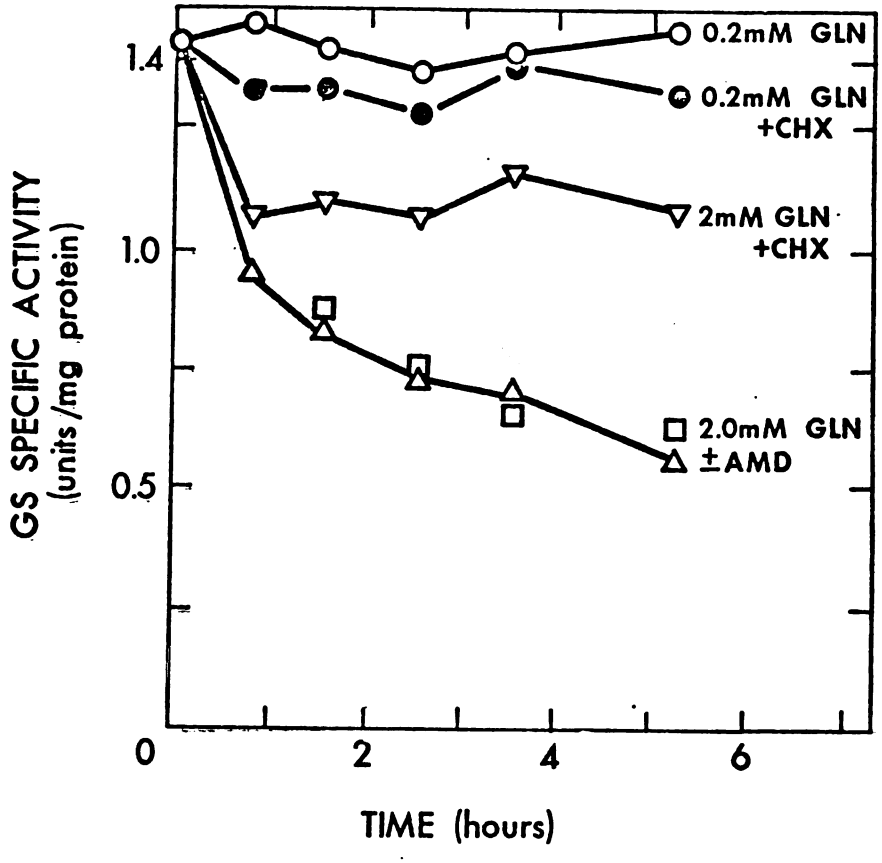

GS SPECIFIC ACTIVITY
(units/mg protein)
1.4
1.0
0.5
0
2
4
6
TIME (hours)
0.2mM GLN
0.2mM GLN
+CHX
2mM GLN
+CHX
2.0mM GLN
±AMD

Fig 8a. Effect of cycloheximide preincubation of glutamine synthetase repression by glutamine. Cells grown in Growth Medium containing 0.2 mM glutamine were split into two sets of cultures. To the first was added 2×10^{-4} M cycloheximide; no additions were added to the other. Two hours later, the set of cultures without cycloheximide were split. To one set was added; △ , 2×10^{-4} M cycloheximide; ● , 2 mM glutamine; ▲ , 2×10^{-4} M cycloheximide plus 2 mM glutamine; and ○ no additions. To the preincubated set of cultures were added: □ , no additions; and ■ , 2 mM glutamine. Enzyme activity was determined after various times.

b. The data from the three sets of cultures given 2 mM glutamine in 8a is expressed percent of the respective controls without glutamine. (same symbols).

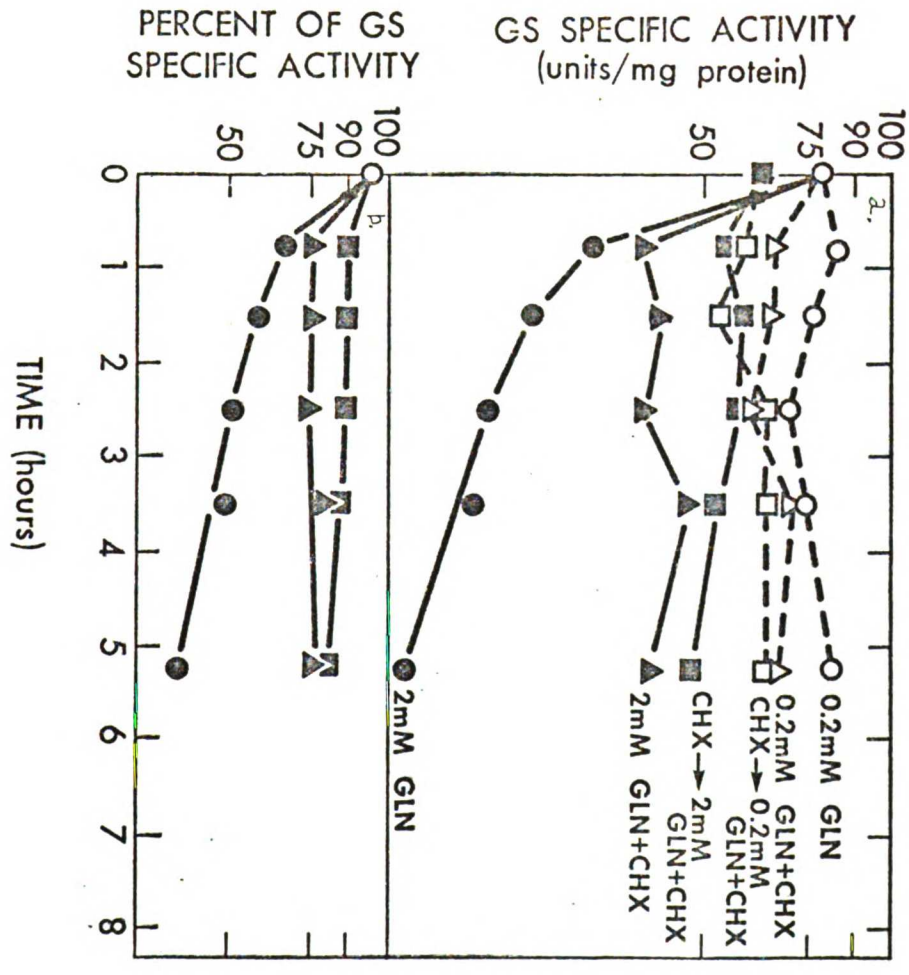

PERCENT OF GS SPECIFIC ACTIVITY
GS SPECIFIC ACTIVITY (units/mg protein)
100
90
75
50
a.
b.
0.2mM GLN
0.2mM GLN+CHX
CHX→ 0.2mM GLN+CHX
CHX→ 2mM GLN+CHX
2mM GLN+CHX
2mM GLN
0 1 2 3 4 5 6 7 8
TIME (hours)

Fig 9. The rate of total protein synthesis and glutamine synthetase specific activity in cells grown in varying concentrations of glutamine. Cells were grown for 20 hours in Growth Medium containing various concentrations of glutamine. Aliquots from each set of cultures were then taken for GS assays and incorporation of $[^3H]$ leucine into protein, both as described in Materials and Methods.

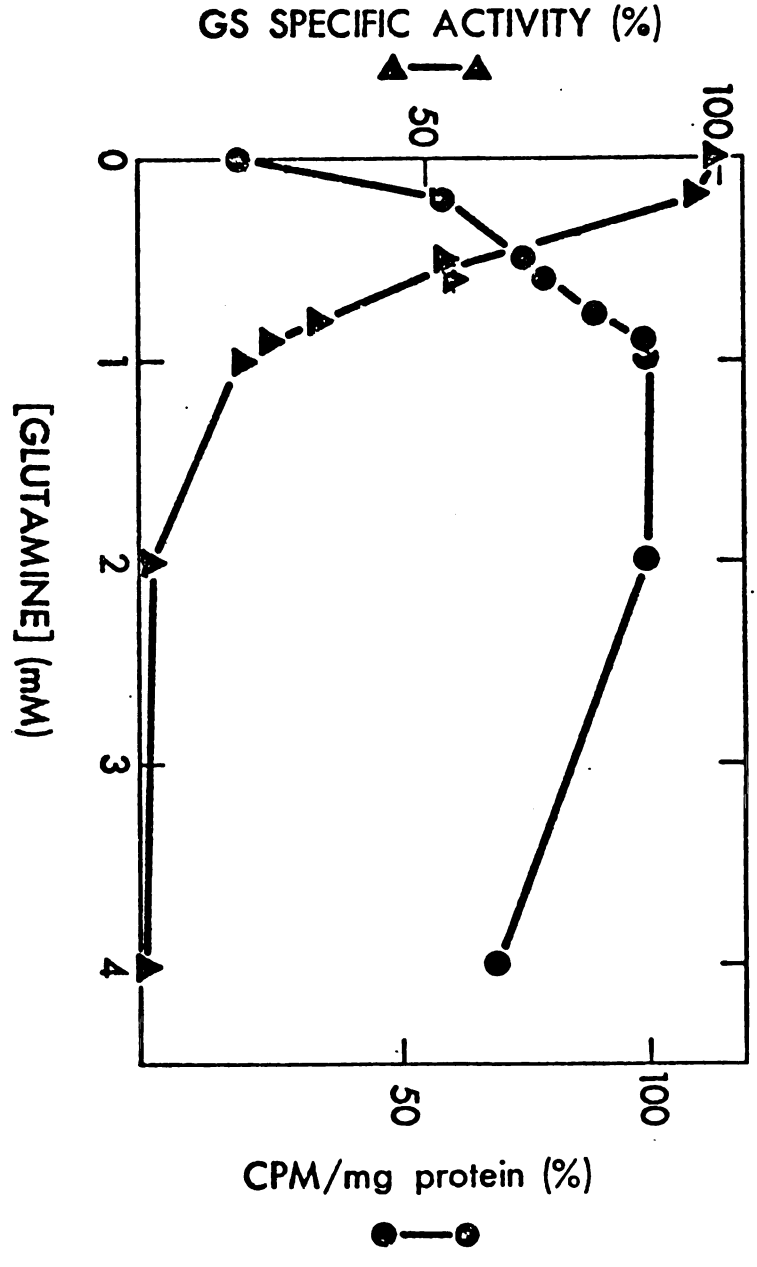

GS SPECIFIC ACTIVITY (%)
50
100
0
1
2
3
4
[GLUTAMINE] (mM)
50
100
CPM/mg protein (%)

Fig 10. Amino acid specificity of glutamine synthesis induction. Cells grown 20 hours in 2 mM glutamine were centrifuged at 160 xg for 5 minutes, the supernatant decanted and the cell pellet resuspended in .15 M NaCl. The cells were then centrifuged again, the supernatant decanted and the cell pellet resuspended in Growth Medium without serum, containing: △, no leucine and 2 mM glutamine; ▲, .25 mM leucine and 2 mM glutamine; □, .5 mM leucine and 2 mM glutamine; or ○, .5 mM leucine and .2 mM glutamine, GS activity was determined at various times.

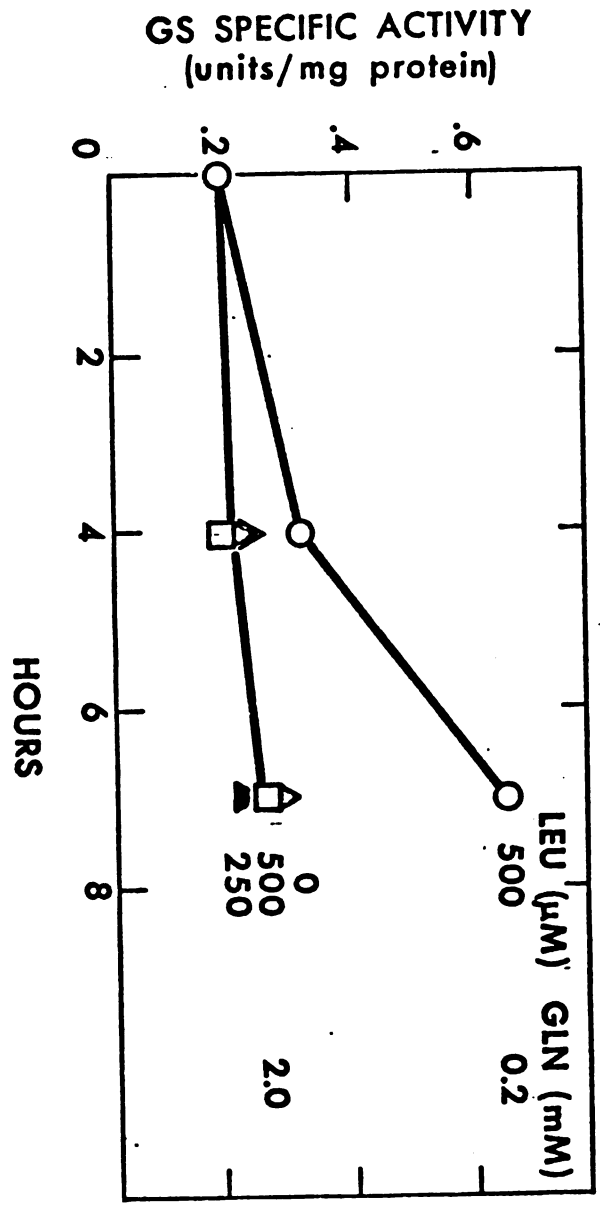
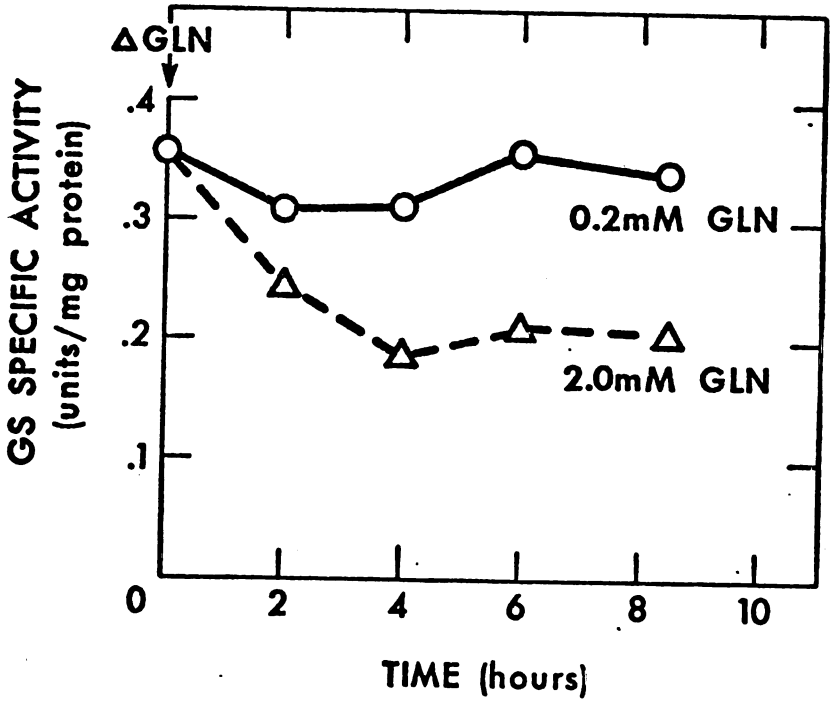

GS SPECIFIC ACTIVITY
(units/mg protein)
.2
.4
.6
0
2
4
6
8
HOURS
LEU (μM)
500
0
500
250
GLN (mM)
0.2
2.0

Fig 11. Repression of glutamine synthetase by 2 mM glutamine in enucleate GM22 cells. Cells were grown in Growth Medium with 0.2 mM glutamine plus 10^{-7} M dex. Enucleation was performed as described in Materials and Methods; then the cultures were split and one set ($\triangle$) given 2 mM glutamine. The other (O) received no additions. Enzyme activities were determined at various times.


ΔGLN
.4
.3
.2
.1
0
2
4
6
8
10
GS SPECIFIC ACTIVITY
(units/mg protein)
TIME (hours)
0.2mM GLN
2.0mM GLN

CHAPTER FOUR

RESULTS

The data presented in Chapter Three imply that the mechanisms by which glutamine and dex regulate the activity of GS are different. The data further suggest that the glutamine mechanism includes an increase in the rate of inactivation of the enzyme. Reduction of the level of glutamine in the medium appears to release GS from rapid inactivation, increasing its half-life in proportion to the glutamine reduction.

The half-life of GS in 0.2 mM glutamine appears to be around 10-20 hours (data not shown). (This is confirmed in Chapter Six.) The long half-life in 0.2 mM glutamine was exploited to gain information about the effect of dex on the rate of synthesis of GS. The rationale is the following: If the half-life of GS is long, then the slope at early times of specific activity as plotted against time under these conditions is a proportional to the rate of synthesis. (But it is not identical. Only in the decay of the enzyme is zero is the accumulation curve an exact reflection of synthesis.) If dex, as the inhibitor experiments suggest, acts by increasing the rate of synthesis of GS (or of another protein which then activates GS molecules) then this increase would be reflected in a change in the slope of GS activity in 0.2 mM glutamine. The experimental design was the following. Cells in 2 mM glutamine were incubated for varying lengths of time in dex. At the end of the incubation, the cells were (1) given AMD to prevent

further RNA synthesis. As was shown in Chapter Three, this has no effect on the induction of GS by glutamine; (2) washed and resuspended in fresh Growth Medium with 0.2 mM glutamine and dex. Dex was kept in the medium to insure that the effect of dex did not diminish in the absence of further RNA synthesis. The slope of the GS activity curve was then measured, and the slopes plotted as a function of a second variable and termed "GS synthesizing capacity" (GSSC).

Figure 12 shows that, when cells are exposed to dex for various times and the above-mentioned protocol is followed, the slope of GS activity accumulation does increase, and that, over a few hours, the increases are linear. When these slopes are plotted against time of steroid incubation, the increase is seen to plateau at between 4-8 hours (Figure 13). This increase is prevented by incubation of the cells in AMD during the dex exposure. The effect of cycloheximide was not determined because it was not possible to wash it out of cells.

A dose-response curve made from cells grown for 12 hours in several concentrations of dex is shown in Figure 14. This curve is quite similar to the dose-response curve measuring GS specific activity as a function of dex concentration (Fig. 3).

When cells which have been grown in dex for 12 hours are washed free of dex, and the GS synthesizing capacity is followed as a function of time after dex washout, it can be seen (Fig 15) that the GSSC falls with a half-time of 8 hours including a lag of 4 hours. However, if AMD is added to cells at the time of dex washout, GSSC is maintained, suggesting that RNA synthesis is required for the fall in the rate of synthesis of GS which occurs with dex washout (Fig 16).

DISCUSSION

This set of experiments suggest that dex causes an increase in the rate of appearance of GS activity proportional to the changes in GS catalytic activity. While these changes are sufficient to account for the change in specific activity observed, they do not exclude the possibility that dex also affects the inactivation of GS. They also do not discriminate between an effect on another protein which in turn activates preexisting GS molecules, and an affect on synthesis of new GS molecules. These questions are addressed on Chapter Six.

Fig 12. The slope of glutamine synthetase specific activity increase with time as a function of preincubation time in dexamethasone. Cultures were grown in Growth Medium containing 2 mM glutamine and 10^{-7}M dex for: ○, 0 hours; △, 2 hours; □, 4 hours; ▼, 8 hours; ●, 12 hours; ▲, 16 hours; ■, 34 hours. The two sets of cultures were exposed to 0.5 µg/ml AMD during the initial dex exposure for: ▽, 4 hours; and ◇, 8 hours. These cultures were then centrifuged at 160 xg for 5 minutes at 25°C, and resuspended in Growth Medium containing 0.2 mM glutamine, 0.5 µg/ml AMD, and 10^{-7} M dex (at a time termed zero time). Aliquots were then taken for enzyme activity determination at 0, 3, and 6 hours.

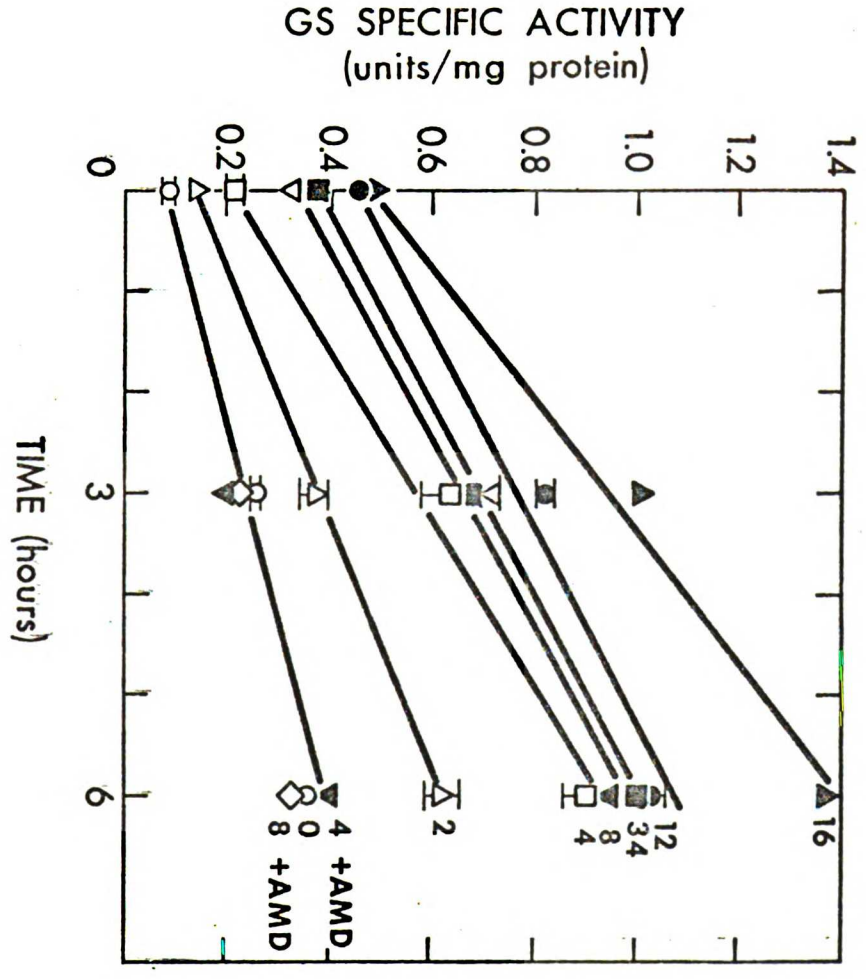

GS SPECIFIC ACTIVITY
(units/mg protein)
0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
TIME (hours)
3
6
16
12
34
8
4
2
4 +AMD
0
8 +AMD

Fig 13. The slopes of glutamine synthetase activity increase expressed as a function of preincubation in dexamethasone. The data of Fig 12 are re-expressed as slopes and termed GSSC (GS synthesizing capacity).

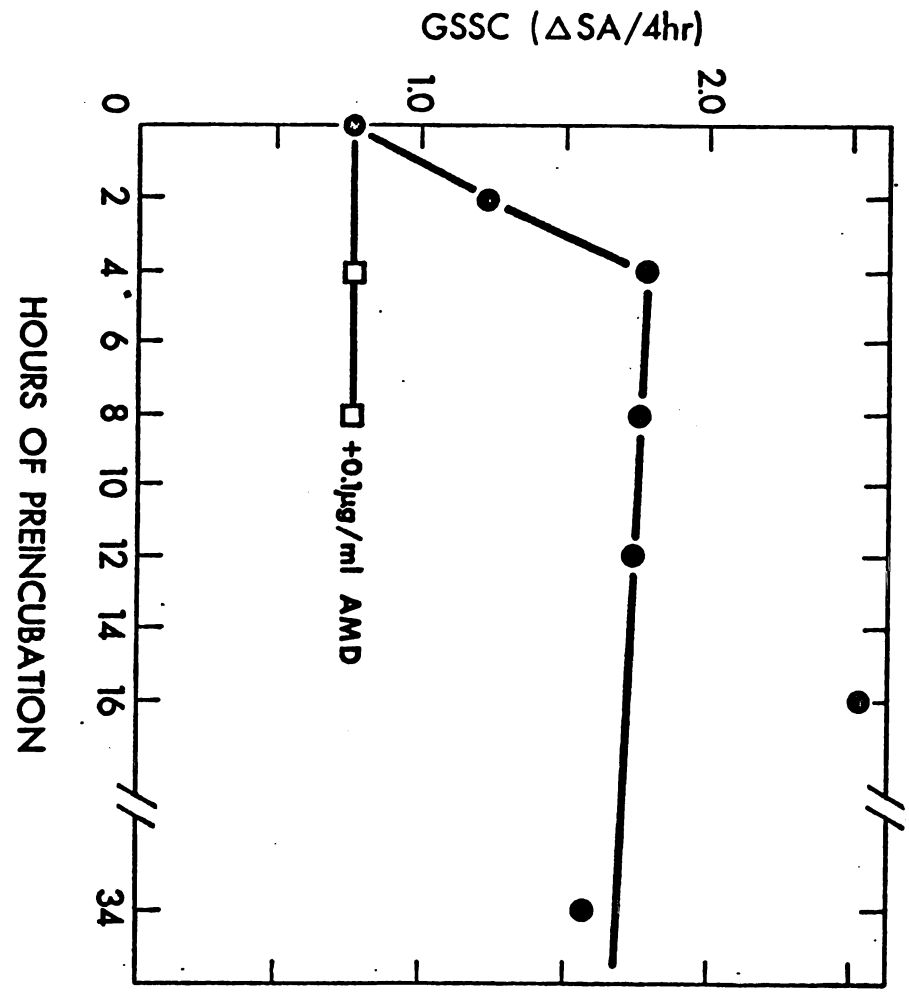

GSSC (ΔSA/4hr)
1.0
2.0
0
2
4
6
8
10
12
14
16
34
+0.1μg/ml AMD
HOURS OF PREINCUBATION

Fig 14. Dose-response of glutamine synthetase synthesizing capacity as a function of dexamethasone concentration. Cultures were preincubated in a variety of dex concentrations, then the procedure described in Materials and Methods followed.

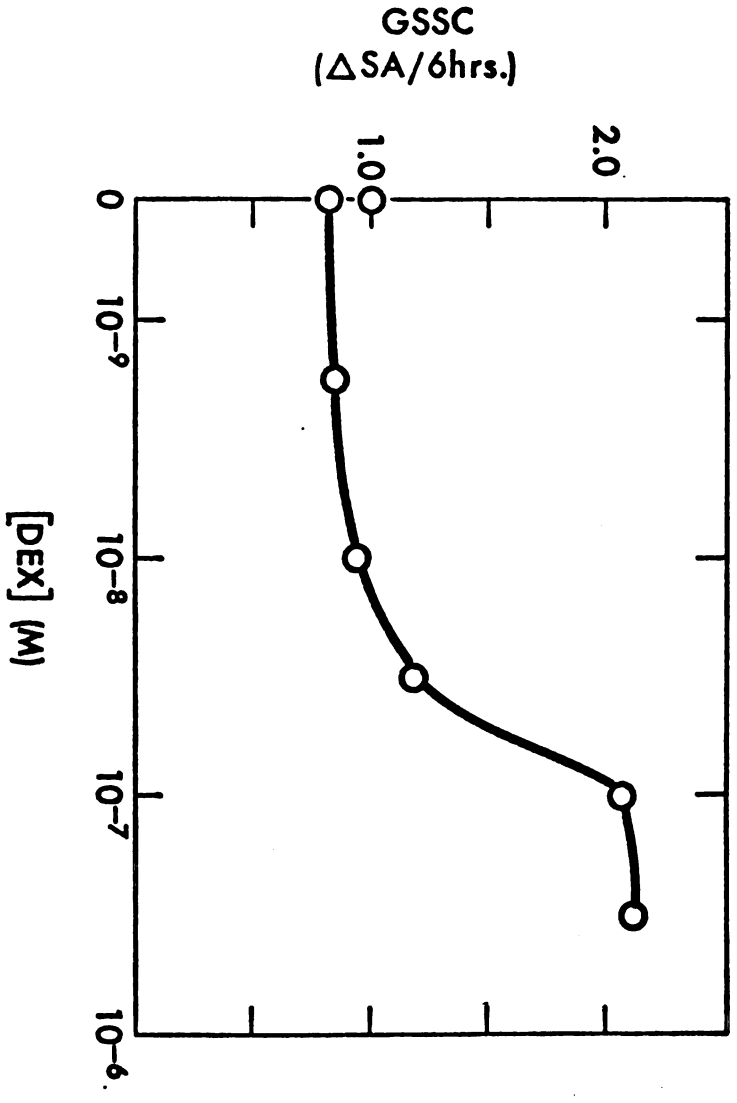

GSSC
(ΔSA/6hrs.)
1.0
2.0
0
10−9
10−8
10−7
10−6
[DEX] (M)

Fig 15. Glutamine synthetase synthesizing capacity as a function of time after washout of dexamethasone. Cells grown in Growth Medium with 2 mM glutamine and 10^{-7} M dex for 22 hours were centrifuged as in Fig. 12 and resuspended in Growth Medium with 2 mM glutamine, without dex. At various times following this step, cells were processed as in Fig. 14. Insert: The glutamine synthetase activity at zero time after various times in 10^{-7} M dexamethasone.

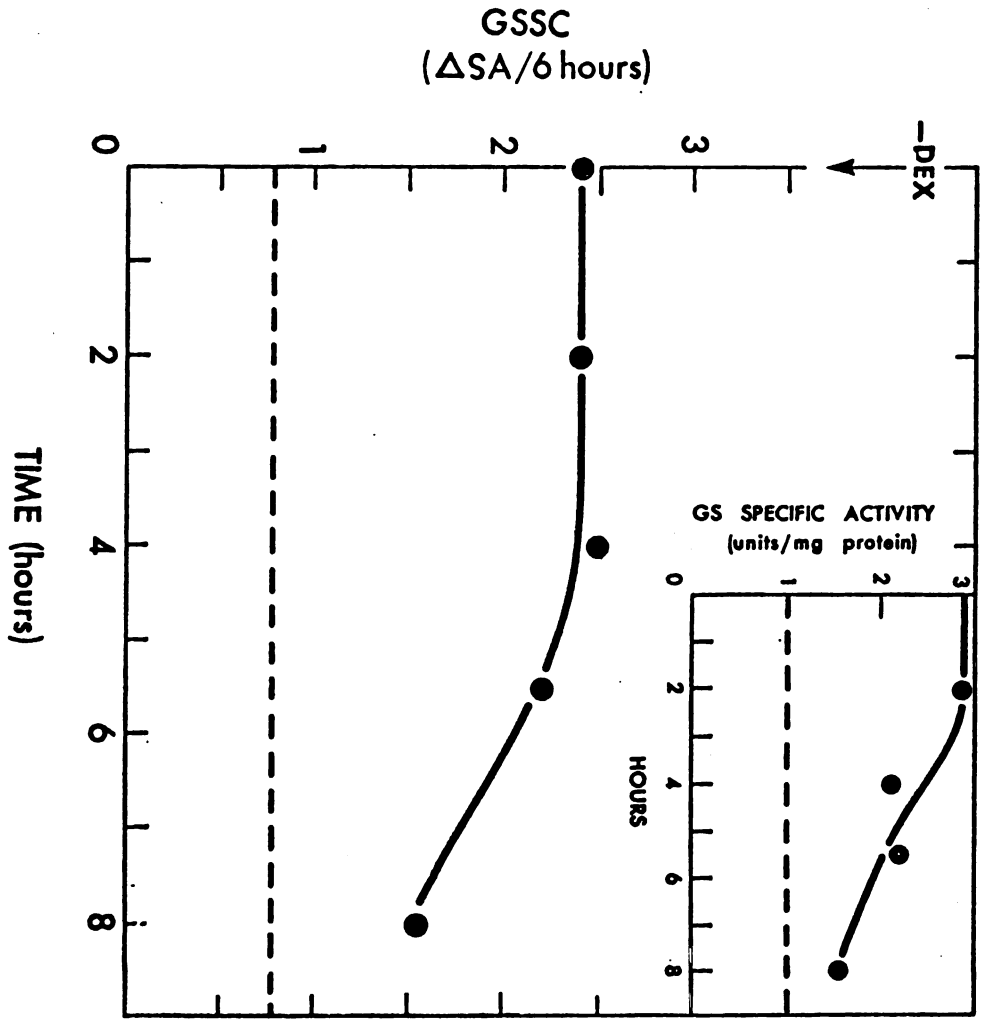
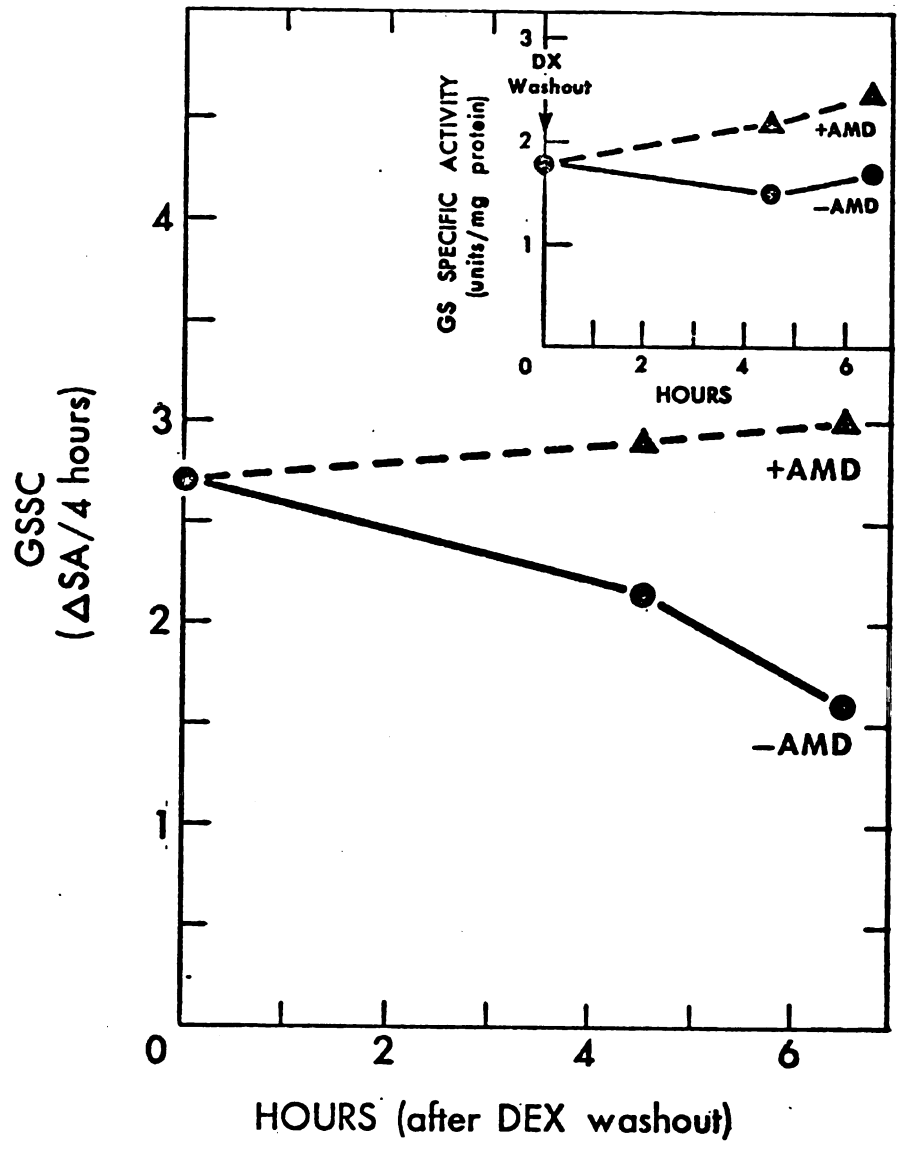

GSSC
(ΔSA/6 hours)
0
1
2
3
−DEX
TIME (hours)
0
2
4
6
8
GS SPECIFIC ACTIVITY
(units/mg protein)
0
1
2
3
HOURS
0
2
4
6
8

Fig 16. The effect of actinomycin D on the fall in glutamine synthetase synthesizing capacity following dexamethasone washout. Cells grown in Growth Medium with 2 mM glutamine and 10^{-7} M dex for 12 hours were centrifuged and resuspended as in Fig 15, except that to one set of cultures (▲) 0.5 µg/ml AMD was added; to the other set (●) no additions were made. The cells were processed as in Fig 14.


GSSC
(ΔSA/4 hours)
0
1
2
3
4
2
4
6
HOURS (after DEX washout)
+AMD
−AMD
GS SPECIFIC ACTIVITY
(units/mg protein)
DX
Washout
1
2
3
0
2
4
6
HOURS
+AMD
−AMD

CHAPTER FIVE

RESULTS

While the experiments in the previous chapters are consistent with the effect of steroids being on synthesis of GS, and the effect of glutamine being to alter the degradation rate, they are far from conclusive. A more rigorous experimental system would be one in which an antibody specific for GS would allow quantitation of the rate at which radioactive amino acids entered and left the pool of GS molecules.

Such an antiserum, raised in rabbits against purified rat liver GS, was provided by Dr. Thomas Deuel. Marjorie Louie, his graduate student, participated in some of the early experiments on equivalence points of GS.

Equivalence titrations were performed to establish whether the GS activity present in 0.2 mM and 2 mM glutamine-grown cells and cells grown with and without dex are identical with respect to the antibody. It was found that the antibody does not inhibit the catalytic activity of GS, and that attempting to remove the antibody-antigen complex after overnight incubation by centrifugation at 30,000 xg for 10 minutes at $4^{o}C$ in some cases left 30% of the activity in the supernatant under conditions of antibody excess (data not shown). Such artifacts could be avoided by using an indirect antibody precipitation, which involves reacting the anti-GS antibody-GS complex with an sheep antiserum raised

against purified rabbit IgG. The resultant aggregate removes no less than 97% of GS activity when in antibody equivalence or excess.

Figure 17a shows the precipitin curve of sheep anti-rabbit IgG against rabbit IgG. Figure 17b is the precipitin curve of sheep anti-rabbit IgG against Deuel's rabbit anti-rat GS serum. These curves were used to determine equivalence points for the two sera.

When an increasing anti-GS concentration is titrated against a fixed extract protein concentration, the results are seen in Figure 18a. The ratio of antibody to antigen at equivalence is 20 µl: unit GS for all extracts. The data are replotted on Figure 18b to correct to the same activity in all cases. Here it can be seen that all curves are identical.

If an increasing GS activity is titrated against a fixed amount of anti-GS, the results are quite different (Fig. 19). The equivalence point for GS from cells grown in high glutamine plus dex is 20 µl : .7 units GS. For cells grown in low glutamine with and without dex, the ratio is the same.

Taken together, these two experiments suggest that there is a difference between the population of GS molecules in cells grown in 2 mM glutamine and that of cells grown in 0.2 mM glutamine. Table 4 is a formal listing of the possible combinations of the three variables in these experiments--number of GS molecules, catalytic activity per molecule, and antigenic reactivity per molecule--that can occur. The equivalence point experiments eliminate all but two: differences exist between GS populations in 0.2 mM and 2 mM glutamine-grown cells in both the catalytic activity per molecule and antigenic reactivity per molecule, and the number of molecules may or may not be the same. Moreover,

the results demand that the ratio of the antigenic reactivity of the GS populations be identical to either the ratio of the catalytic activities (row 3 of Table 4), or to the ratios of the products of catalytic activities x number of molecules (row 8).

If more than one form of GS exists in GM22 cells, it seemed possible to detect them by separation procedures. Accordingly, 300-400 µg of extracts of cells grown in 0.2 mM and 2.0 mM glutamine, with and without dex, were each layered on a 5-20% sucrose gradient and centrifuged for 5 hours at 50,000 rpm at 4°C. Fractions were collected and assayed for GS activity. The results are shown in Figure 20a. All extracts contain one activity of GS, with an S value of 15. Sedimentation in sucrose gradients containing 0.4 M NaCl gave the same result (Figure 20b). Centrifuging for 1 hour failed to reveal a heavier sedimenting peak (data not shown). Thus, no aggragates of GS are detectable under these conditions.

Extracts were heated at 60°C for 5 minutes to determine whether more than one GS activity could be detected on the basis of heat denaturation profiles. Figure 21 shows that all extracts gave similar curves.

DEAE cellulose chromatography with elution with a 0-0.5 M NaCl gradient is shown in Figure 22 a-d. All extracts displayed the same profile eluting at .10 M NaCl. Occasionally a smaller trailing peak was observed, but it occurred in all extracts simultaneously, and in the same proportion. Therefore it probably does not constitute a difference among extracts. Thus no differences in GS binding to the ion exchange resin can be seen among the four extracts.

Isoelectric focussing in glycerol gradients gave results complicated by artifacts (data not shown). However, the best data, which were

from extracts heated to 60°C for 5 minutes under conditions where all of the catalytic activity is retained, gave the results shown in Figure 23 a-c. It can be seen that the three extracts all have iso-electric points at pH 7.5, suggesting that no detectable differences in charge exist among these populations of GS.

DISCUSSION

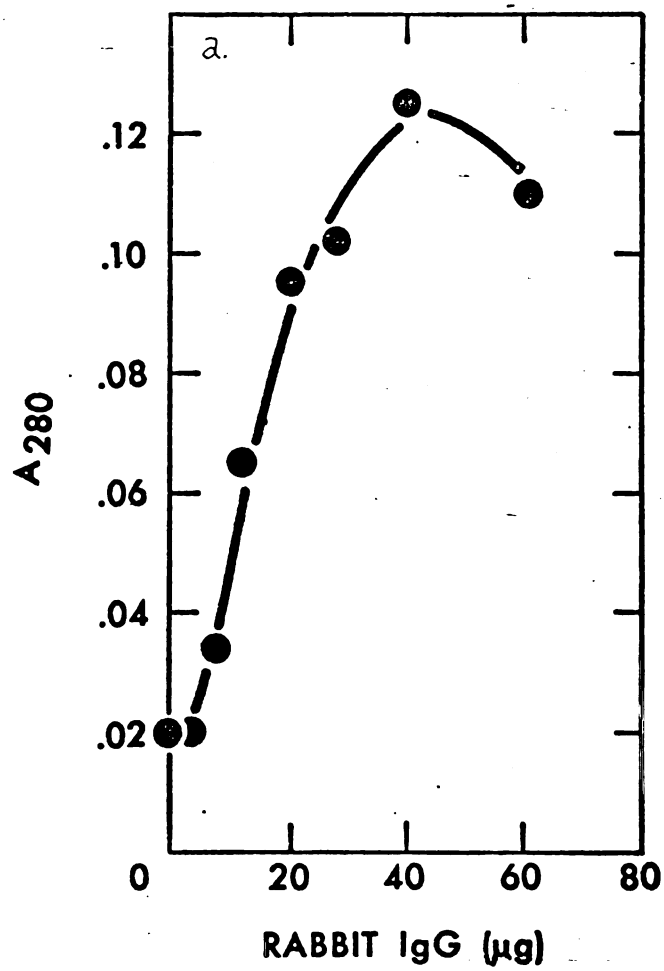
GS activity in cells grown in the presence or absence of dex, in 0.2 mM or 2.0 mM glutamine, appears to behave like a single species whose physical properties are not influences by dex or glutamine. The possibility of catalytically active forms of GS of greater or lesser molecular weight--for example, dissociation or association products--was ruled out by the sucrose density gradient data. A modification which results in an alteration in heat stability of one species of catalytically active GS appears unlikely from the heat denaturation data. And finally, a charge change which might result from a covalent modification of the GS polypeptide, such as occurs in prokaryotes, could not be detected either by ion-exchange chromatography or iso-electric focussing.

With respect to these last two techniques, it should be pointed out that the larger the total number of charged side groups in a protein, the smaller the effect of a fixed number of charge changes will have on the net charge of the molecule. Stadtman et al. were not able to detect a difference between the pI's of fully adenylylated (i.e. 12 ademylyl groups perdodecamer) and fully unadenylylated GS in native iso-electric focussing gels (45). Thus, with an octamer such as rat lever GS, such a charge change might also go undetected.

In sum, the evidence presented in this chapter suggests that only one physically distinguishable form of GS is catalytically active. Therefore, it seems likely that the equivalence titration data is due to the presence of a catalytically inactive form of GS which exists, alone with the active form, in cells grown in 2 mM glutamine, and cross-reacts with anti-GS (although to a different extent from the active form.)

The modification of GS which renders it inactive must alter the catalytic sites and the antigenic sites on the oligomer, which are different by two criteria: (1) interaction of the enzyme with its anti-serum does not inhibit catalytic activity; and (2) the presence of $MnCl_2$, ATP, NH_4Cl, and glutamate inhibit the immunologic reaction, but not the catalytic reaction. The alteration of catalytic activity, moreover, is proportional to the alteration of antigenicity. Such a set of results could result from a dissociation of GS into oligomers of less than eight subunits, which are catalytically inactive but which provide, either by their increased number or by an alteration of the antigenic determinants of each subunit caused by dissociation, an increasing number of antigenic sites for the antibody. A promising experiment would be to immunoprecipitate fractions from a sucrose gradient to see if fractions lacking GS activity contain the isotopically labelled GS monomer.

Fig 17a. Precipitin curve of sheep anti-rabbit IgG. The samples were prepared and analyzed as described in Materials and Methods. b. Precipitin curve of sheep anti-rabbit IgG against purified rabbit anti-rat liver glutamine synthetase. The samples were prepared and analysed as in 16a.


a.
.12
.10
.08
.06
.04
.02
0
20
40
60
80
A280
RABBIT IgG (µg)

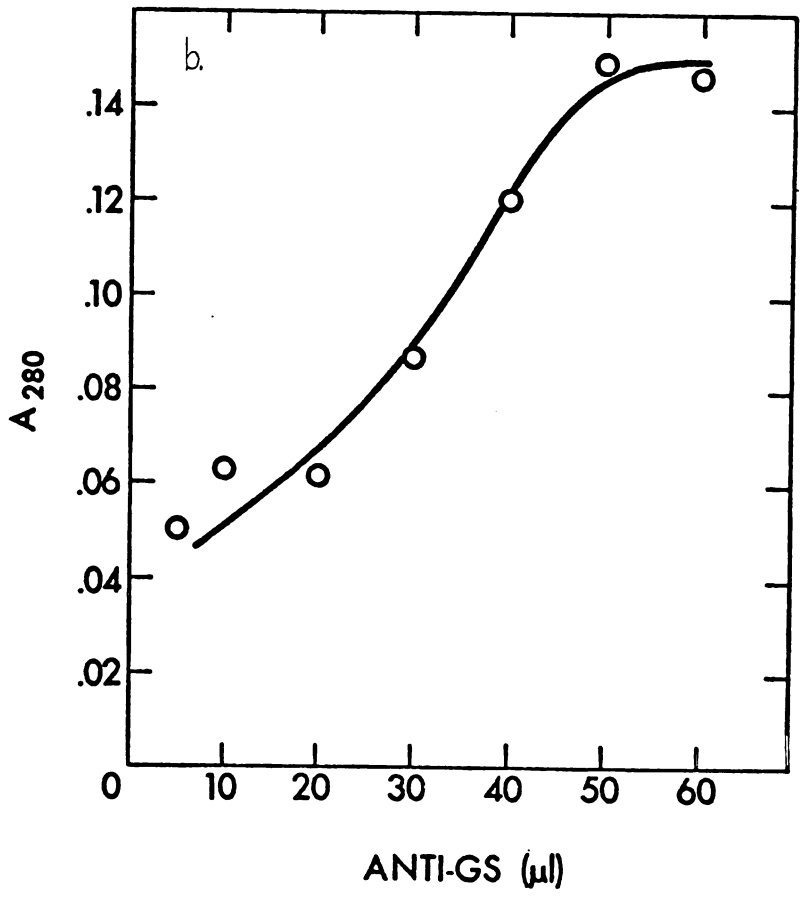

b.
.14
.12
.10
.08
.06
.04
.02
0
10
20
30
40
50
60
A 280
ANTI-GS (µl)

Fig 18a. Equivalence titrations varying antibody. Cells were grown for 24 hours in Growth Medium containing ○ , 2 mM glutamine; ▲, 2 mM glutamine plus 10^{-7} M dex; △ , 0.2 mM glutamine; and ●, 0.2 mM glutamine plus 10^{-7}M dex. Extracts were then prepared and analyzed as described in Materials and Methods. b. The results in 18a are normalized to the control value of the 0.2 mM glutamine plus 10^{-7} M dex extract (same symbols).

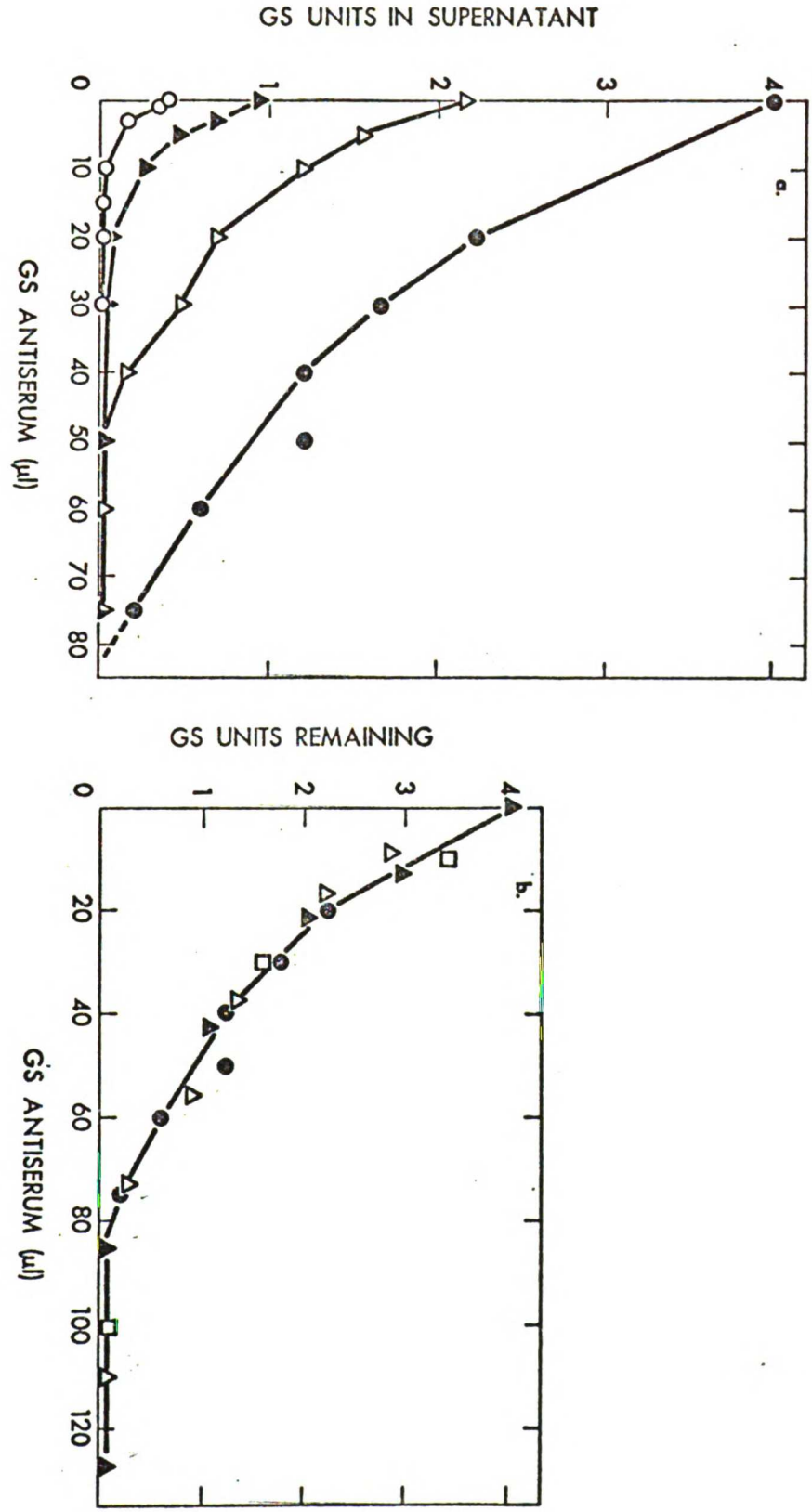

GS UNITS IN SUPERNATANT
GS ANTISERUM (µl)
0
10
20
30
40
50
60
70
80
1
2
3
4
a.
GS UNITS REMAINING
GS ANTISERUM (µl)
0
20
40
60
80
100
120
1
2
3
4
b.

Fig 19. Equivalence titrations varying antigen. Cells were grown as in Fig 17a and denoted by the same symbols. Extracts were then made and analyzed as described in Materials and Methods.

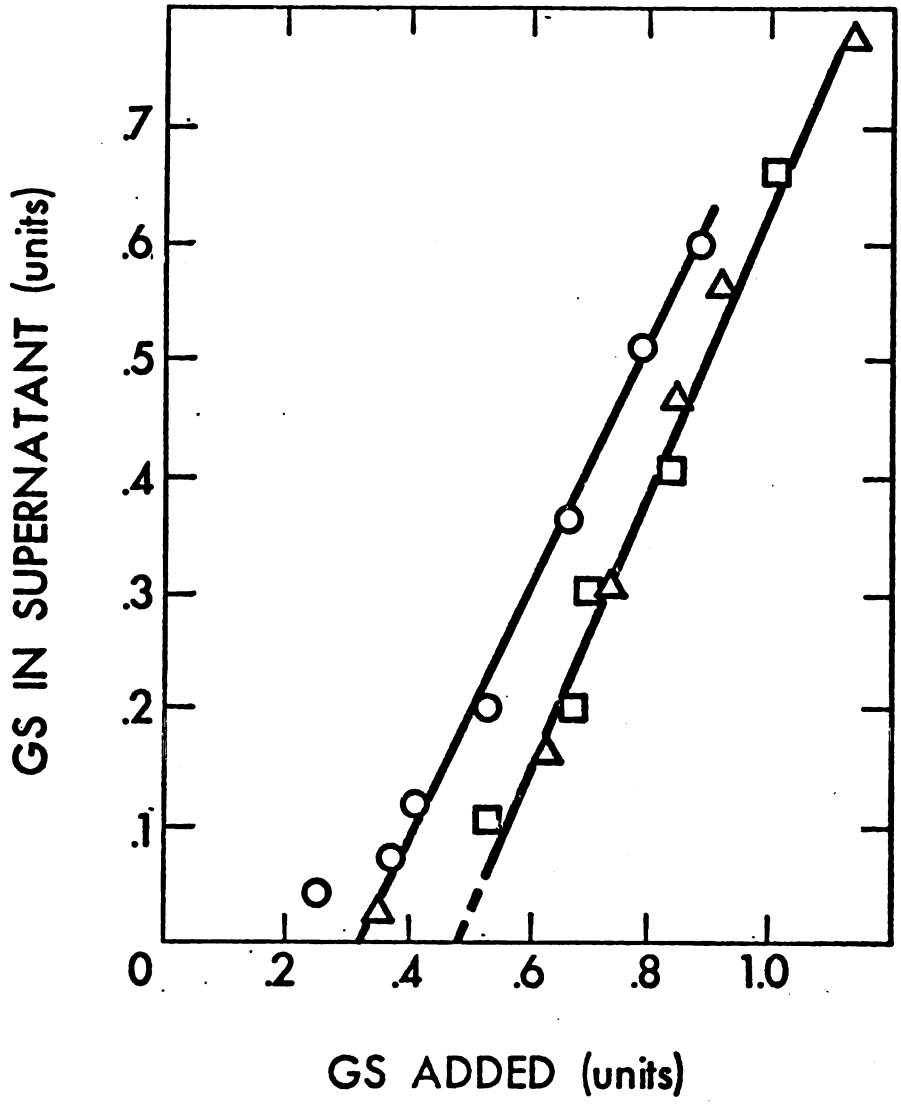
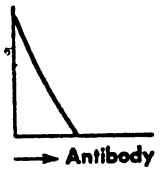

GS IN SUPERNATANT (units)
.7
.6
.5
.4
.3
.2
.1
0
.2
.4
.6
.8
1.0
GS ADDED (units)

Table 4. Possible combinations of the variables of the equivalence titration experiments. S=same, D=different. Column Four indicates which experiment if any, eliminates the combination of variables in a given row, and why.

I



II

	# MOLECULES	SA PER MOLECULE	ANTIGENIC REACTIVITY/MOL.		REASON NOT POSSIBLE
1.	S	S	S	..... II:	Should see same equivalence points
2.	S	S	D	..... I:	Should see different equivalence points
3.	S	D	D	.....	
4.	S	D	S	..... I:	Should see different equivalence points for the same activities
5.	D	S	D	..... I:	Same as #4
6.	D	D	S	..... II:	Should see same equivalence points
7.	D	D	S	..... I:	Curves should not be identical
8.	D	D	D	.....	

Fig 20a. Sucrose gradient sedimentation of glutamine synthetase. Cells were grown as in Fig 17a. 300-450 µg of extracts from each was applied to a gradient made , run, and analyzed as described in Materials and Methods. b. Sedimentation of GS in sucrose gradients containing 0.4 M NaCl. Extracts prepared from cells grown as in Fig 17a and centrifuged as in Fig 20a, except that the sucrose gradients contained 0.4 M NaCl. ● , 2 mM glutamine; ▲ , 2 mM glutamine plus 10^{-7} M dex; ■ , 0.2 mM glutamine; ○ , 0.2 mM glutamine plus 10^{-7} M dex.

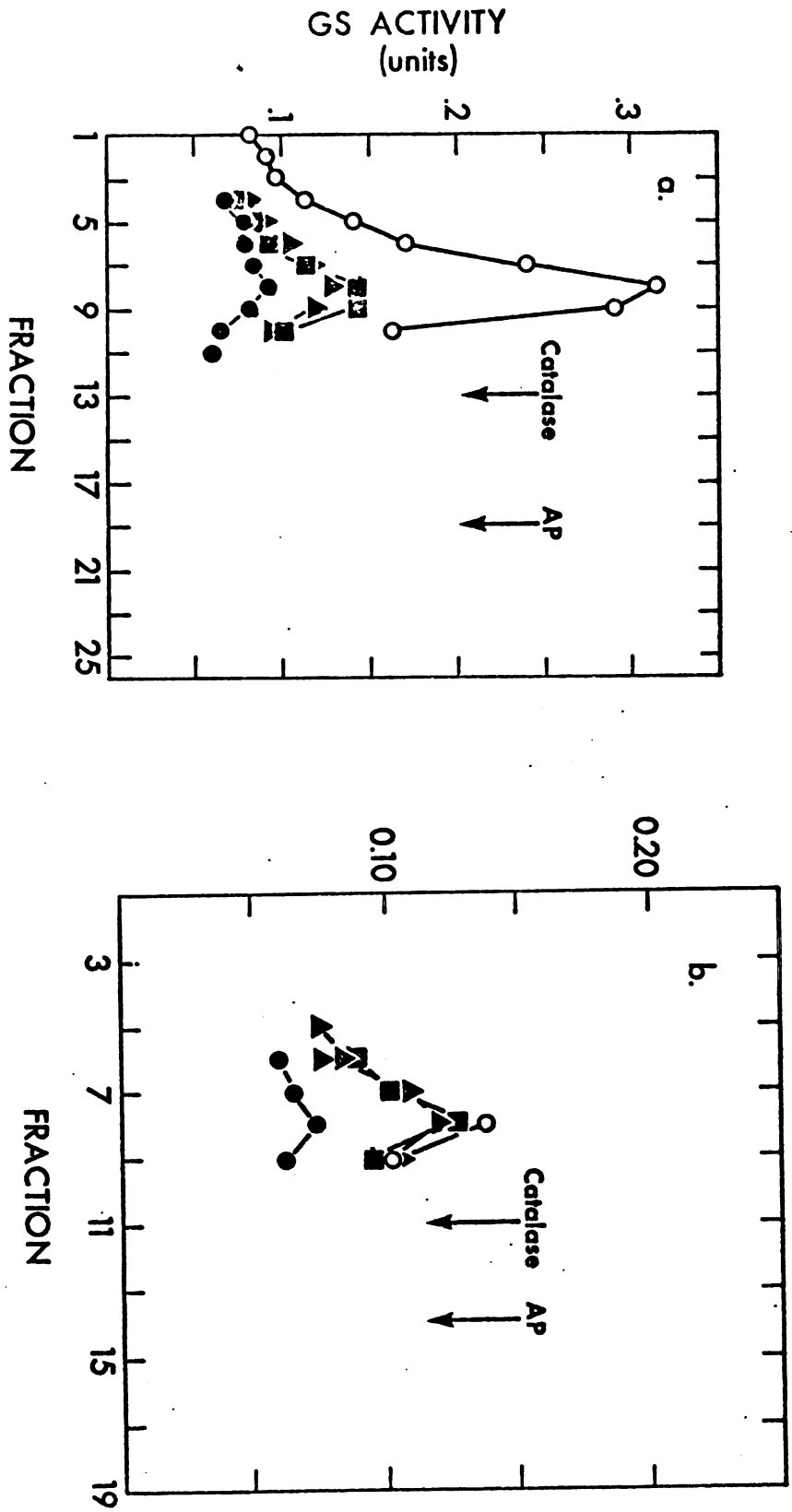

GS ACTIVITY
(units)
.1
.2
.3
a.
Catalase
AP
FRACTION
1
5
9
13
17
21
25
0.10
0.20
b.
Catalase
AP
FRACTION
3
7
11
15
19

Fig 21. Heat denaturation of glutamine synthetase. Cells grown as in Fig 17a were heated to various temperatures for 5 minutes and then analyzed as described in Materials and Methods.

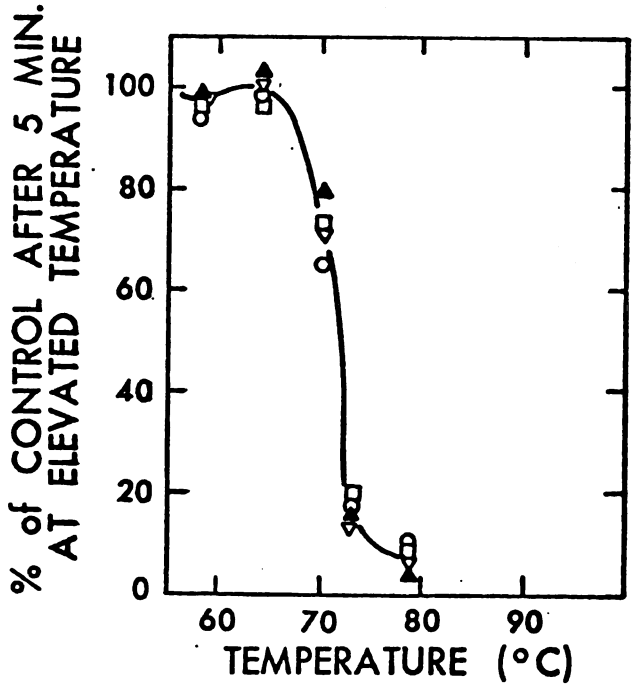

% of CONTROL AFTER 5 MIN.
AT ELEVATED TEMPERATURE
100
80
60
40
20
0
60
70
80
90
TEMPERATURE (°C)

Fig 22. DEAE cellulose chromatography of glutamine synthetase. Cells were grown as in Fig 17a. Extracts were prepared, and 6 mg of each extract was applied to a 6 ml DEAE cellulose column. The extract was equilibrated and eluted as described in Materials and Methods. Cells grown in (a) 2 mM glutamine; (b) 2 mM glutamine plus 10^{-7} dex; (c) 0.2 mM glutamine (d) 0.2 mM glutamine plus 10^{-7} M dex.

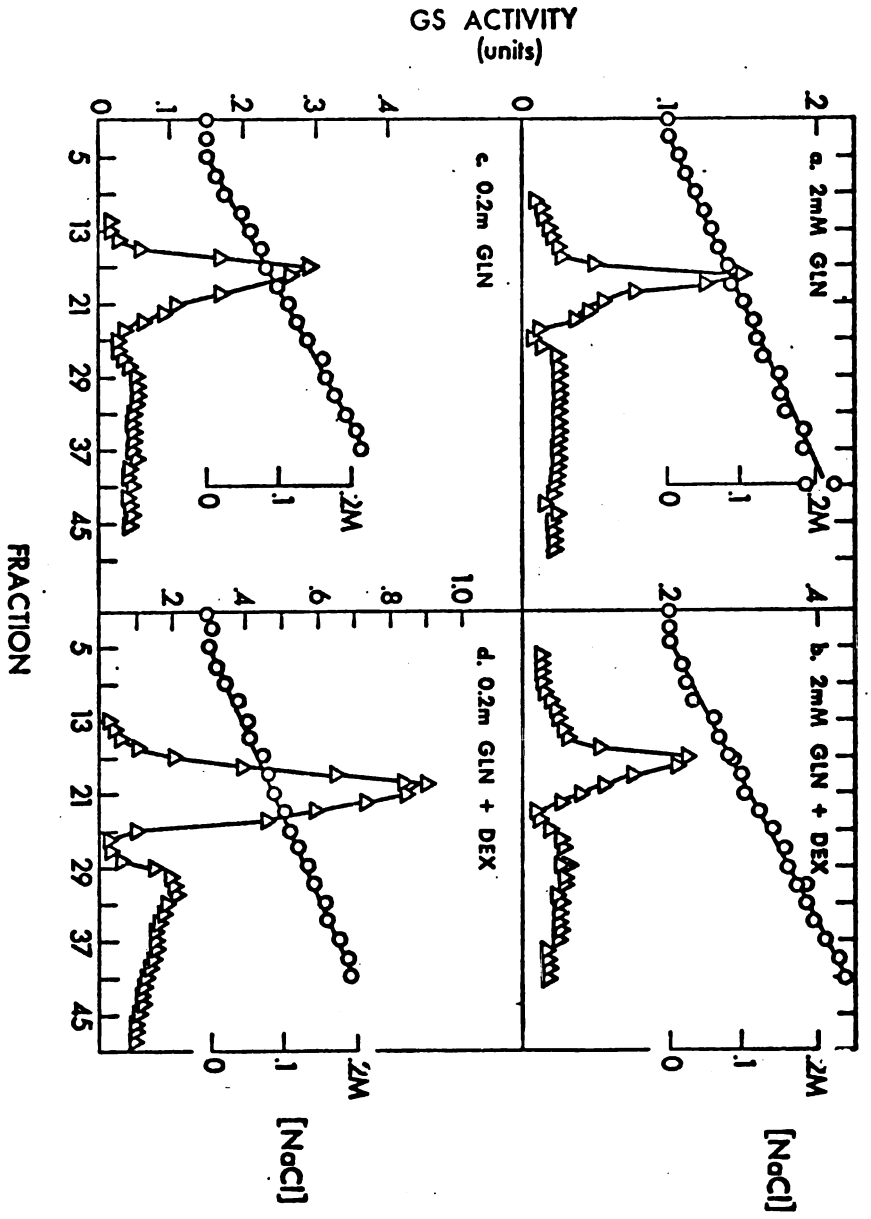
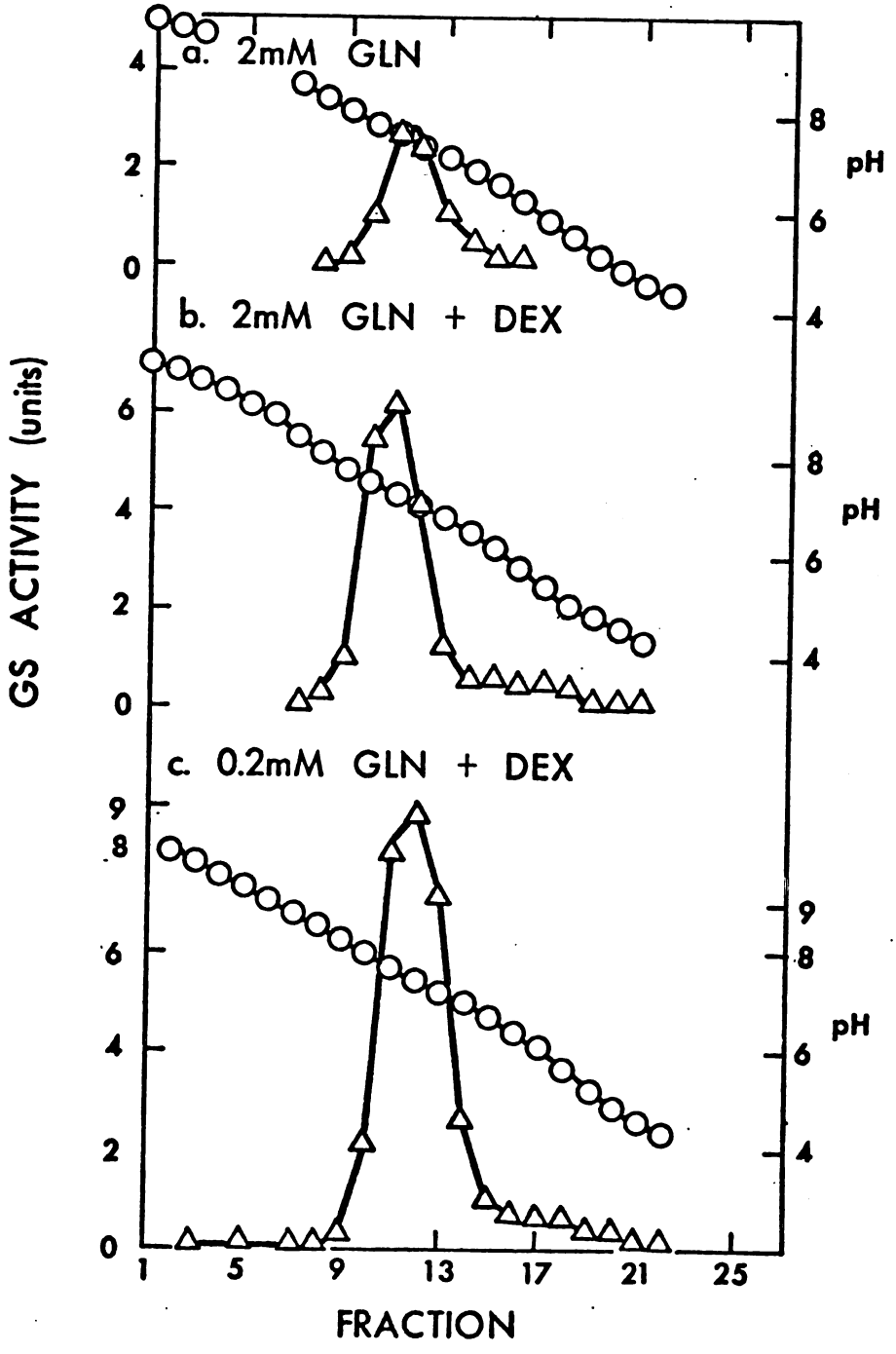

GS ACTIVITY
(units)
a. 2mM GLN
b. 2mM GLN + DEX
c. 0.2m GLN
d. 0.2m GLN + DEX
[NaCl]
FRACTION

Fig 23. Isoelectric focussing of glutamine synthetase. Cells were grown for 24 hours in Growth Medium containing; a., 2mM glutamine; b., 2 mM glutamine plus 10^{-7} M dex; c., 0.2 mM glutamine plus 10^{-7} M dex. 1 mg of each extract was run as described in Materials and Methods.


a. 2mM GLN
b. 2mM GLN + DEX
c. 0.2mM GLN + DEX
GS ACTIVITY (units)
pH
FRACTION
1
5
9
13
17
21
25

CHAPTER SIX

RESULTS

IDENTIFICATION OF IMMUNOPRECIPITATED GS

When an extract of GM22 cells is labelled for 30 minutes with [^{35}S] methionine, and the GS immunoprecipitated and electrophoresed on a 10% SDS slab gel as described in Materials and Methods, the pattern seen on X-ray film exposed to the gel is complex (Fig 24). A variety of proteins over a wide range of molecular weights are present. That all but one of the bands are not specifically precipitated by anti-GS, and that one band is GS, are suggested in the following results.

(1) In the absence of anti-GS, with purified rabbit IgG added as antigen, the indirect precipitate of a labelled extract of dex induced cells contains all but one band of the set seen in the presence of anti-GS (Plate 1, columns a-d, and Figure 26). In this set of gels, another band appears to be absent in the absence of anti-GS; however, this is not a reproducible finding. Thus in a heated S-30, anti-GS is monospecific.

(2) The band specific for anti-GS coelectrophoreses with purified rat liver GS (Plate 2).

(3) This band is inducible by dex (Fig 24, column e and f and Figure 27a). In cells exposed to dex for 12 hours, it is the only band which is induced. Moreover, it is induced about 3 fold as determined

by weighing the peak on a gel scan corresponding to this band, This is the induction predicted by previous experiments (see Figure 1).

(4) A cloned HTC cell line auxotrophic for glutamine, called ST3, possesses a barely detectable GS activity which is not inducible by dex or glutamine (see Table 5, Chapter Seven). A GS immunoprecipitate made from this cell line (grown 12 hours in dex) possesses reduced amounts of this band (Figure 25b).

Further purification of the extract from GM22 was not attempted in the experiments which follow in spite of the nonspecific background of labelled protein, because the putative second form of GS might be lost in the process.

DEX INDUCTION OF GS

The effect of dex on the rate of synthesis of GS is shown in Figure 26. The rate is approximately 2-2.5 times as great for GS in cells exposed for 12 hours to dex as compared to the control culture not exposed to dex. When peak weight is converted to radioactive decay, the rate of incorporation into GS is about 4,000 cpm/hr for induced cells, and 1,400 cpm/hr for uninduced cells. The rate of general protein synthesis is not affected by dex (insert, Figure 26).

The effect of dex on the rate of turnover of GS was next investigated. Induced and uninduced cells were labelled for 35 minutes and then chased with Growth Medium containing 2 mM glutamine with or without dex, for varying lengths of time. The results (Figure 27) show that the half-time for GS degradation is about 1.7 hours in the presence or absence of dex. Thus, dex appears to increase the synthetic rate of GS

but not affect the rate of degradation. The turnover of total protein is not affected by dex (insert, Figure 27).

The time course of dex induction is shown in Figure 28. The rate of GS synthesis increases approximately 3 fold, reaching a new steady state at 4-6 hours after the addition of dex. The specific activity of GS in these cultures can be seen to rise, after a lag of about 30 minutes, and plateau somewhat later, after 8 hours.

The time course of dex deinduction is shown on Figure 29. When dex is washed out of these cultures, the rate of GS synthesis falls with a half-time of roughly 3-4 hours. Thus the continued presence of dex is required for the elevated rate of GS synthesis. If 0.5 μg/ml AMD is added at the time of dex washout to one set of cultures, maintenance of the elevated rate continues in the absence of dex for 6 hours. This result suggests that RNA synthesis is required for the normal fall in the rate of GS synthesis to the basal rate seen when dex is washed out of the cells.

GLUTAMINE INDUCTION OF GS

To investigate how glutamine influences the change in GS specific activity, rates of synthesis of GS in 0.2 mM and 2 mM glutamine were measured (Figure 30). The rate of synthesis of GS in 0.2 mM glutamine appears to be about 80% that in 2 mM glutamine where the specific activity of GS in .2 mM glutamine-grown cells is 5-fold higher. That the rate of GS synthesis on 0.2 mM glutamine is lower may be attributable to the fact that general protein synthesis in 0.2 mM glutamine-grown cells is about 30% that of 2 mM glutamine-grown cells (Fig. 30, insert).

The rate of turnover of GS in 0.2 mM and 2 mM glutamine-grown cells was next investigated. Cells grown 12 hours in 0.2 mM and 2 mM glutamine were pulsed with [^{35}S] methionine, then chased with Growth Medium containing the original concentrations of glutamine, for varying lengths of time. The results (Figure 31) indicate that the rate of turnover of GS proceeds with a half-time of about 1.7 hours in cells grown in 2 mM glutamine, but with a half-time of 8-10 hours in cells grown is 0.2 mM glutamine. The ratio of these rates corresponds to the ratio of specific activities of GS from the two cultures. By comparison, the turnover of general protein can be seen to be unaffected by the glutamine concentration.

To investigate the dependence of GS turnover of glutamine more fully, cells grown for 12 hours in various concentrations of glutamine were pulsed for 30 minutes with [^{35}S] methionine and then chased for three hours in Growth Medium containing the original concentrations of glutamine. The data, shown in Figure 34, suggest that the rate of turnover of GS is a function of glutamine concentration, and is directly paralleled by the specific activity of GS in these extracts. The rates of GS synthesis is at the various glutamine concentrations are shown in the insert. Figure 35a is a replotting of the date to show that the half-times of GS decay are a function of the glutamine concentration. Figure 35b is the relation between GS specific activity and GS degradation half-time. That the plot in the last does not extrapolate to zero specific activity when the $t_{\frac{1}{2}}$ is zero is probably a comment on the sensitivity of the enzyme assay.

The kinetics of the shift in the degradation rate of GS which occurs when grown in 0.2 mM glutamine are given 2.0 mM glutamine was

next investigated. Cells grown in 0.2 mM glutamine were pulsed for 40 minutes with [^{35}S] methionine in 0.2 mM glutamine and then chased in medium containing no isotope and 2 mM glutamine for various length of time. The results (Figure 36) indicate that a period of roughly 30 minutes elapses before the new rate of GS degradation characteristic of 2 mM glutamine-grown cells is established. The specific activity of GS turnover rate, (1.25 hours vs. 1.9 hours) presumably reflects the contributing of ongoing synthesis of catalytically active GS to the total GS pool assayed at each time.

DISCUSSION

These results demonstrate that dex treatment of GM22 cells results in an increase in the rate of GS synthesis, and that the rate of degradation of GS is not affected by dex. A new steady state concentration of GS molecules occurs in 8-10 hours which is about 2.5-3 fold greater than that of cells without dex.

The maintenance of induced GS synthesis in the absence of steroid, in the presence of AMD suggests that some gene product is required for the reduction in the rate of GS synthesis, and that this gene product turns over rapidly. A simple model would be that a labile RNA (or the protein that it codes for) affects the concentration of specific RNA which causes the increase in rate of synthesis of GS. This RNA could be GS mRNA or an RNA which enhances translation of GS in some other way. When steroid is present, the concentration of this RNA increases. When AMD is present, the labile degrading function disappears and the synthesis of new GS-specific RNA ceases. With the synthetic and degradative rates of GS RNA reduced to zero, the concentration of GS remains constant.

Such a model has been proposed to account for the regulation of TAT by steroids in HTC cells (81). In fact, the similarities in the dex response of TAT and GS are quite similar. Both depend upon RNA and protein synthesis for induction (95,101); both require RNA synthesis for deinduction, and the kinetics of both processes are roughly similar for both enzymes (102). The only salient difference is in the extent of induction, which is 8-10 fold for TAT but 2.5-3 fold for GS.

The dose-response curves for TAT and GS are identical (Figure 3). This fact suggests that the steroid receptor implicated in the TAT response is the same in the GS response.

These similarities lead to the view that the control mechanism by which steroids act to induce TAT and GS are functionally quite similar. That TAT and GS both deinduce with half-times of between 1½-4 hours suggests that the degradation mechanism of RNA for each enzyme are also similar. It may be that the same specific nuclease recognized the RNA of both TAT and GS due to similarities in their sequence and/or tertiary structure.

The difference in induction ratio between TAT and GS might be due to the rate of synthesis or processing and transport of these two gene products. However, the steroid deinduction data presented here are not detailed enough, nor have appropriate precautions been taken with variables such as serum, which influence the lag period in deinduction, to say that the half-times are identical. Thus, the difference in extent of induction between TAT and GS could be due to differences in the degradation rate. This might be an interesting subject for a future study.

The studies of the effect of glutamine on the rate of GS synthesis show that a ten-fold reduction of glutamine, which leads to an increased GS specific activity (Figure 5) results in a slight decrease in the synthetic rate. This is probably due to a general decrease in protein synthesis caused by reduction of the concentration of glutamine, although the effect on GS synthesis (about a 20% reduction) is much less than the general effect.

The effect of glutamine on GS degradation on the other hand, is to increase the rate of degradation as an direct function of the glutamine concentration, from .3 mM to at least 2 mM glutamine. The specificity of the effect is illustrated by the fact that general protein synthesis turnover proceeds with a half-time of greater than 10 hours in both cases. These data are consistent with those of Hershko and Tomkins (89) who found in HTC cells that general protein turnover proceeds at a rate of about 3% per hour under normal conditions and 5% per hour under various conditions of nutritional stepdown.

What is the immediate fate of GS when it is rendered catalytically inactive? The equivalence point titrations together with the single identity of GS observed during various separation techniques, suggests a catalytically inactive, immunologically cross-reactive form of GS is present in cells grown in high glutamine. No evidence, however, was found for a second band of GS on SDS gels, by the following criteria: no second band was specifically precipitated by anti-GS, or was seen to increase in radioactivity as counts were chased out of the main GS band. Also, no other band was induced by dex, though this would not necessarily be proof of identity.

A second form of GS may exist, but not have been detected on gels for several reasons.

(1) The gel procedure involves a heat step. This step allows for a 3-4 fold purification of the GS activity, with no loss in activity. For technical reasons, it has not been possible to do an equivalence point titration on heated extracts. Therefore it is possible that heating denatures a second GS form and it is lost in the 30,000 xg centrifugation step which follows heating. Gels of unheated extracts have been run, with and without a cold chase, with and without anti-GS in the immunological reaction. No second GS band has been seen.

(2) A second form may be on the gels but not detectable because of the background protein pattern. This is especially possible in gels of unheated extracts, where background protein is more prominent.

(3) The second form may be a fragment which runs at the leading end of the gel, where resolution is reduced.

(4) The second form may not enter the gel.

A more thorough investigation, involving partial purification of GS using methods which produce extracts which maintain immunilogocal differences but cleaner gel patterns is an important next step.

What is the mechanism of GS inactivation? These data shed little light on the subject. It could be a co-valent modification, such as occurs in prokaryotes (see Chapter 1). If this process is reversible, protein synthesis is required for the production of catalytically active from inactive GS. Alternatively, the mechanism could be proteolytic cleavage, followed by more extensive hydrolysis to amino acids. (For a discussion of this as general mechanism, see ref. 103). Since the enzymatic function which inactivates GS is not known, it will be

referred to as GIF (GS Inactivating Factor). Whether GS is hydrolysed by a proteolytic cleavage or modified by covalent reaction, or some other mechanism, glutamine must either identify GS molecules as available for inactivation by GIF, which is catalytically active and merely seeks a substrate; or, glutamine activates GIF by non-covalent interaction; then GIF specifically interacts with GS. Both mechanisms may exist, as well. Freikopf and Kulka (69) have eliminated the GS active site as the position of glutamine binding by the identification of glutamine analogues which inhibit degradation but not enzyme activity.

An example of a catalytically active protease which requires substrate indentification is provided by Katunuma and his colleagues (104), who have shown that there exist in several mammalian organs a protease which is specific for pyridoxal requiring enzymes. In the absence of pyridoxal, the target enzymes are degraded by the group specific protease, whereas the presence of the co-factor stabilized the enzymes against degradation. Non-pyridoxal requiring enzymes are not affected by the protease.

There is genetic evidence that a degrading function exists in mice which is specific for catalase, but is not dependent on catalase activity (105,106). A mutation which inactivates this function has no effect on the degradation of other cellular proteins.

An interesting characteristic of GIF is that, unlike the proteases responsible for general protein degradation, and unlike those responsible for "enhanced" degradation observed when cells are deprived of amino acids or serum, it is inactivated by glutamine step-down (89). Moreover, it is rendered inactive by cycloheximide treatment of the

cells. In the latter respect, GIF bears resemblance to the degradation system of "enhanced" degradation, which is also inhibited by cycloheximide (89).

The cycloheximide result may have other explanations than GIF turnover. The inhibition of GS inactivation by cycloheximide may be due to the turnover of some other protein which activates GIF. Alternatively, the pool of a molecule which modifies GIF, such as an amino-acyl tRNA, might change with the blockage of protein chain elongation, thus modifying the rate of GS inactivation. Charged amino-acyl tRNA has been implicated in the regulation of the histidine operon in Salmonella (107).

Certainly the logic of degradation control in the case of GS seems reasonable. Under glutamine starvation condition, the organism seeks to increase its glutamine level by increasing enzyme activity. This would be difficult through the mechanism of increasing synthesis of the protein, since the effect of amino acid starvation on cells is to slow protein synthesis. Decreasing the rate of degradation of GS can presumably be accomplished with little energy requirement, and no additional protein synthesis, thus increasing the GS concentration in the cells which ultimately results in an increase in intracellular glutamine. Moreover, the lability of GIF insures that if protein synthesis halts for reasons other than glutamine deprivation, GS, though no longer synthesized, will not be continuously degraded, so that the level of GS will not fall by this specific mechanism far below its level at the time of protein synthesis cessation.

Other enzymes in animals have been reported to display control which modulates both their synthesis and degradation. The rate of

synthesis of tryptophan oxygenase is reported to be increased 4-5 fold in rat liver by steroid hormones, while the presence of the substrate, tryptophan, causes a decrease in the rate of degradation of the enzyme (108,109). Acetyl Co A carboxylase, the rate limiting enzyme in fatty acid synthesis, in rat liver, is responsive to dietary conditions both it its rate of synthesis and degradation (110). In a fat free dietary state, the enzyme is degraded with a half-time of 48 hours relative to total protein, whereas under starvation conditions the half-time of 48 hours. If the diet is shifted from fat-free to fat-containing (Purina rat chow), the rate of synthesis of the enzyme appears to increase 2-fold.

Plate 1. SDS acrylimide gel electrophoresis of glutamine synthetase immunoprecipitates. Cells were grown in 2 mM glutamine plus 10^{-7} M dex (columns a-c and f-i) and without dex (columns c-e). Cells were labelled with $[^{35}S]$ methionine for 30 minutes as described in Materials and Methods. GS immunoprecipitates were made using anti-GS as the antibody (columns a-e, f,h) or without anti-GS with 4 µg rabbit IgG as the antigen (columns g and i). Immunoprecipitate preparation, electrophoresis, and film development were as described in Materials and Methods.

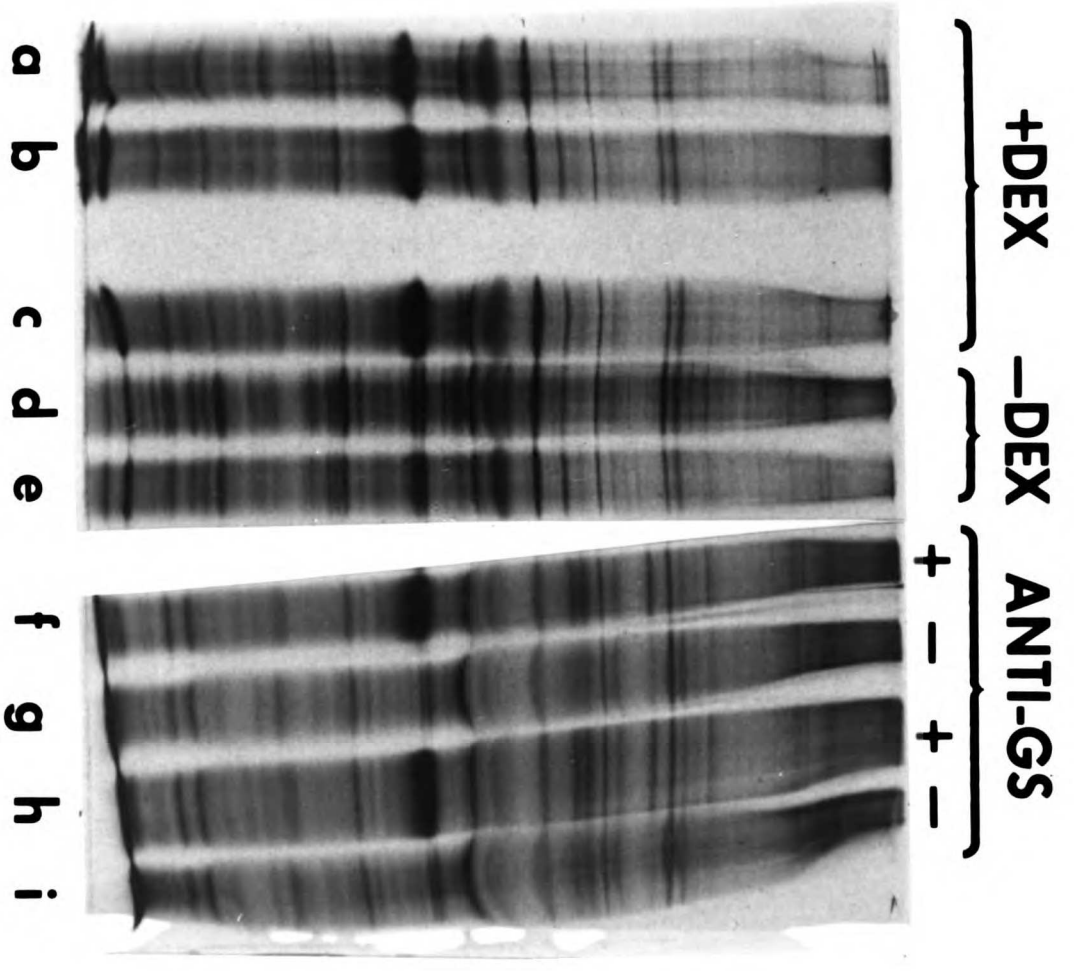

a b c d e f g h i
+DEX
−DEX
ANTI-GS
+ − − + + −

Fig 24. SDS acrylimide gel electophoresis of immunoprecipitates. Gels shown in Plate 1, columns 1 and 2, were scanned with a microdensitometer as described in Materials and Methods. Upper scan: with anti-GS; lower scan: without anti-GS.

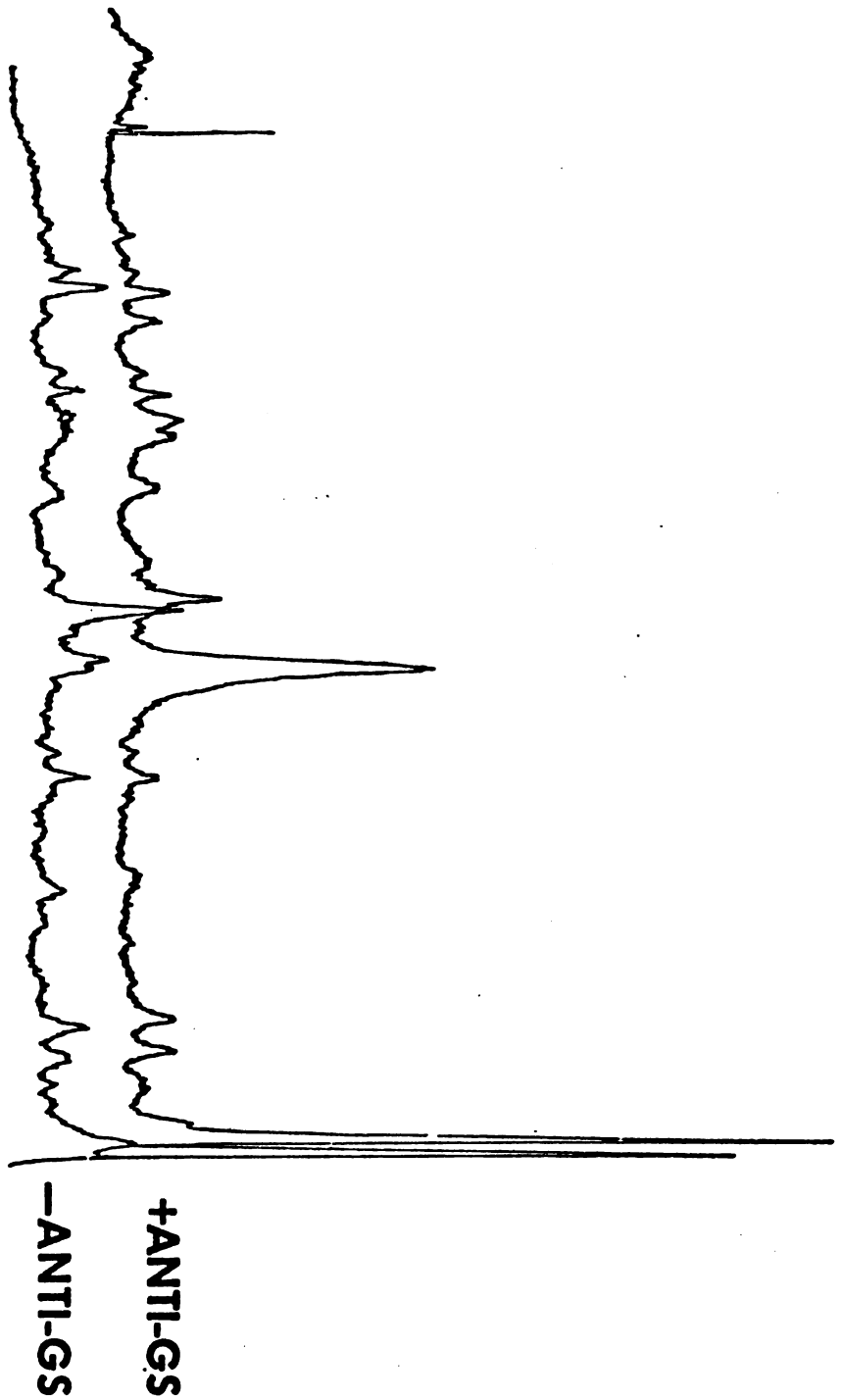

+ANTI-GS
—ANTI-GS

Plate 2. SDS acrylimide gel electrophoresis of purified rat liver glutamine synthetase and glutamine synthetase immunoprecipitate of cells grown, and labelled, as in Plate 1. Rat liver GS was diluted with SDS sample buffer to a concentration of 1 mg/ml. Electrophoresis and film development were as described in Plate 1. Columns: (a) rat liver GS; (b) X-ray film of GS immunoprecipitate; (c) Comassie blue-stained gel of the same GS immunoprecipitate (for orientation of the film in b.)

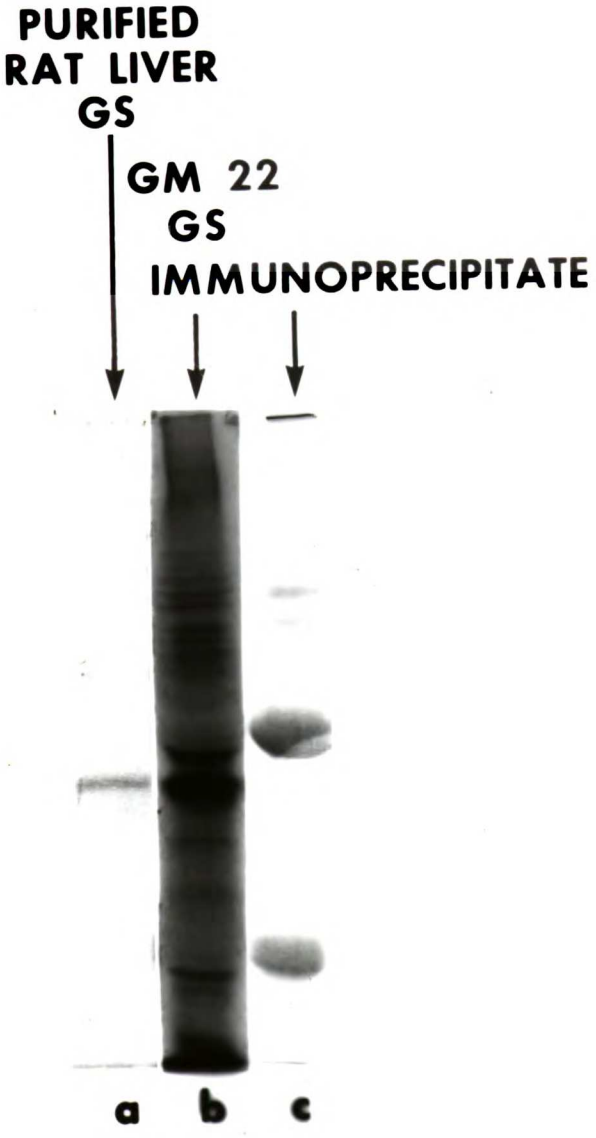

PURIFIED
RAT LIVER
GS
GM 22
GS
IMMUNOPRECIPITATE
a
b
c

Fig 25. SDS acrylimide gel electrophoresis. (a) The gel shown in Plate 2 columns were scanned as in Fig 24. Upper scan: plus dex. Lower scan: minus dex. (b) SDS acrylimide gel electrophoresis of a GS immunoprecipitate from ST3 cells. ST3 cells were grown 12 hours in Growth Medium with 2 mM glutamine plus 10^{-7} M dex. They were then labelled, and an immunoprecipitate was prepared and analyzed as in Fig 25a. Arrow indicates the predicted position of GS.

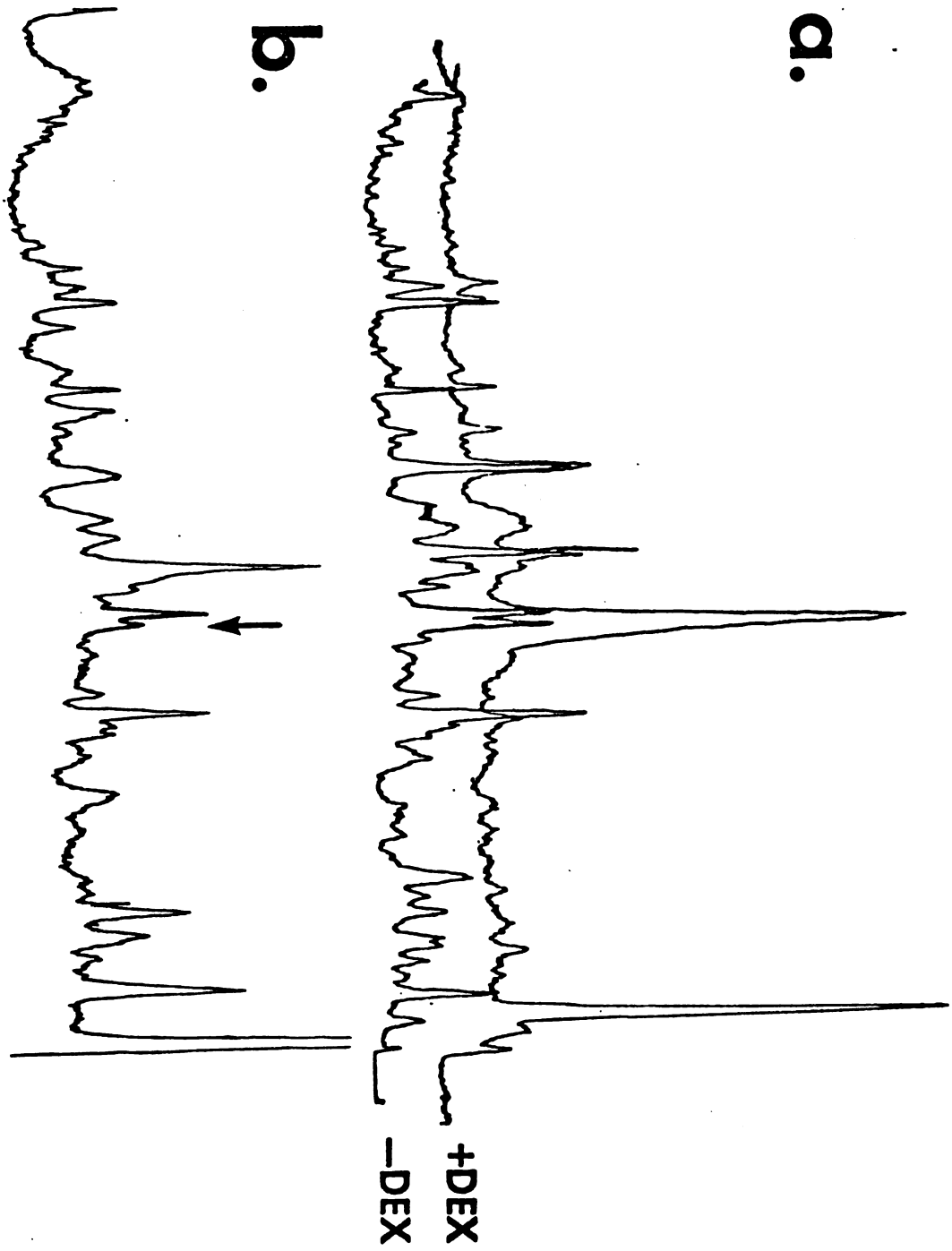

a.
+DEX
—DEX
b.

Fig 26. Effect of dexamethasone on the rate of synthesis of glutamine synthetase. Cells were grown in monolayer as described in Materials and Methods in Growth Medium containing 2 mM glutamine either (△) with or (□) without 10^{-7} dex. They were then washed and resuspended in Cloning Medium containing 2 mM glutamine, with and without 10^{-7} M dex, and allowed to incubate for various lengths of time. The incubation was stopped, extracts were then made and analyzed as described in Materials and Methods. Insert: the rate of total protein synthesis in cells grown with or without dexamethasone. 2 µl aliquots were taken from the extract and analyzed for isotope incorporation into total protein as described in Materials and Methods.

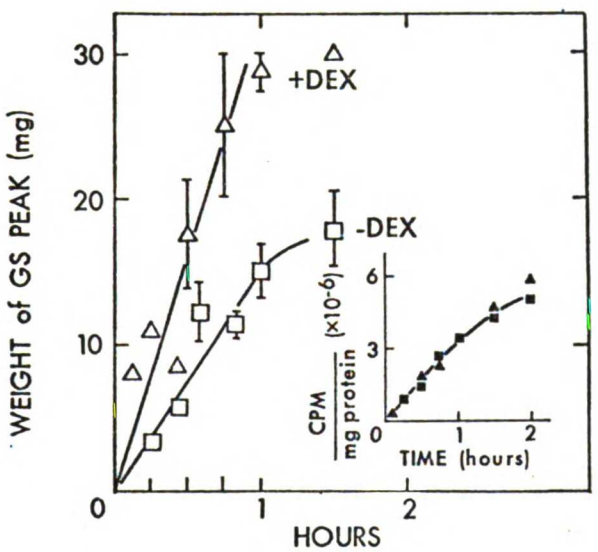

WEIGHT of GS PEAK (mg)
30
20
10
0
1
2
HOURS
+DEX
-DEX
CPM / mg protein (×10⁻⁶)
6
3
0
1
2
TIME (hours)

Fig 27. Effect of dexamethasone on the turnover of glutamine synthetase. Cells grown as in Fig 26 were incubated in [^{35}S] methionine as in Fig 26 for 35 minutes, then washed and resuspended in Growth Medium containing 2 mM glutamine with or without 10^{-7} M dex, for various lengths of time. The cells were then processed as in Fig 26. Δ , plus dex; O , minus dex. Insert: the rate of degradation of general protein in the same cells. The procedure in insert, Fig 26, was followed.

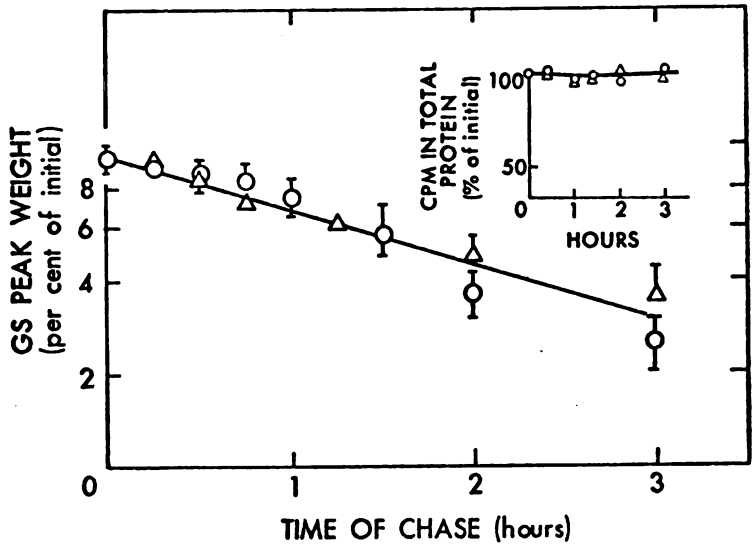

GS PEAK WEIGHT
(per cent of initial)
8
6
4
2
0
1
2
3
TIME OF CHASE (hours)
CPM IN TOTAL
PROTEIN
(% of initial)
100
50
0
1
2
3
HOURS

Fig 28. Kinetics of the dexamethasone induction of glutamine synthetase rate of synthesis. Cells grown in Growth Medium with 2 mM glutamine were exposed to 10^{-7} M dex for various length of time. The cells were then washed and resuspended in Labelling Medium with 2 mM glutamine, 170 μCi/ml $[^{35}S]$ methionine, and 10^{-7} M dex (except zero time cultures which did not receive dex) for 30 minutes. Extracts were prepared as in Fig 26, 25 μl was taken from each for GS assays, and immunoprecipitates were made and analyzed as in Fig 26. O , GS peak weight; ▲ , GS specific activity.

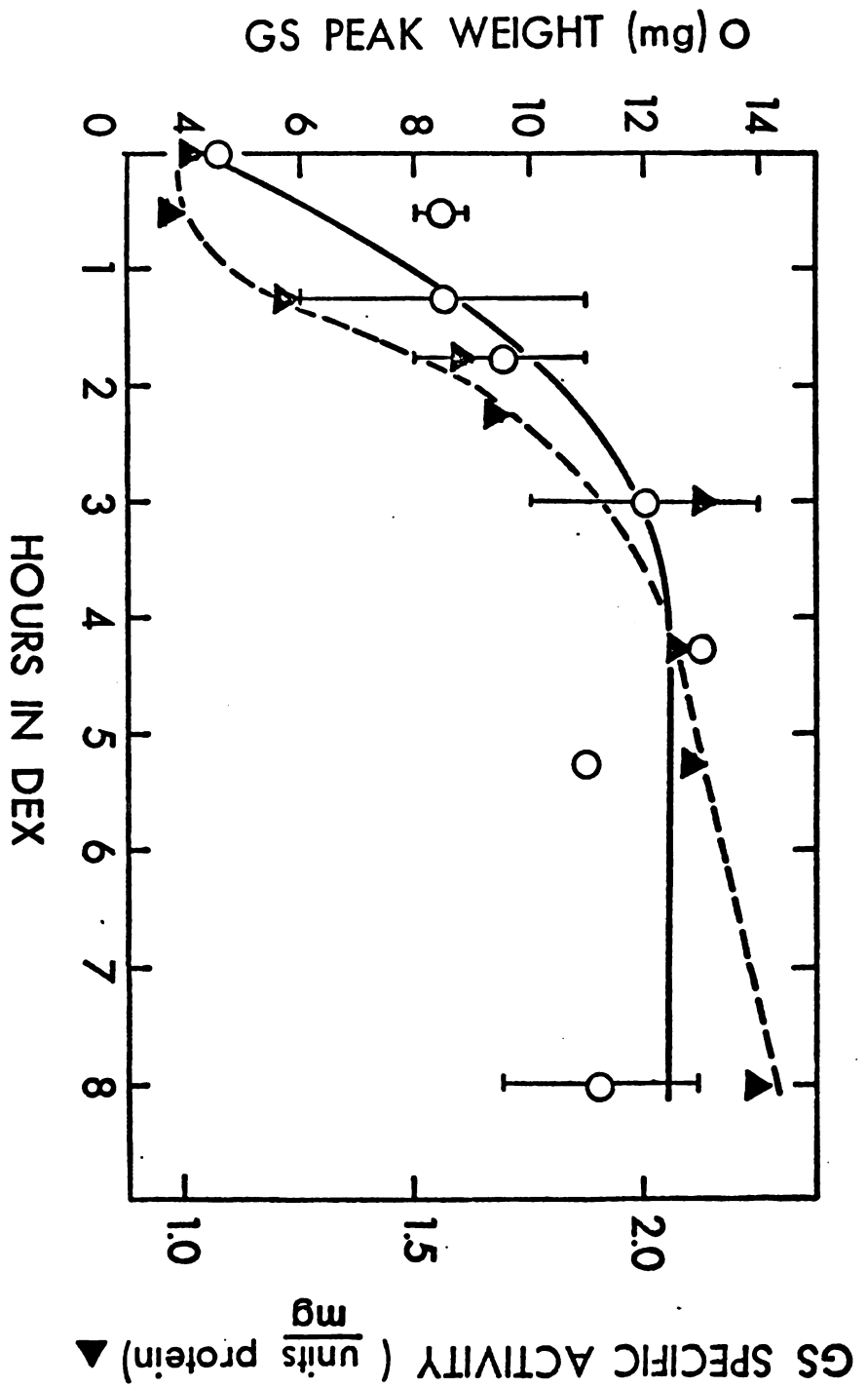

GS PEAK WEIGHT (mg) O
HOURS IN DEX
GS SPECIFIC ACTIVITY (units/mg protein) ▲
O
O
4
6
8
10
12
14
0
1
2
3
4
5
6
7
8
1.0
1.5
2.0

Fig 29. Effect of the removal of dexamethasone, in the presence and absence of actinomycin D, on the rate of synthesis of glutamine synthetase. Cells were grown in Growth Medium containing 2 mM glutamine and 10^{-7} M dex for 12 hours. They were then washed and resuspended in Growth Medium with 1 mM glutamine without dex and either (Δ) with or (○) without 0.5 µg/ml AMD for various times. Cells were labelled, immunoprecipitates were prepared, and analyzed as described in Fig 26.

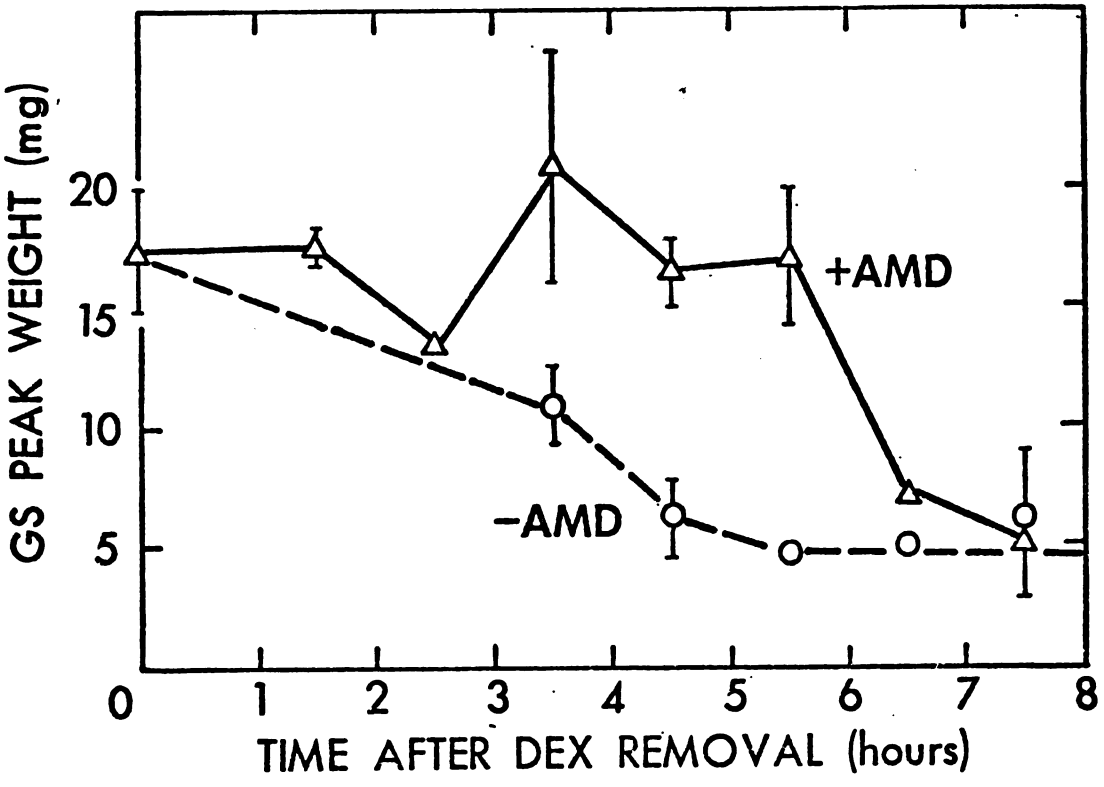

GS PEAK WEIGHT (mg)
20
15
10
5
0
1
2
3
4
5
6
7
8
+AMD
-AMD
TIME AFTER DEX REMOVAL (hours)

Fig 30. The rate of synthesis of glutamine synthetase in cells grown in 0.2 mM and 2 mM glutamine. Cells were grown for 20 hours in Growth Medium containing 10^{-7} M dex and either (○) 0.2 mM or (△) 2 mM glutamine. They were then labelled with [^{35}S] methionine as in Fig 26 for varying lengths of time. Immunoprecipitates were made and analyzed as in Fig 26. Insert: the rate of total protein synthesis in these cultures. 2 µl aliquots were taken from the extracts and analyzed for incorporation of isotope into total protein as described in Fig 26. (Same symbols as in main figure.)

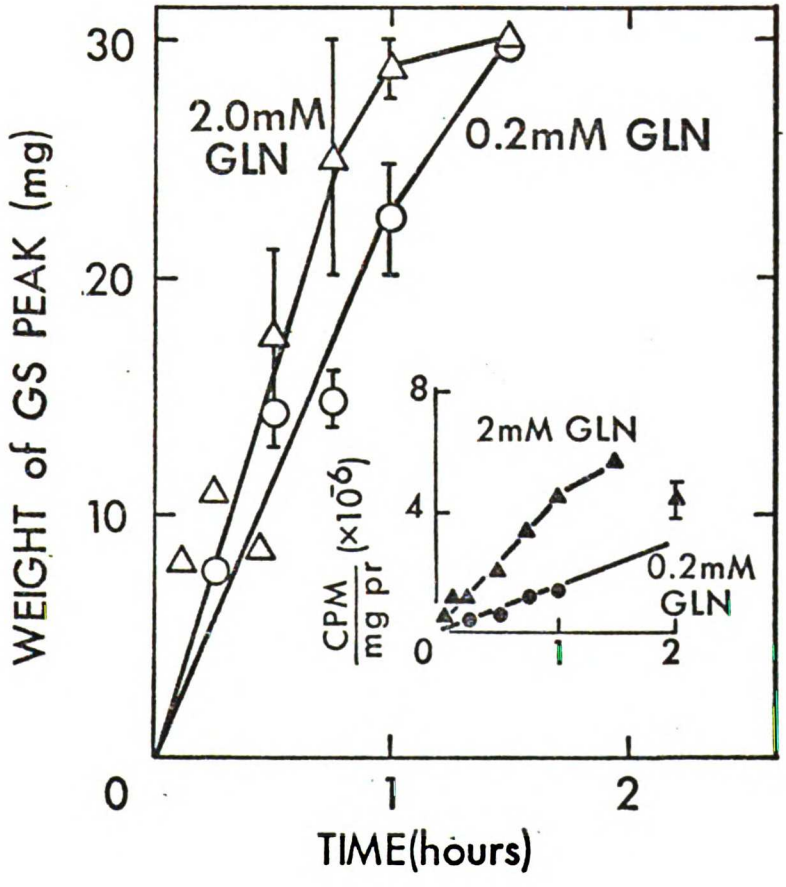

30
20
10
0
1
2
WEIGHT of GS PEAK (mg)
TIME(hours)
2.0mM GLN
0.2mM GLN
8
4
0
1
2
2mM GLN
0.2mM GLN
CPM / mg pr (×10⁻⁶)

Fig 31. Rate of glutamine degradation in cells grown in 2 mM and 0.2 mM glutamine. Cells grown in Growth Medium containing 10^{-7} M dex, and either ○, 0.2 mM glutamine of □, 2 mM glutamine, were incubated in [^{35}S] methionine for 30 minutes as in Fig 30, and then washed and resuspended in Growth Medium containing either 2 mM or 0.2 mM glutamine, for various lengths of time. Immunoprecipitates were prepared and analyzed as in Fig 26. 2 µl samples were taken and analyzed for the rate of isotpoe incorporation into total protein as in Fig 26. (same symbols). GS specific activity from unlabelled samples: 0.2 mM glutamine, SA= 21+2 units/mg protein; 2 mM glutamine, SA=5+1 units/mg protein.

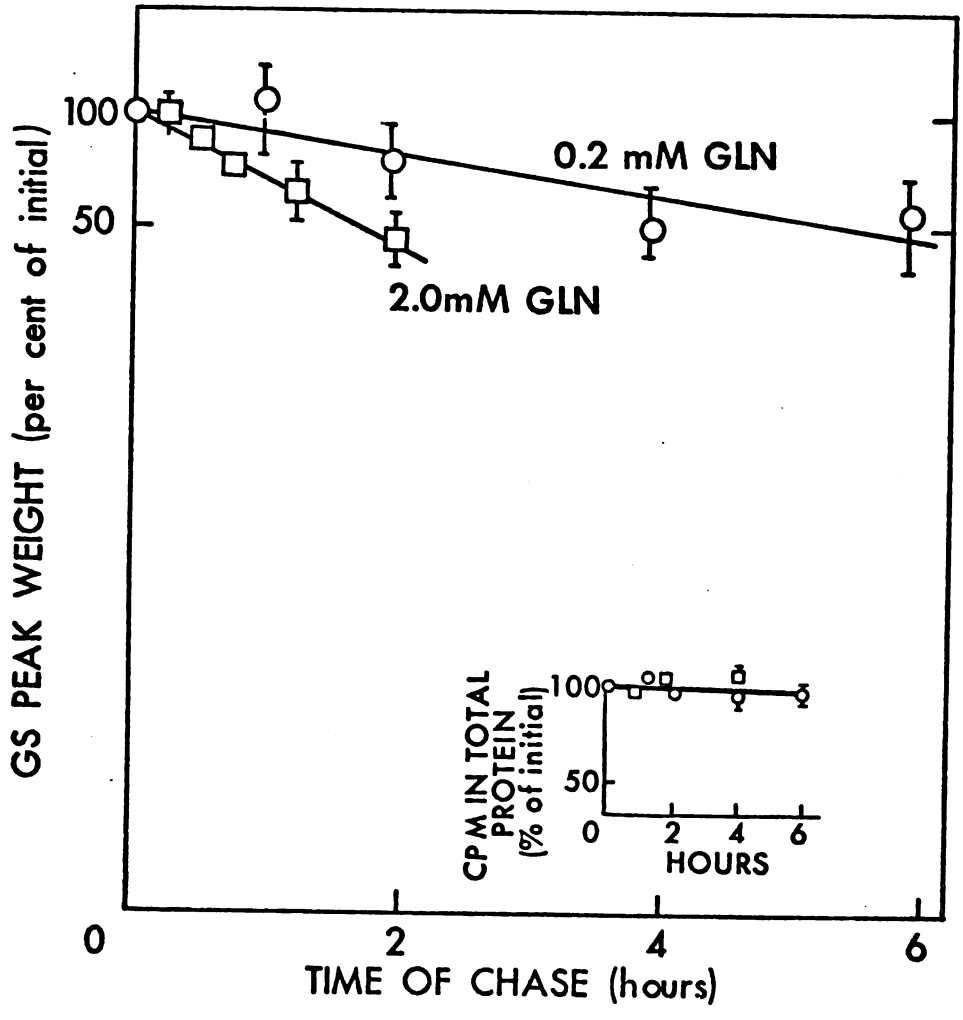

GS PEAK WEIGHT (per cent of initial)
100
50
0
0.2 mM GLN
2.0mM GLN
2
4
6
TIME OF CHASE (hours)
CPM IN TOTAL PROTEIN (% of initial)
100
50
0
2
4
6
HOURS

Fig 32. Rate of glutamine synthetase degradation in cells grown in various concentrations of glutamine. Cells were grown in Growth Medium containing 10^{-7} M dex and various concentrations of glutamine for 12 hours. They were then incubated in $[^{35}S]$ methionine as in Fig 30 and chased for 3 hours as in Fig 31, or not chased (see insert). Immunoprecipitates were prepared and anlayzed as in Fig 26. ● , weight of GS peak; Δ, GS specific activity. Insert: rate of GS synthesis at various concentrations of glutamine.

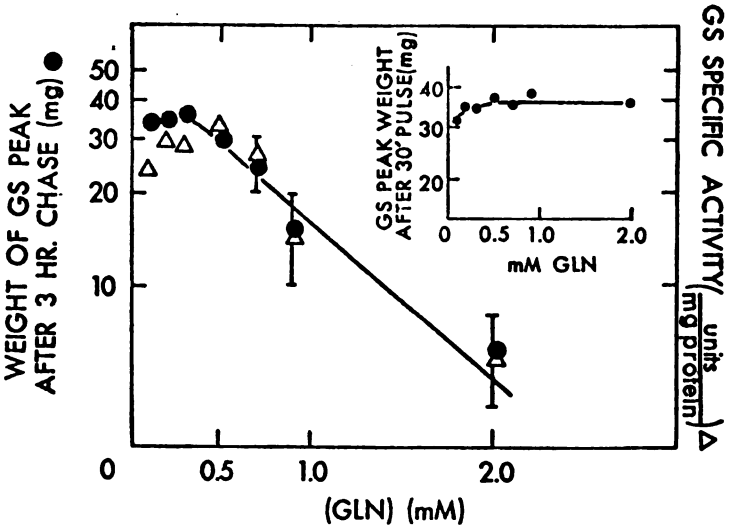
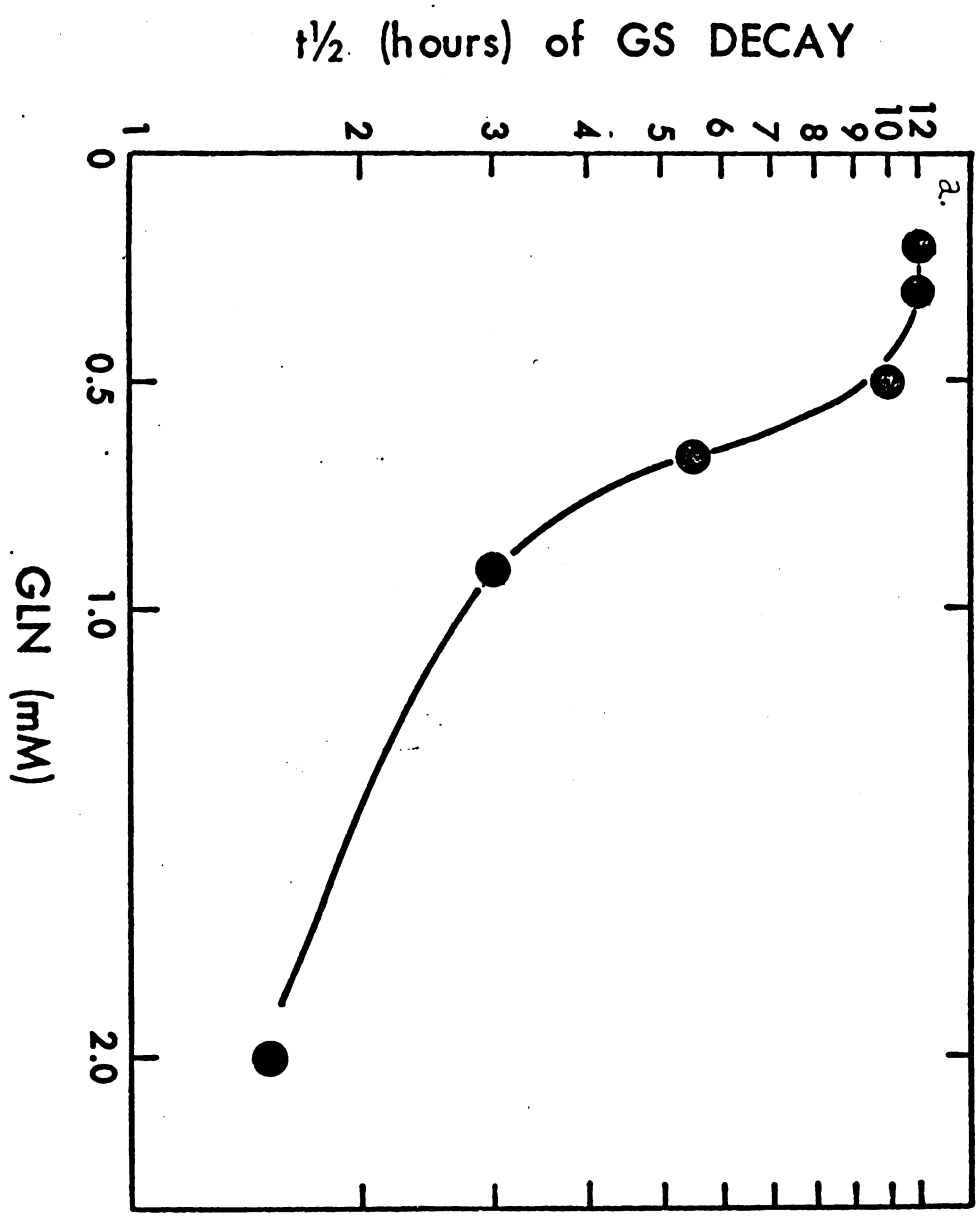

WEIGHT OF GS PEAK
AFTER 3 HR. CHASE (mg) ●
50
40
30
20
10
0
0.5
1.0
2.0
(GLN) (mM)
GS SPECIFIC ACTIVITY (units/mg protein) △
GS PEAK WEIGHT
AFTER 30' PULSE(mg)
40
30
20
0
0.5
1.0
2.0
mM GLN

Fig 33a. Half-time of glutamine synthetase degradation as a function of glutamine concentration. The slopes of the plots of each glutamine concentration in Fig 32 were used to determine half-times of degradation. (b) Glutamine synthetase specific activity as a function of the half-time of GS degradation. Data from Fig's 32 and 33a were replotted.


t½ (hours) of GS DECAY
1
2
3
4
5
6
7
8
9
10
12
a.
0
0.5
1.0
2.0
GLN (mM)

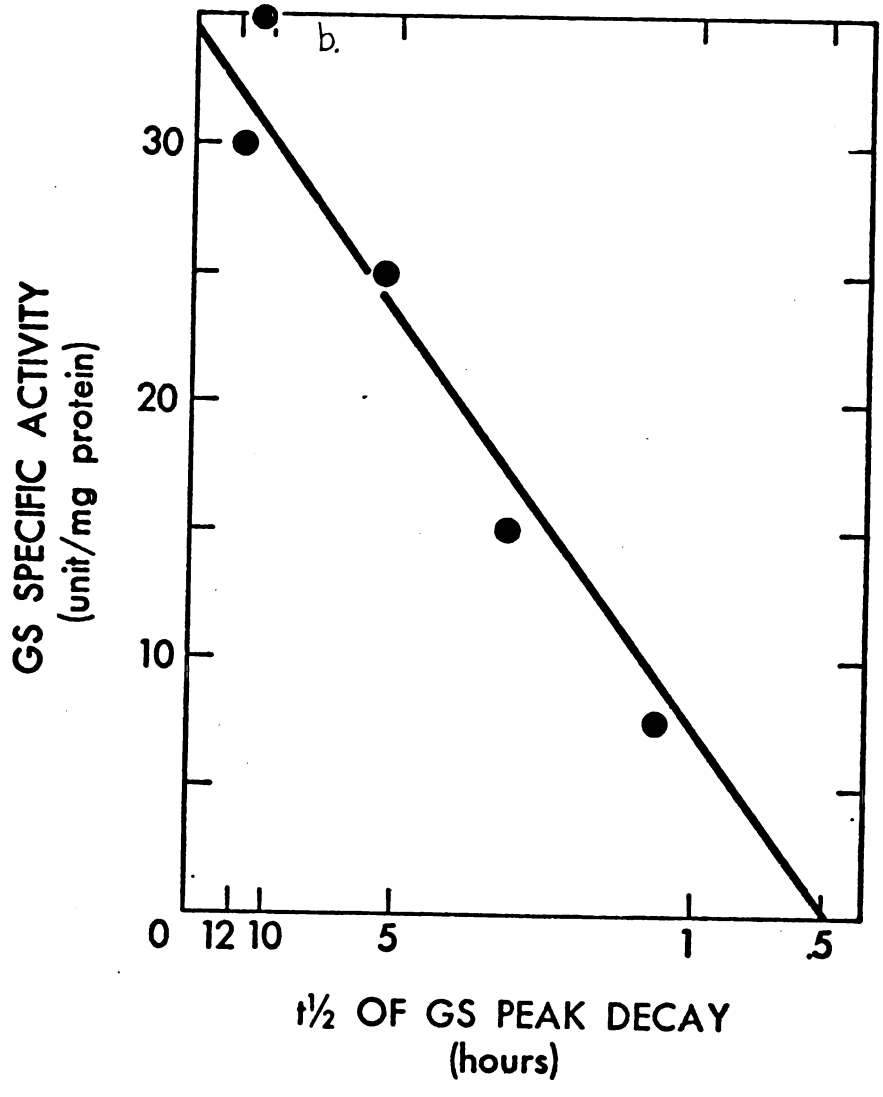

b.
30
20
10
0
12
10
5
1
.5
GS SPECIFIC ACTIVITY
(unit/mg protein)
t½ OF GS PEAK DECAY
(hours)

Fig 34. Kinetics of glutamine synthetase degradation in cells shifted from 0.2 mM to 2 mM glutamine. Cells grown for 20 hours in Growth Medium containing 10^{-7} dex and 0.2 mM glutamine were incubated in [^{35}S] methionine for 30 minutes as in Fig 30. They were then washed and resuspended in Growth Medium containing 1 x 10^{-7} M dex and 2 mM glutamine for various times. Immunoprecipitates were made and analyzed as in Fig 26, and GS activity determined as in Fig 28. ○, GS peak weight; △, GS specific activity.

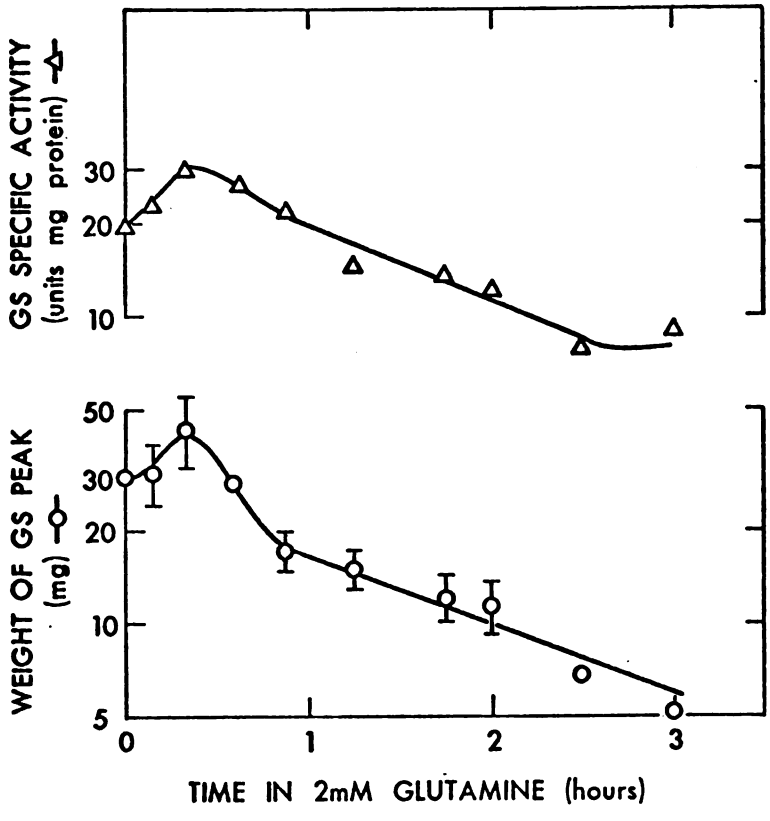
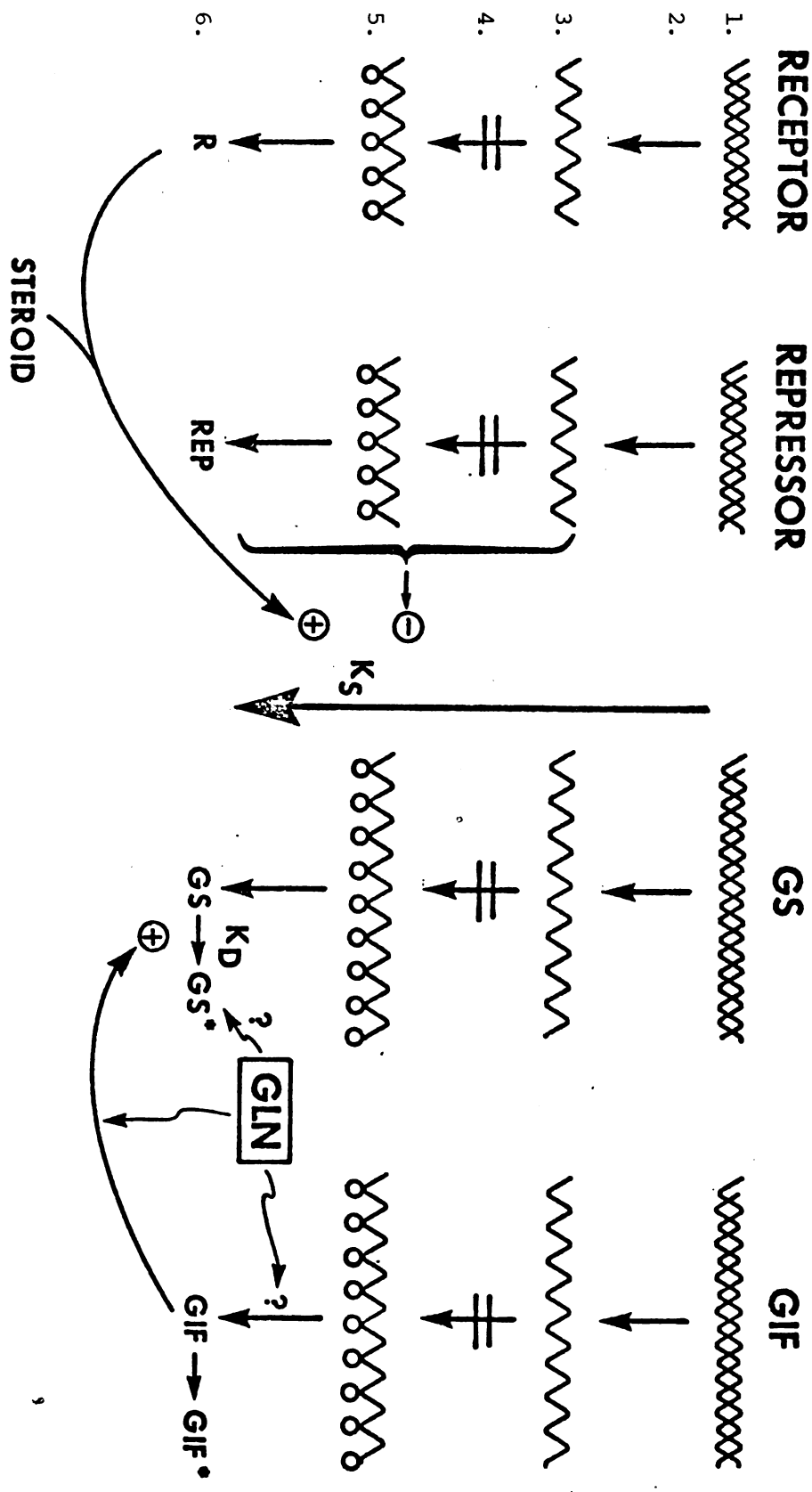

GS SPECIFIC ACTIVITY
(units mg protein)
30
20
10
WEIGHT OF GS PEAK
(mg)
50
30
20
10
5
0
1
2
3
TIME IN 2mM GLUTAMINE (hours)

Fig 35. Model of control of glutamine synthetase by dexamethasone and glutamine. R = steroid receptor protein; REP = GS repressor; GS = glutamine synthetase; GIF = GS inactivating factor; 1. gene; 2. transcription; 3. mRNA; 4. transport out of nucleus; 5. translation; 6. protein


STEROID RECEPTOR
GS REPRESSOR
GS
GIF
1.
2.
3.
4.
5.
6.
R
REP
STEROID
⊕
⊖
K_S
GS → GS*
K_D
?
GLN
GIF → GIF*

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CHAPTER SEVEN

Evidence has been accumulating for some time that the occurence of many altered phenotypes in cultured animal cells is due to mutations, a chemical modification of the chromosomal DNA (either small nucleotide alterations or segmental alterations such as chromosome translocations). Siminovitch has recently reviewed the evidence for this view (1). Examples now exist of markers which dispaly frequencies compatible with theoretical considerations (2-4). Also, most markers' frequencies are enhanced by mutagens (1). More convincing is evidence for alterations in the protein responsible for a given phenotypic change. HGPRTase, the enzyme whose inactivity shows resistance to the purine analogues 6-thioguanine and 8-azaguanine, can be demonstrated (a) to exist as non-catalytically active, cross-reactive material (CRM) in 6TG resistant lines (5,6); and (b) to display altered thermal stability in a 6TG sensitive line derived from a resistant line, compared to the original sensitive line (7). Other strong evidence for mutational change is temperature-sensitive lethal phenotypes such as one in CHO cells which has been identified as due to an altered leucine tRNA synthetase (8). Other temperature sensitive mutations exist as well which have been ascribed to a specific gene product (91). It seems clear that mutation is responsible for many of the phenotypic changes studied.

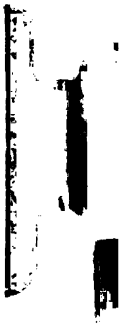
There is, however, evidence of markers which display behavior difficult to reconcile with a mutational mechanism. This behavior is of two types. (1) some markers evince mutation rates which are much

higher than predicted theoretically (10,11). Such an example is thymidine transport in CHO cells (1.6×10^{-4}/cell/gen)(10). (2) The frequencies of some markers appear not to be a function of cell ploidy. The appearance of a recessive or hemizygous trait would theoretically be expected to diminish as the ploidy, and copy number of the marker gene, increases. The frequency of a dominant marker would be expected to increase as the gene copy number increased with ploidy. 8-azaguanine resistance behaves as a recessive trait in cell-cell hybrids (12). In 8-aza sensitive pseudotetraploids and pseudooctoploids, where the HGPRTase gene copy number would be several times that in pseudodiploids, however, the frequency of 8-aza sensitive revertants is the same as in the pseudodiploids.

In a pseudodiploid line of frog embryo cells, the frequency of appearance of $BrdU^r$ colonies is the same or slightly higher than in a haploid line (13). Since the defect appears to be at the level of transport rather than thymidine kinase, it is not clear whether the alteration is recessive: an increase in frequency with increasing ploidy in fact suggests dominance. However, mutagensis with ethyl methane sulfonate or nitrosoguanidine of haploids gave two to three orders of magnitude increase in resistant variants, whereas no effect was detected with pseudodiploids, a result consistent with a recessive phenotype.

Thus, there seems to be data which are not consistent with change by a mutational mechanism. Certainly, precedent for stable, non-mutational phenotypic change exists in the developmental patterns of most eukaryotes. In view of the multiplicity of developmental "programs" which any animal cell must contain, assuming all cells of an organism contain the same genetic information (14), and in view of the irrever-

sible shifts of expression which charaterize developmental change (15), regulatory mistakes which, once made, are stable and heritable seem a possibility. Of these changes it would be predicted that (1) they very well might be random (i.e. demonstrate random occurence in a fluctuation analysis); (2) all variants would display identical enzyme (or other functional behavior (e.g. same Km's for substrate, same thermal stability, isoelectric point); (3) their frequencies might be higher (or lower) than that expected for mutations; and (4) their frequencies would not necessarily be enhanced by mutagens. This chapter reports on results of the third and fourth predictions.



MATERIALS AND METHODS

Cloning. Cloning medium is Growth Medium without glutamine, with the following modifications: .02 Mtricine, .017 M $NaHCO_3$, 10% fetal calf serum (Gibco) instead of calf serum, and pH 7.6. 2 mM glutamine was added where desired.

The appropriate number of cells was centrifuged at 160 xg for 5 minutes, then resuspended in Cloning Medium and mixed with 3% agar (Difco Purified) making a final agar concentration of 0.3%. The mixture was then poured into a 100 x 20 mm polystyrene tissue culture dish (Corning) and allowed to cool. The plates were kept in a 37°C water-jacketed incubator in 5% CO_2. Cloning efficiency was routinely between 50-100%.

Colony counting. Colonies were counted after 2-4 weeks against a cross-hatched background. Only colonies larger than 10-15 cells in diameter (roughly the limit of confidence for positive indentification of an HTC clone by eye.) Occasionally clones were counted by first staining with neutral red, a vital dye which is taken up by active transport into living cells. Red colonies were then counted.

Picking of Clones. Clones were picked with an 18 gauge needle and syringe, The clone was worked gently back and forth in the needle to disaggragate the cells, then ejected into a 25 cm^2 sterile T-flask (Falcon) containing 5 mls of Growth Medium. The flasks were then kept in a 5% CO_2 37°C incubator.

Mutagenesis. Nitrosoguanidine (Sigma) or ethyl methane sulfonate (Sigma) were made up to a 1,000x concentration and added to cells at between 3 and 5 x 10^5 cells/ml growing in suspension. After 2-4 hours exposure to the drug, the cells were centrifuged at 160 xg for 5 minutes, resuspended in .15 M NaCl, centrifuged again, and then resuspended in fresh Growth Medium. Samples were taken for killing curves within an hour.

RESULTS

The cell line used in this study, called ST3, is a clone picked from a soft-agar plate of HTC cells. These cells had been plated in Cloning Medium containing 2 mM glutamine (i.e. non-selective conditions.) Table 5 shows that ST3 has barely detectable GS activity, about 10 times lower than the basal activity on GM22, and that this activity is not influenced by growth in 0.2 mM glutamine or 10^{-7}M dex. Other enzymes have been reported to have glutamyl transferase activity (16), so it is possible that this activity is caused by an enzyme other than GS. In an GS immunoprecipitate from ST3 (Fig 25b), a small component which is not specifically precipated by anti-GS (data not shown) is present only. No other component of the gel pattern compared to that of GS22 is greatly changed, making it unlikely that GS is somewhere else on the gel. The same results are seen if the extract is not heated.

When ST3 is plated in the absence of glutamine, its growth characteristics as compared to GM22 are shown in Figure 36. ST3 and GM22 plate with identical efficiencies in medium containing 2 mM glutamine, whereas ST3 gives colonies at about 3 in 10^5 in the absence of glutamine, while the plating efficiency of GM22 is reduced about 20%. This can be raised to 100% of controls with glutamine by preincubating the cells with dex for 8 hours before plating.

The gln^+ phenotype is a stable, heritable trait. GM22 has been carried for 5 years under non-selective conditions with no detectable

changes in specific activity of inducibility by dex or glutamine step-down.

The effects of nitrosoguanidine (NTG) and ethyl methane sulfonate (EMS) on the frequencies of appearance of isolates of the gln^+ phenotype from ST3 were investigated. These two chemicals are reported to be potent mutagens in both bacteria and animal cells. In order to have an internal control for the mutagenic effect of these compounds, 6-thioguanine (6TG) resistance, a phenotype extensively studied in other established cell lines (17-19), and one whose frequency is reported to be increased by mutagens, was also monitored. (ST3 is killed by 17 µg/ml 6TG).

Figure 37 shows the effect of exposure to various concentrations of NTG and EMS for 2 hours on the subsequent plating efficiency of ST3 in non-selective medium. Survival after NTG treatment ranges from 60% at .15 µg/ml to less that 5% at 1-2 µg/ml. Exposure to EMS at 50 µg/ml results in roughly 60% of the cells surviving, while 500 µg/ml results in 15% surviving. Thus, a 2 hour exposure to these mutagens can result in changes which lead to eventual cell death.

Next the effect of the mutagens on the frequencies of gln^+ and $6TG^r$ phenotypes was studied. After cells were exposed to NTG or EMS for 2 hours, they were grown in Growth Medium containing no mutagens and 2 mM glutamine for one week. This period of time was chosen to allow expression of new or altered gene products and the dilution of gene products no longer being synthesized. Since the doubling time for ST3 is about 24 hours, the possibility that a mutant would over grow or be over grown by the rest of the population in 7 doublings seemed remote. Then cells were plated in Cloning Medium containing soft agar as described in Materials and Methods. For the gln^+ phenotype selection,

the medium also contained 10^{-7} M dex and no glutamine. For selection of the $6TG^r$ phenotype, medium contained 2 mM glutamine and 17 ug/ml 6-thioguanine. At this concentration, 6-thioguanine prevents any growth of cells not able to grow at rates approximating that of controls without the purine analogue. Controls lacking the selective condition were also plated in each experiment and cloning efficiency determined.

Table 6 is a summary of the data. Neither EMS or NTG, at concentrations which result in a wide range of killing, has a significant effect on the frequency of appearance of the gln^+ phenotype, compared to unmutagenized controls. The $6TG^r$ phenotype, in contrast, is increased up to 50 times the unmutagenized control when exposed to NTG. EMS, on the other hand, appears to have little, if any, effect on the $6TG^r$ locus.

DISCUSSION

The data presented in this chapter, that the frequency of the gln^+ phenotype is 3×10^{-5}, while 6 TG resistance is $1\text{-}2 \times 10^{-8}$ and ouabain resistance is 2×10^{-8} (data not shown), and that this frequency is not affected by mutagenesis while 6TG resistance is; together with the observation that GS is not present in ST3 GS immunoprecipitates, are consistent with a mechanism which does not include covalent alteration of the genome. However, other explanations may account for these findings. The absence of CRM could be due to (a) a point mutation which produces a polypeptide which does not cross-react with the antibody; or (b) a mutation in a locus other than the structural gene of the GS protein which affects its synthesis or degradation.

The frequency of gln^+ variants in ST3, while not a rate, is probably not far from the rate because ST3 was frozen away after about 25 doublings and unfrozen samples were grown up for each experiment. The same approximation applies to 6TG and ouabain resistance.

HTC cells are hypotetraploid, with a mean chromosome number of 66. In such a genetic configuration, recessive (or hemizygous) traits like 6TG resistance might be expected to exhibit a frequency lower than in a diploid cell (theoretically the sum of the powers of the diploid frequency). Likewise, a dominant trait, such as the gln^+ phenotype would be if it were a mutation in the structural gene of GS, might be expected to be slightly higher than the diploid frequency. Thus the numbers can, at least roughly, be accomodated by a conventional explanation.

The absence of an effect of NTG or EMS is more difficult to explain. Other examples of differential effects of mutagens on a eukaryotic locus do exist in the literature.(16). The fact that EMS does not increase the frequency of 6TG resistance, weakens the argument that no effect indicates a non-mutational mechanism. A more convincing case could be made for the origin of the gln^+ phenotype were several more mutagens tested.

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Table 5. Glutamine synthetase specific activity of ST3 and GM22 cells. Cells were grown 20 hours in 2 mM glutamine, 2 mM glutamine plus 10^{-7} M dex, and 0.2 mM glutamine plus 10^{-7} M dex.

CELL LINE	GS SPECIFIC ACTIVITY (units/mg protein) OF CELLS GROWN 20h IN GROWTH MEDIUM CONTAINING: 2mM GLN	2mM GLN + 10^{-7} m DEX	0.2mM GLN + 10^{-7} m DEX
ST3	.020	.018	.017
GM 22	.15	.38	2.80

Fig 36. Cloning efficiency of ST3 and GM22. ST3 and GM22 were plated as described in Materials and Methods in 0.3% agar and Cloning Medium containing: △ , □ ;2mM glutamine; ● , ■ : no glutamine plus 10^{-7} M dex; and: ○ , 17 ug/ml 6-thioguanine plus 2 mM glutamine. GM22: □ , ■ ; ST3: △ , ○ , ● .

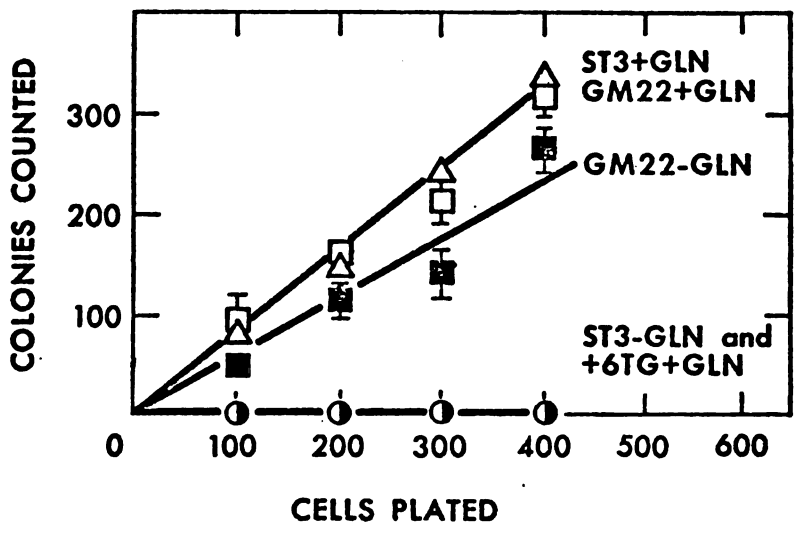
COLONIES COUNTED
300
200
100
0
100
200
300
400
500
600
CELLS PLATED
ST3+GLN
GM22+GLN
GM22-GLN
ST3-GLN and
+6TG+GLN

Fig 37. Effect of mutagens on the cloning efficiency of ST3. Cells were mutagenized with various concentrations of EMS and NTG and plated as described in Materials and Methods. Each set of symbols represents a separate experiment.

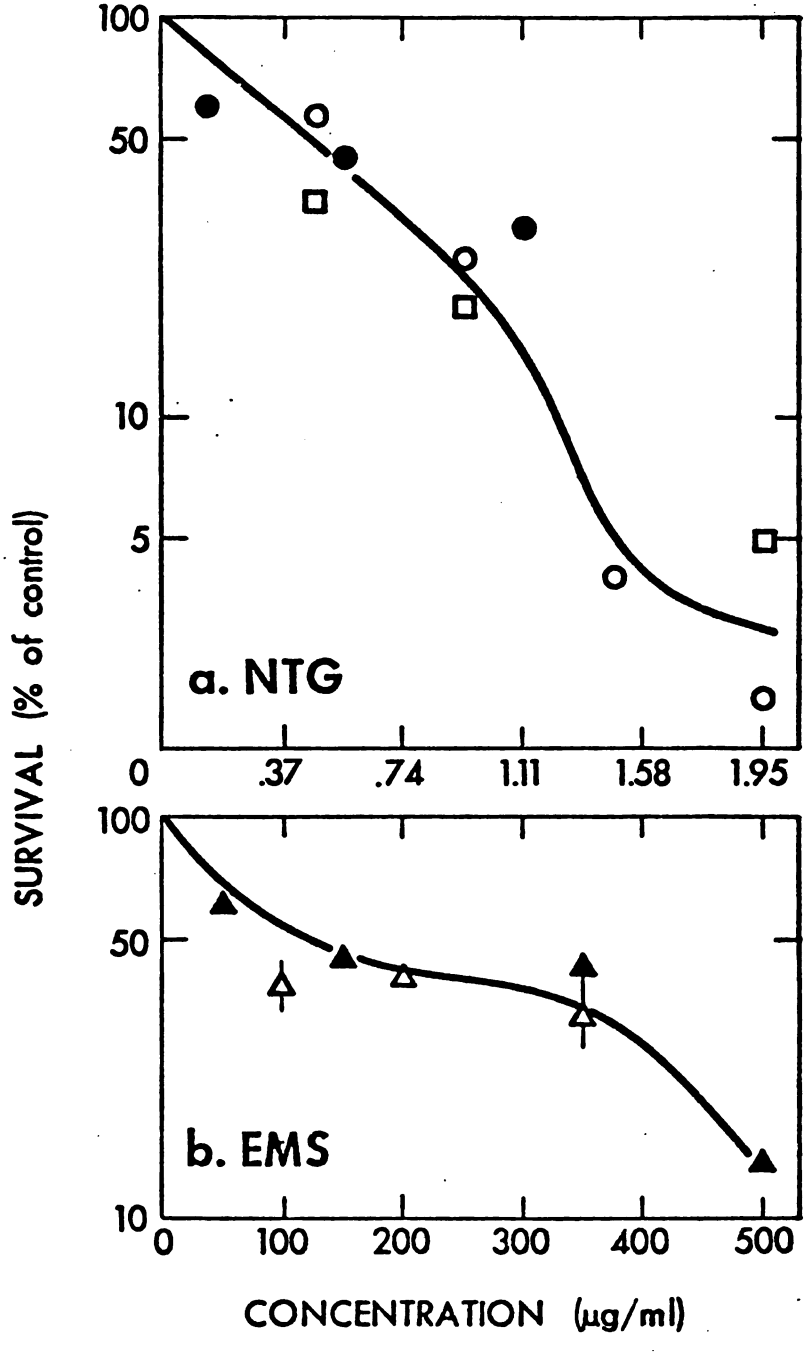
100
50
10
5
0
a. NTG
.37
.74
1.11
1.58
1.95
100
50
10
b. EMS
0
100
200
300
400
500
SURVIVAL (% of control)
CONCENTRATION (µg/ml)

Table 6. Effect of mutagens on the frequency of gln^+ and $6TG^r$ phenotypes from ST3. Cells were exposed to various concentrations of EMS and NTG for 3 hours as described in Materials and Methods, then washed and allowed to grow in spinner culture under non-selective conditions for one week. Cells were then plated in 0.3% agar and Cloning Medium containing: 2 mM glutamine (control); no glutamine plus 10^{-7} M dex (gln^+ phenotype selection); and 17 µg/ml 6-thioguanine plus 2 mM glutamine ($6TG^r$ phenotype selection). 2×10^8 cells were plated for each mutagen concentration and phenotype selection.

Mutagen	Mutagen Concentration (μg/ml)	Cell Survival (%)	PHENOTYPE GLN+ ×10⁵	GLN+ Fold Increase	6.TG^r ×10⁸	6.TG^r Fold Increase
—	—	100	3 ± 1	1	2 ± 1	1
NTG	.15	(70)	3.5 ± .5	1.2		
	.37	35	1.8	0.6	4 ± 3	2
	.88	(10-20)	1.9 ± 1	0.6	15 ± 10	7
	1.10	13	2 ± .5	0.7	25 ± 5	12
	1.50	5	4.5 ± 2	1.5	100 ± 30	50
	3.70	(< 5)	2.8	0.9		
EMS	50	62	3.5 ± 1	1.2	5	~2
	150	45	2.5 ± 1	0.8	4	~2
	200	40	1.8 ± .5	0.6		
	350	43			4	~2
	400	(25-30)	2.5 ± .5	0.8		
	500	14			5	~2
	600	(<12)	2.2	0.7		