Regulation of Glutamine Synthetase. XI. The Nature and Implications of a Lag Phase in the *Escherichia coli* Glutamine Synthetase Reaction*

Henry S. Kingdon,† Jerry S. Hubbard,‡ and E. R. Stadtman§

ABSTRACT: Relaxed *Escherichia coli* glutamine synthetase is catalytically inactive at pH 7.1, but regains activity under conditions of assay, in a time-dependent process. The lag phase associated with this activation can be eliminated by preliminary incubation of the enzyme at pH 6.5–7.0 with 0.5 mM concentrations of either Mg^{2+} or Mn^{2+}, or with higher concentrations of Ca^{2+}. The lag was also eliminated by prior incubation of the enzyme at alkaline pH (7.5–8.0) and high ionic strength, or at pH 7.1 in the presence of 0.1 M glutamate. These effects are attributed to the concerted action of glutamate or alkaline buffers and the trace amounts of divalent cations that contaminate these reagents. This follows from (a) the fact that glutamate and alkaline buffers were ineffective if 5 mM EDTA was included in the prior incubation mixtures, and (b) prior incubation of relaxed enzyme at neutral pH with 2 μM concentrations of divalent cations (*i.e.*, with concentrations shown to contaminate the glutamate and buffer solutions) had no significant effect on the lag. The effect of glutamate is highly specific; prior incubation of relaxed enzyme with analogs of glutamate or a variety of other amino acids did not decrease the lag. When divalent cations are added to relaxed enzyme there is a nearly instantaneous exposure of hydrophobic groups as indicated by the abrupt increase in fluorescence intensity when the reaction is carried out in the presence of the hydrophobic probe, 2-*p*-toluidinylnaphthalene-6-sulfonate; this is followed by slower conformational changes and restoration of catalytic activity. Although the exposure of hydrophobic groups can be achieved by the addition of any one of a large number of divalent cations, the restoration of catalytic activity requires either Mn^{2+}, Mg^{2+}, or Ca^{2+}. The implication of the reversible relaxation phenomenon in the cellular regulation of glutamine metabolism is discussed.

Cumulative feedback inhibition of the *Escherichia coli* glutamine synthetase by eight different end products of glutamine metabolism has been described (Woolfolk and Stadtman, 1964, 1967). Since only a limited degree of inhibition (10–50%) was obtained with each of the feedback inhibitors when tested individually, a detailed analysis of the kinetics of inhibition was restricted by the low sensitivity of the standard assay procedures commonly used to measure glutamine synthetase activity. To overcome this difficulty, a highly sensitive spectrophotometric assay procedure was devised. This involved coupling the glutamine synthetase reaction with reactions catalyzed by pyruvate kinase and lactate dehydrogenase. In this optical assay, which permits continuous monitoring of glutamine synthetase activity, it was discovered that, with certain enzyme preparations, the initial rate of glutamine synthesis was not maximal, but that the rate increased over a period of several minutes before maximal velocity was attained. The present report is concerned with the results of studies to determine the basis for this lag and the conditions that affect it. It will be shown that the lag is a manifestation of the time-dependent conversion of catalytically inactive enzyme, produced by the removal of divalent cations, into a catalytically active form during exposure to the divalent cations in the standard assay mixture. Conversion of the inactive enzyme into an active form also can be achieved by prior incubation with high concentrations of L-glutamate, or by prior incubation at a high pH at relatively high ionic strengths. The significance of the presumed conformational alterations to the cellular regulation of the enzyme is discussed. A preliminary report of this work has been published (Kingdon, 1967).

Materials and Methods

Chemicals. Adenosine 5′-triphosphate disodium salt (ATP) and pyruvate kinase (rabbit skeletal muscle, type II, crystalline) were obtained from Sigma Chemical Co. Phosphoenolpyruvate (PEP),[1] monosodium, and lactate dehydrogenase, crystalline, were obtained from C. F. Boehringer und Soehne. Reduced nicotinamide–adenine dinucleotide (NADH) was obtained from Calbiochem. Monosodium L-glutamate was ob-

* From the Laboratory of Biochemistry, Section on Enzymes, National Heart Institute, National Institutes of Health, Bethesda, Maryland. *Received January 31, 1968.*

† Present address: Department of Medicine, Section of Hematology, University of Chicago.

‡ Present address: Jet Propulsion Laboratory, Pasadena, Calif.

§ To whom requests for reprints should be addressed.

[1] Abbreviations used that are not given in *Biochemistry 5*, 1445 (1966), are: PEP, phosphoenolpyruvate; TNS, 2-*p*-toluidinylnaphthalene-6-sulfonate.

tained from Nutritional Biochemicals Corp. and was recrystallized once from 0.01 M EDTA and twice from deionized water before use as an activator. All other chemicals were reagent grade. Crystalline *Escherichia coli* glutamine synthetase was prepared as previously described (Woolfolk *et al.*, 1966), from *E. coli* W grown on glycerol and glutamate. This preparation corresponds to the synthetase II described elsewhere (Kingdon and Stadtman, 1967). The enzyme was a generous gift from Dr. Bennett M. Shapiro.

2-*p*-Toluidinylnaphthalene-6-sulfonate (TNS) was prepared as described by McClure and Edelman (1966).

Relaxed Glutamine Synthetase. Glutamine synthetase, at a concentration of 2–5 mg/ml was incubated overnight at 4° in 10 mM EDTA–10 mM imidazole, adjusted to pH 7.1 with HCl. The protein fraction obtained by filtration of this incubation mixture through Sephadex G-25 which had been previously equilibrated with 10 mM imidazole (pH 7.1) constitutes the relaxed enzyme; it had less than 1 equiv of Mn^{2+}/mole of enzyme.

Metal content of solutions of enzyme, buffers, or monosodium glutamate was determined by atomic absorption spectrometry using the Perkins-Elmer atomic absorption spectrometer.[2]

Protein concentration was estimated by ultraviolet absorbancy measurement. A value for the specific absorbancy $A_{cm}^{0.1\%}$ 0.77 was reported previously (Shapiro and Stadtman, 1967).

Fluorescence intensity was measured with an Aminco-Bowman spectrophotofluorometer, and is expressed as arbitrary scale units without corrections.

The Coupled Assay of Glutamine Synthetase. In this assay the ADP produced during glutamine synthesis (reaction 1) is measured by the procedure of Kornberg and Pricer (1951). In this method, the ADP is converted into ATP by reaction with PEP catalyzed by pyruvate kinase (reaction 2), and the pyruvate thus produced is reduced by NADH in the presence of lactate dehydrogenase (reaction 3). The over-all reaction (4) is measured spectrophotometrically by following the change in absorbance at 340 mμ, due to the oxidation of NADH.

$$\text{ATP} + \text{NH}_4^+ + \text{glutamate} \xrightarrow{\text{glutamine synthetase}} \text{ADP} + \text{P}_i + \text{glutamine} \quad (1)$$

$$\text{ADP} + \text{PEP} \xrightarrow{\text{pyruvate kinase}} \text{ATP} + \text{pyruvate} \quad (2)$$

$$\text{pyruvate} + \text{NADH} \xrightarrow{\text{lactate dehydrogenase}} \text{lactate} + \text{NAD}^+ \quad (3)$$

$$\text{sum: NADH} + \text{NH}_4^+ + \text{glutamate} + \text{PEP} \longrightarrow \text{lactate} + \text{NAD}^+ + \text{glutamine} + \text{P}_i \quad (4)$$

The pyruvate kinase (free of adenylate kinase) was added in about 10-fold excess, and the lactate dehydrogenase in about 100-fold excess, so that the reaction rate was dependent solely on the amount of glutamine synthetase added. Absorbancy measurements were made at 30° with the Cary Model 11 double-beam recording spectrophotometer. The reaction mixture contained 50 mM imidazole-HCl buffer (pH 7.1), 7.6 mM ATP, 1.0 mM PEP, 50 mM $MgCl_2$, 10 mM KCl, 40 mM NH_4Cl, 0.35 mM NADH, 0.1 M monosodium glutamate, 25 μg of pyruvate kinase, and 50 μg of lactate dehydrogenase, in a volume of 1.0 ml. This reaction mixture was allowed to equilibrate at 30° for 10 min, during which time traces of ADP and pyruvate in the reactants were consumed, and about 0.1 μmole of NADH was oxidized. When a stable base line was reached (at optical density 1.5–1.7) glutamine synthetase was added in a small volume (10–25 μl) as a drop on a bent glass stirring rod. The change in absorbance at 340 mμ, due to oxidation of NADH, was followed until all of the NADH was consumed.

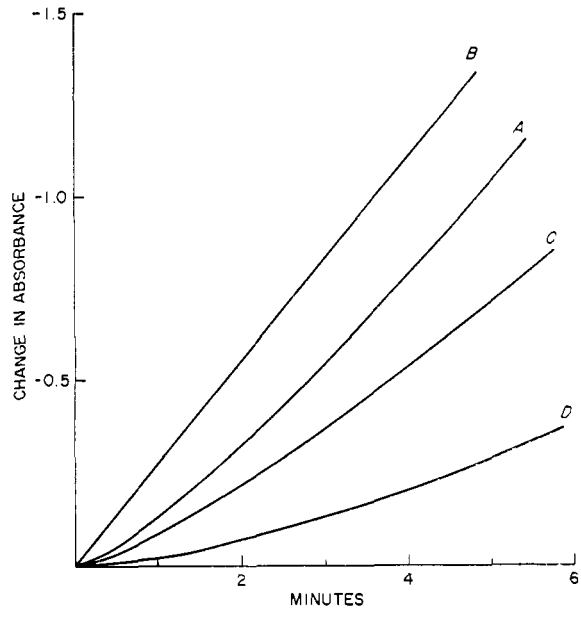

FIGURE 1: Effect of preliminary incubation of glutamine synthetase with components of the assay. Coupled assays were performed, as described under materials and methods, on glutamine synthetase which had been preincubated with assay components as indicated, each component being present in the same concentration used in the assay. In each case 5 μg of glutamine synthetase was assayed; the amount of the assay component used in preincubation was reduced in the assay by the amount transferred from the preincubation, so that all assays were run at standard conditions. (A) Preliminary incubation with imidazole alone, or with NH_4Cl or ATP. (B) Preliminary incubation with $MgCl_2$ alone, glutamate alone, or $MgCl_2$ in combination with any of the other components. (C) Preliminary incubation with glumate plus ATP. (D) Preliminary incubation with glutamate plus ATP NH_4Cl.

Results

Initial Observations on the Lag Phenomenon. Examination of several different, apparently homogeneous, glutamine synthetase preparations disclosed a marked variation in the extent of the lag obtained when

[2] Dr. Gerald Aurbach kindly made this instrument available to us.

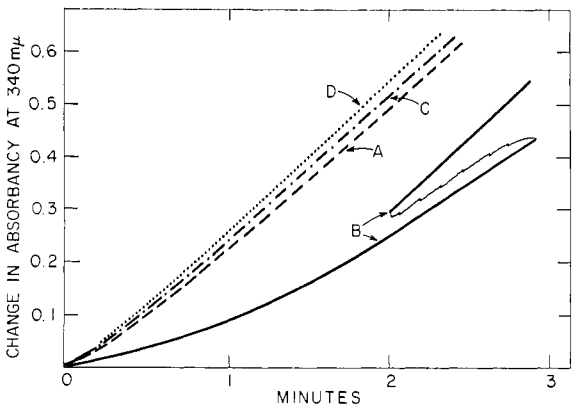

FIGURE 2: Role of divalent cations in lag phenomenon. The curves show the time course of the change in absorbancy at 340 mμ due to DPNH oxidation in the coupled assay as described in Materials and Methods. Various glutamine synthetase preparations were used as follows. Curve A: 3.5 μg of taut enzyme (*i.e.*, untreated glutamine synthetase II containing 40 moles of Mn^{2+}/mole of enzyme); curve B: 3.5 μg of relaxed enzyme (*i.e.*, taut enzyme that had been incubated with 0.01 M EDTA at pH 7.1, 0°, for 2 hr). Curve C: 3.5 μg of *tightened* enzyme (obtained by incubating relaxed enzyme with 50 mM $MgCl_2$ at pH 7.1 for 20 min); curve D: 3.5 μg of taut enzyme that had been incubated with 50 mM $MgCl_2$ for 20 min at pH 7.1. The jagged line connecting the two segments of curve B represents a 3-min period of discontinuity in the time course during which the assay mixture was supplemented with 0.2 μmole of DPNH. The upper straight-line segment in curve B therefore represents the time course of DPNH oxidation during the 6–7-min time interval after initiation of the reaction. The fact that this segment is parallel to the terminal portions of curves A–D shows that all enzyme preparations finally achieve the same specific activity in the coupled assay.

TABLE I: Effects of Preliminary Incubation with Divalent Cations on the Lag Phase of Glutamine Synthetase.[a]

Salt Added	Fractional Reduction in Lag Phase
$CuCl_2$	0.01
$NiCl_2$	0.00
$CoCl_2$	0.04
$FeSO_4$	*b*
$ZnSO_4$	0.00
$BaCl_2$	0.00
$SrCl_2$	0.00
$HgCl_2$	*b*
$Pb(NO_3)_2$	*b*
$CaCl_2$	0.79
$CdCl_2$	0.00
$SnCl_2$	0.00
$MgCl_2$	0.99
$MnCl_2$	0.90

[a] Relaxed glutamine synthetase was preincubated at a concentration of 0.54 mg/ml for 25 min at 25°, in 0.25 M imidazole-HCl (pH 7.1) with or without 5 mM salt of divalent cation as indicated. Amount of lag and fractional reduction of the lag, as compared with four controls, were estimated as described in the preceding section. [b] Inhibits over-all assay.

they were assayed in the coupled assay system. To determine if this lag was due to slow activation of the enzyme by its reaction with one of the components in the assay mixture, an enzyme preparation exhibiting a pronounced lag was incubated with each component separately prior to its addition to the complete assay mixture. The results are shown in Figure 1. Prior incubation of the enzyme with either ATP, NH_4Cl, or imidazole buffer alone had no effect (curve A), but, prior incubation with either $MgCl_2$ or with high levels of glutamate abolished the lag (curve B). Furthermore, incubation with $MgCl_2$ together with any of the other components abolished the lag. However, prior incubation of enzyme with a mixture of ATP and glutamate (curve C) or with a mixture of ATP, glutamate, and NH_4Cl (curve D) accentuated the lag; the maximal activity ultimately attained by prior incubation with these mixtures was never as great as that obtained by prior incubation with either $MgCl_2$ or glutamate alone. Conversion of the enzyme into a less active form by incubation with the above mixtures has not been explained and was not investigated further.

The role of divalent cations in the lag phenomenon is further demonstrated by the data in Figure 2. Curve A illustrates the time course of glutamine synthesis by an untreated enzyme preparation exhibiting only a slight lag. When treated with 10 mM EDTA to remove divalent cations, this enzyme was converted into a form exhibiting a very marked lag (curve B). However, when this EDTA-treated enzyme was then incubated with 50 mM $MgCl_2$ at pH 7.0, it was converted back into a form that exhibited little or no lag (curve C). In fact, it could not be distinguished from a preparation of the original enzyme that had been treated only with 50 mM $MgCl_2$ at pH 7.0 (compare curve C with curve D).

In parallel studies (Shapiro and Stadtman, 1967; Shapiro and Ginsburg, 1968) it was found that the three types of enzyme illustrated by curves A, B, and C in Figure 2 differ from each other with respect to their reactivity with sulfhydryl reagents and to their susceptibility to inactivation by mild denaturants. These considerations led to the following definition of terms (Shapiro and Stadtman, 1967). (1) Enzyme preparations of the type illustrated by curve A of Figure 2 are called *taut* enzymes. They are obtained from crude extracts by the standard isolation procedure and presumably represent native enzyme. Such taut enzyme preparations contain variable amounts of protein-bound Mn^{2+}, and they exhibit little or no lag in the coupled assay; they are not inhibited by sulfhydryl group reagents, and they are resistant to denaturation by exposure to alkaline pH or by treatment with 1.0 M urea or 0.1% dodecyl sulfate. (2) Enzyme preparations of the type illustrated by curve B of Figure 2 are called *relaxed* enzymes. They are readily obtained by treating taut enzyme with EDTA and may be freed of the latter by filtration through Sephadex. Such preparations contain little or no divalent

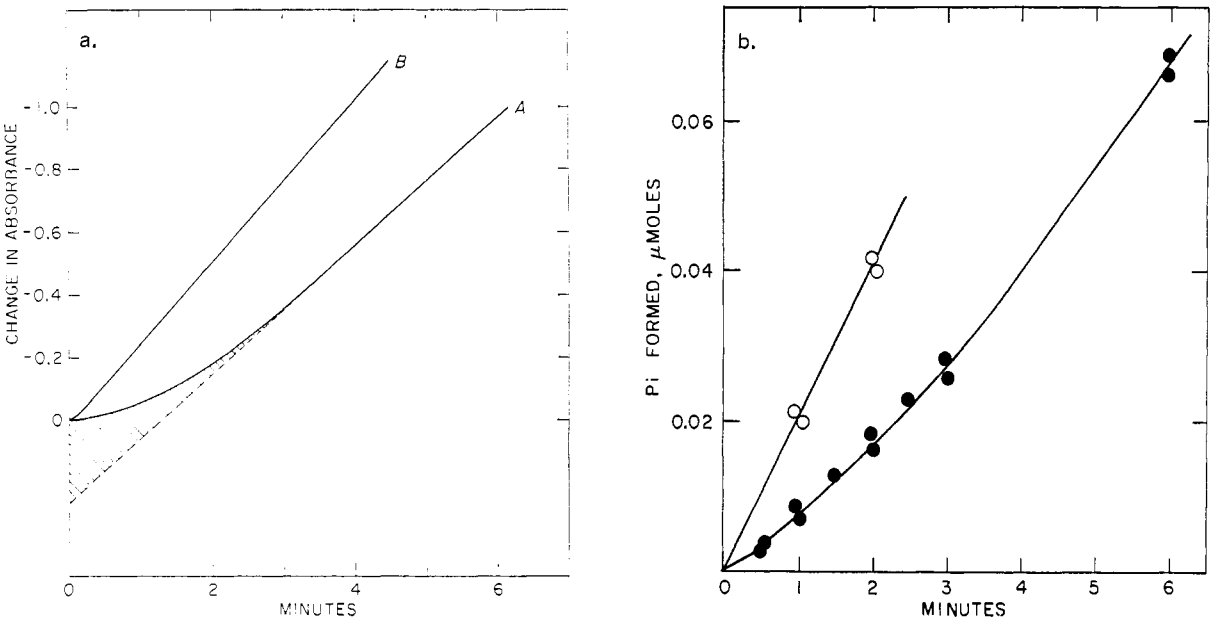

FIGURE 3: Lag-phase studies. (a) Estimation of extent of lag phase. Coupled assay performed as described under Materials and Methods. (A) 4 μg of relaxed enzyme and (B) 4 μg of the same relaxed enzyme which had been preincubated for 30 min at room temperature with 10 mM $MgCl_2$ (tightened enzyme). (b) Effect of prior incubation with L-glutamate on the lag observed in the standard biosynthetic assay. The reaction was followed by measuring the P_i released (Woolfolk and Stadtman, 1967). (●—●) 20 mM imidazole-HCl buffer (pH 7.0), 7.5 mM ATP, 100 mM L-glutamate, 50 mM NH_4Cl, and 5 μg of partially relaxed enzyme, temperature 21°; (○—○) same as above except the enzyme was first incubated with 0.2 M glutamate for 30 min.

cation and they exhibit a marked lag in the coupled assay system. They react readily with various sulfhydryl group reagents and are easily denatured by exposure to alkaline pH or by treatment with 1.0 M urea or 0.1% dodecyl sulfate. All of these treatments cause dissociation of the subunits. (3) Finally, preparations of enzyme of the type illustrated by curve C of Figure 2 are called *tightened* enzymes. They are obtained by incubating relaxed enzyme with either Mg^{2+}, Mn^{2+}, or Ca^{2+}. Tightened enzyme shows little or no lag in the coupled assay and is indistinguishable from taut enzyme preparations in any of the above properties. However, it is less soluble than the taut enzyme in dilute buffer solutions (Valentine *et al.*, 1968).

Measurements of the Extent of Lag. In order to study the factors effecting lag reduction, a standard procedure was adopted for preparing relaxed enzyme, which is described in Methods.

From preliminary studies it was evident that the kinetics of the lag phase are complex and cannot be described by simple rate functions or by simple functions of the time required to obtain maximal velocity. Therefore, for comparative purposes, a rough quantitative estimate of the extent of the lag phase was obtained by measuring the area under the lag portion of the curve (change in absorbance *vs.* time) in the following manner. A straight line was drawn through the recorded tracing in the later stages of the reaction and extrapolated back to zero time. The roughly triangular area bounded by this line, the zero-time line, and the observed reaction rate line was estimated by tracing it on Whatman No. 3MM filter paper and weighing the paper. This area is shown as the shaded area under curve A in Figure 3a. In experiments where preliminary incubation of the glutamine synthetase reduced the lag phase, the results thus obtained are expressed in terms of fractional reduction of the lag, arbitrarily taking the condition with the most marked lag as zero lag reduction. Curve B of Figure 3a shows a typical result with tightened enzyme, which shows virtually no lag. The fractional reduction in lag as outlined above would be 1.0 with this preparation.

It should be emphasized that the extent of the lag as measured by this procedure is very dependent upon the concentration and on the extent of adenylylation (*i.e.*, specific activity) of the enzyme used (Shapiro and Ginsburg, 1968). The method is therefore useful only for studying factors that influence the lag characteristics of a given enzyme preparation examined at a fixed concentration under otherwise carefully standardized conditions.

The kinetic difference between the relaxed and tightened enzymes shown in Figure 3a is not apparent in the standard biosynthetic assay procedure which measures P_i release due to reaction 1, since the time required to achieved complete reactivation of relaxed enzyme at 37° is short (1–2 min) in comparison with the total incubation time normally used (15 min). However, the lag phenomenon is not a unique property of the coupled assay system. As shown in Figure 3b, the lag is demonstrable in the standard biosynthetic assay when the time course of the reaction is measured over sufficiently short time intervals.

Reduction of the Lag by Preliminary Incubation with Divalent Cations. The effects of incubating relaxed glutamine synthetase with various divalent cations are summarized in Table I. The only cations among those tested which reduced the lag phase significantly and

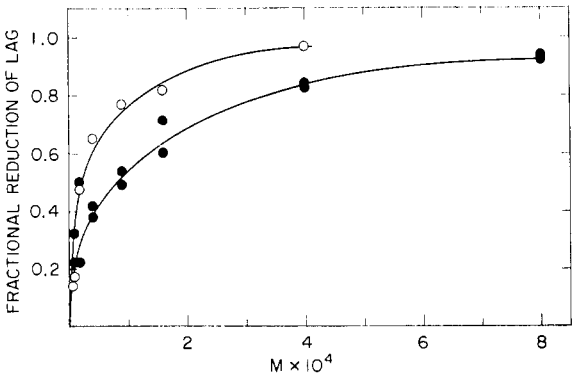

FIGURE 4: Effect of prior incubation with varying concentrations of Mg^{2+} or Mn^{2+} on reduction of the lag phase. Relaxed glutamine synthetase was incubated at 25° for 25 min in 0.2 M imidazole (pH 7.1) at the indicated divalent cation concentrations, and at a protein concentration of 0.4 mg/ml. Coupled assays were then performed as described in Methods, and fractional reduction in lag phase estimated as described in Figure 3. (●—●) $MgCl_2$ and (○—○) $MnCl_2$.

reliably were Mg^{2+}, Mn^{2+}, and Ca^{2+}. All three of these ions are active in the standard assay, but Ca^{2+} gives only about 1% of the rate seen with Mg^{2+} and Mn^{2+} (Kingdon and Stadtman, 1967). Cobalt, which can replace Mn^{2+} or Mg^{2+} in the standard assay (Woolfolk *et al.*, 1966; Kingdon and Stadtman, 1967) has no demonstrable effect on the lag.

Effects of Varying Concentrations of Divalent Cations. Preliminary incubations of relaxed glutamine synthetase were carried out at a variety of Mn^{2+} and Mg^{2+} concentrations. Fractional reduction of the lag as a function of divalent cation concentration is shown in Figure 4. From these data a concentration of divalent cation which reduces the lag by one-half can be estimated. For Mn^{2+} this concentration is 2.5×10^{-5} M, which is similar to the concentration that causes half-maximal burial of the aromatic amino acid residues that are exposed

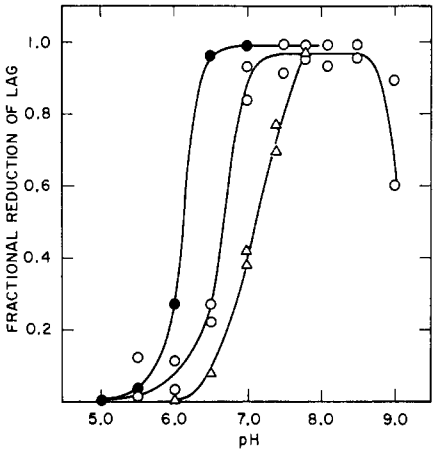

FIGURE 5: Effect of pH during prior incubation on reduction of lag phase. Relaxed enzyme was incubated at 25° for 25 min at a protein concentration of 0.3 mg/ml, at the indicated pH, and was then assayed as described in Methods. (○—○) 67 mM sodium acetate, 67 mM potassium phosphate, and 67 mM Tris-HCl; (●—●) same buffer, containing 2.7 mM $MgCl_2$; (△—△) 0.2 M imidazole, without $MgCl_2$.

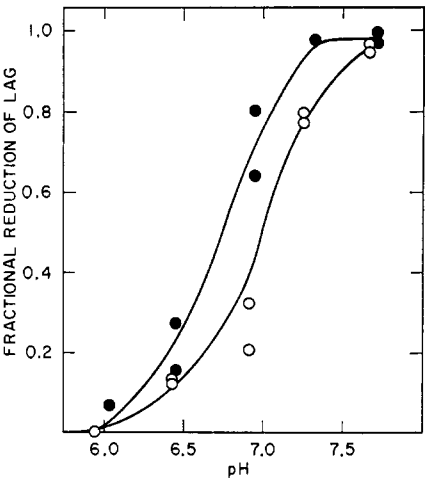

FIGURE 6: Effect of preliminary incubation with glutamate on reduction of the lag phase. Relaxed enzyme was incubated at 25° for 25 min at a concentration of 0.3 mg/ml, in 0.2 M imidazole. (○—○) Imidazole alone; (●—●) imidazole containing 65 mM glutamate. The pH of the preliminary incubation mixtures was measured directly after addition of glutamate and enzyme.

during relaxation at a comparable protein concentration (Shapiro and Ginsburg, 1968).

Effect of pH of the Preincubation Mixture. Relaxed glutamine synthetase was subjected to prior incubation at a variety of pH values at relatively high ionic strength. The fractional reduction of the lag as a function of pH is shown in Figure 5. It can be seen that even in the absence of added divalent cation, preliminary incubation at low pH values yields an enzyme preparation which demonstrates a marked lag phase, and preliminary incubation at high pH values yields an enzyme preparation which demonstrates no lag phase.

In the presence of added $MgCl_2$, the plot of fractional lag reduction *vs.* pH is shifted to a lower pH range. That is, the effect of added $MgCl_2$ is to decrease the pH required for a given fractional reduction in the lag.

It is also evident from the data in Figure 5 that the relationship between the lag phase and pH is influenced by the nature of the buffer system used. Thus, in the pH range of 6.5–7.5 a complex buffer mixture containing phosphate, acetate, and Tris salts was more effective in reducing the lag than was an imidazole buffer system. Other studies show that phosphate buffer alone (0.1 M) is more effective than is imidazole buffer in reducing the lag.

From considerations to be presented below, the effects of buffer ions and of alkaline pH on lag reduction may be attributable to the capacity of these effectors to increase the affinity of the relaxed enzyme for divalent cations. The trace levels of divalent cations that contaminate the buffer mixtures thus become sufficient to activate the enzyme.

Effect of Preliminary Incubation with Glutamate. As noted in Figure 1, prior incubation of relaxed enzyme with L-glutamate abolished the lag. The concentration of L-glutamate required to produce one-half reduction of the lag is 8 mM. A concentration of 0.1 M is required to effect essentially complete (>90%) reduction of the

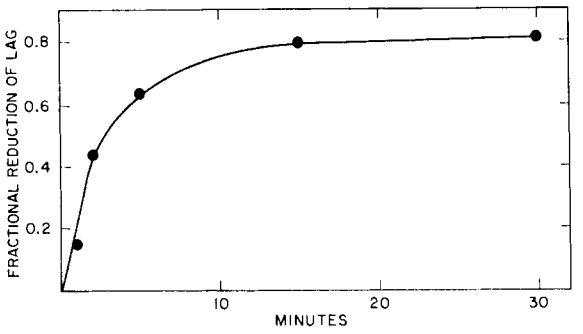

FIGURE 7: Time course of lag-phase reduction by $MgCl_2$. Relaxed enzyme was incubated at 25° at a concentration of 0.4 mg/ml, in 0.2 M imidazole (pH 7.1) containing 0.4 mM $MgCl_2$. Samples were removed for assay at the indicated times following addition of $MgCl_2$, and fractional reduction of lag calculated by comparison with a zero-time assay.

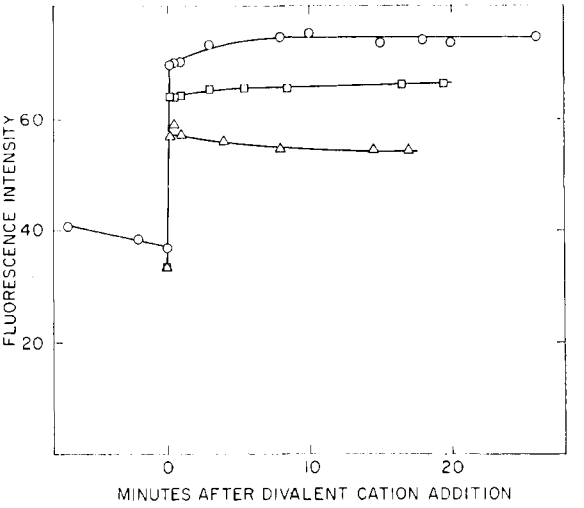

FIGURE 8: Effect of divalent cations on TNS-enzyme fluorescence. Relaxed enzyme at 1.54 mg/ml (2.5×10^{-6} M) in 0.01 M imidazole (pH 7.1) containing 10^{-6} M TNS was equilibrated for 10–20 min in the spectrophotofluorometer, and then divalent cation was added at zero time. Exciting wavelength, 365 mμ; emitting wavelength, 460 mμ. (○—○) 3 mM $MgCl_2$, (□—□) 3 mM $MnCl_2$, and (△—△) 3 mM $CaCl_2$.

lag. This effect appears to be highly specific for L-glutamate. Prior incubation of relaxed enzyme at pH 7.0 with 0.1 M concentrations of various analogs of glutamate or with other amino acids failed to abolish the lag. Compounds tested included DL-β-hydroxyglutamate, *N*-acetylglutamate, 2-methylglutamate, L-isoglutamine, L-glutamine, D-glutamine, α-amino-*n*-butyrate, α-ketoglutarate, L-alanine, L-valine, L-isoleucine, L-leucine, L-serine, L-methionine, and NaCl. Prior incubation with D-glutamate produced slight reduction in the lag, but the possibility that this effect was due to contamination of the D-glutamate with divalent cations was not excluded.

The effect of pH on the fractional reduction in lag obtained by prior incubation of relaxed enzyme in the presence and absence of L-glutamate is illustrated in Figure 6. As was the case with $MgCl_2$ the effect of L-glutamate is to lower the pH required to abolish the lag; *i.e.*, the curve obtained by plotting the fractional lag reduction against pH is shifted to a lower pH range when L-glutamate was present in the prior incubation mixtures.

The data reported in Figures 5 and 6 suggest that divalent cations are not needed to abolish the lag when relaxed enzyme is preincubated with 0.1 M glutamate or in the absence of glutamate at high pH (>7.5). This conclusion is probably not correct, however, since in other experiments no reduction in lag was achieved by either treatment when 5 mM EDTA was included in the prior incubation mixtures. Thus, there is either a direct effect of EDTA on the relaxed enzyme that prevents activation (tightening) by glutamate or high pH, or else the activation observed with these treatments is dependent upon the presence of trace amounts of divalent cations that contaminate the glutamate and buffer solutions. The latter is a reasonable possibility since direct binding studies (D. Denton and A. Ginsburg, unpublished data) have shown that the affinity of the enzyme for Mn^{2+} is increased very greatly by increasing the pH over the range of 7.0–8.5. Whether or not the tightening effect of glutamate can be explained by its ability to increase the affinity of the enzyme for divalent cations has not been investigated; however, this seems to be the most reasonable explanation for the fact that glutamate is without effect in the presence of EDTA. Nevertheless, the effects of pH and glutamate shown in Figures 5 and 6 cannot be due exclusively to contamination of the reagents with divalent cations. It was established by direct determination that the total concentration of divalent cations (Mg^{2+}, Mn^{2+}, and Ca^{2+}) in the buffers used for the experiment described in Figure 5 was less than 1 μM, yet the addition of twice this concentration of Mn^{2+} to a prior incubation mixture containing only 50 mM imidazole buffer (pH 6.7–7.0) did not have significant effect on the length of the lag. Similarly, at the high concentration employed, the glutamate used for these studies was contaminated with 1.9 μM Mg^{2+}, 9.7 μM Ca^{2+}, and less than 0.1 μM Mn^{2+} (none detected). By direct experiment, a mixture of $MgCl_2$ and $CaCl_2$ at these concentrations had no effect on the lag phase at pH 7.1. Most probably, the observed effects, are manifestations of pH-dependent multiple interactions involving glutamate, and possibly specific buffer ions as well as the divalent cations.

Time Course of Interaction with $MgCl_2$. Preliminary incubation of relaxed glutamine synthetase with $MgCl_2$ was carried out for varying lengths of time. Fractional reduction of lag as a function of time is shown in Figure 7. About 3-min prior incubation with $MgCl_2$ is required to obtain half-maximal reduction of the lag.

Effect of Divalent Cations on Fluorescence due to a Hydrophobic Probe. TNS has been shown to fluoresce strongly when in contact with hydrophobic solvents or aqueous solutions of hydrophobic proteins, but virtually not at all in water (McClure and Edelman, 1966). When a mixture of relaxed glutamine synthetase and TNS was mixed with $MgCl_2$, the fluorescence intensity instantaneously doubled and then did not change further (Figure 8). Similar changes were induced by $CaCl_2$ and $MnCl_2$. In repeated experiments the only constant finding was the instantaneous sharp rise on divalent cation

addition. The subsequent drift of the curves is not consistently in one direction or the other, and is probably due to lack of temperature control and/or electronic drift. NaCl and KCl (5.2 mM) produced a change in fluorescence intensity similar in direction but much smaller in magnitude. $BaCl_2$, which does not reduce the lag phase, increased the fluorescence intensity in much the same manner as did $MgCl_2$; in this respect the action of $BaCl_2$ on the enzyme is similar to its action in preventing inhibition by sulfhydryl reagents (Shapiro and Stadtman, 1967). The above salt solutions produced a very slight decrease in the fluorescence intensity of TNS–albumin mixtures. Although other possibilities have not been excluded, these observations are interpreted to indicate that the addition to divalent cations to relaxed enzyme causes a nearly instantaneous conformational change that involves the exposure of hydrophobic groups. The possibility that $MgCl_2$ simply changes the apparent binding constant for the TNS–enzyme interaction was excluded in the case of chymotrypsin, and various of its ligands (McClure and Edelman, 1967), and by analogy this is not considered a likely explanation for the present effect.

Discussion

The presence of a lag phase in the time course of glutamine synthesis as catalyzed by relaxed enzyme indicates that the relaxed enzyme is catalytically inactive, but becomes active under the conditions of assay. The fact that relaxation of glutamine synthetase is achieved by removal of divalent cations and the demonstration that prior incubation of the relaxed enzyme with either Mg^{2+}, Ca^{2+}, or Mn^{2+} results in its conversion into a catalytically active form, exhibiting no lag, indicates that the relaxation and tightening processes involve divalent cation–protein interactions. Moreover, these interactions are clearly influenced by pH, by inorganic orthophosphate, and possibly other buffer salts and especially by glutamate.

From other studies reported in the accompanying paper (Shapiro and Ginsburg, 1968) it was established that relaxation involves only a small decrease in the sedimentation constant and a slight increase in the intrinsic viscosity. It therefore appears that relaxation involves only subtle conformational changes in protein structure; dissociation of subunits clearly is not involved since there is no change in molecular weight. It is further evident that relaxation does not involve a rearrangement of subunits since the taut and relaxed forms of the enzyme are virtually indistinguishable in the electron microscope; both forms of the enzyme are composed of 12 subunits arranged in two superimposed hexagonal layers (Valentine *et al.*, 1968).

Nevertheless, however subtle the conformational changes may be, relaxation does result in a loss in catalytic activity and in substantial changes in the stability of the enzyme toward mild protein denaturants, and in the exposure of sulfhydryl groups to reaction with various alkylating agents and organic mercurials (Shapiro and Stadtman, 1967; Shapiro and Ginsburg, 1968). Moreover, the tightening of relaxed enzyme in the presence of divalent cations appears to be a complicated process, involving at least two phases. First there appears to be an essentially instantaneous exposure of hydrophobic groups as evinced by the instantaneous increase in fluorescence when divalent cation is added to relaxed enzyme in the presence of the hydrophobic probe, TNS. This is accompanied by concealment of the exposed sulfhydryl groups so that they no longer react with SH-specific reagents (Shapiro and Stadtman, 1967). These instantaneous changes are then followed by slower conformational changes that are attended by the burial of tryptophan and tyrosine residues (Shapiro and Ginsburg, 1968) and by restoration of catalytic activity. Although almost any divalent cation will cause the first rapid changes to occur, the second, slow conformational changes are caused only by Mg^{2+}, Mn^{2+}, or not of Ca^{2+}.

The existence of catalytically inactive and active forms of glutamine synthetase which can be rapidly interconverted *in vitro* by manipulations of pH and divalent cation and glutamate concentrations suggests that fluxes in these parameters could play an important role in the regulation of glutamine metabolism *in vivo*. However, very little is known about the changes in intracellular concentrations of divalent cations, hydrogen ion, or of glutamate, and so the physiological significance, if any, of the relaxation phenomenon remains to be determined. The fact that glutamate can stimulate the tightening of relaxed enzyme at low divalent cation concentrations suggests that the binding of this substrate affects the conformation of the enzyme. This may be responsible for the previously reported potentiation by glutamate of AMP and L-alanine inhibition of glutamine synthetase (Kingdon *et al.*, 1967).

References

Kingdon, H. S. (1967), *Federation Proc. 26*, 842.

Kingdon, H. S., Shapiro, B. M., and Stadtman, E. R. (1967), *Proc. Natl. Acad. Sci. U. S. 58*, 1703.

Kingdon, H. S., and Stadtman, E. R. (1967), *J. Bacteriol. 94*, 949.

Kornberg, A., and Pricer, W. E., Jr. (1951), *J. Biol. Chem. 193*, 481.

McClure, W. O., and Edelman, G. M. (1966), *Biochemistry 5*, 1908.

McClure, W. O., and Edelman, G. M. (1967), *Biochemistry 6*, 559.

Shapiro, B. M., and Ginsburg, A. (1968), *Biochemistry 7*, 2153 (this issue; paper after following paper).

Shapiro, B. M., and Stadtman, E. R. (1967), *J. Biol. Chem. 242*, 5069.

Valentine, R. C., Shapiro, B. M., and Stadtman, E. R. (1968), *Biochemistry 7*, 2143 (this issue; following paper).

Woolfolk, C. A., Shapiro, B. M., and Stadtman, E. R. (1966), *Arch. Biochem. Biophys. 116*, 177.

Woolfolk, C. A., and Stadtman, E. R. (1964), *Biochem. Biophys. Res. Commun. 17*, 313.

Woolfolk, C. A., and Stadtman, E. R. (1967), *Arch. Biochem. Biophys. 118*, 736.