Some Characteristics of the Binding of Substrates to Glutamine Synthetase from *Escherichia coli**

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ABSTRACT: The binding of adenosine triphosphate to the glutamine synthetase from *Escherichia coli* has been investigated by equilibrium and kinetic studies. Enzyme preparations containing different amounts of covalently bound 5′-adenylyl groups were used to determine some characteristics of the binding of substrates to adenylylated and unadenylylated enzyme forms. A Mn^{2+}-activated form of glutamine synthetase binds 12 equiv of adenosine triphosphate/600,000 g of enzyme, or one adenosine triphosphate per subunit, with an apparent association constant of 2×10^5 $mole^{-1}$ estimated for the equilibrium binding of adenosine triphosphate–Mn at 4°. The addition of ethylenediaminetetraacetate in excess of divalent cation produces as enzyme form (*relaxed*) that is unable to bind adenosine triphosphate in the same concentration range (unless Mn^{2+} or Mg^{2+} are readded—conditions shown previously to convert the inactive relaxed enzyme into an active tightened enzyme form). The activation and inhibition of glutamine synthetase at low and high free Mn^{2+} concentrations, respectively, are apparent also from kinetic studies with varying adenosine triphosphate and divalent cation concentrations. The binding and parallel kinetic results indicate that there is a negative type of interaction involved in the binding of adenosine triphosphate to Mn^{2+}-activated glutamine synthetase, and that this interaction is a function of the adenylylation state of the enzyme. Kinetic studies suggest that a similar type of interaction occurs when the other substrates (glutamate and ammonia) react with the Mn^{2+}-activated enzyme forms. (In these cases, approximate bimodal substrate saturation curves are obtained with the apparent affinity of the enzyme for substrates greater at low than at high substrate concentrations.) The Mg^{2+}-activated enzyme forms have apparent hyperbolic substrate saturation curves, but slightly different K'_m values for glutamate (ammonia not measured). The kinetic results indicate that subunit interactions may occur between adenylylated (Mn^{2+} specific) and unadenylylated (Mg^{2+} specific) subunits in hybrid glutamine synthetase molecules. Interactions between subunits appear to affect the affinity of the subunits for substrates in such a way that catalytically inactive subunits, in either the Mn^{2+} or Mg^{2+} biosynthetic assay systems, partially stabilize active, high-affinity enzyme forms at low substrate concentrations. The binding results suggest also that catalytically inactive enzyme-substrate complexes may be formed, since a partially adenylylated, Mn^{2+}-activated glutamine synthetase preparation had the capacity to bind one adenosine triphosphate per subunit even though only an average of 2.3 out of 12 subunits are catalytically active in a Mn^{2+}-dependent biosynthetic assay. The possible physiological significance of the type of subunit interactions observed is discussed. The reversibility of the biosynthetic reaction catalyzed by the glutamine synthetase from *E. coli* has been demonstrated. Under optimum conditions, the reverse reaction is catalyzed at only about $^1/_{50}$th the rate of the biosynthetic reaction, with the divalent cation specificity of one enzyme preparation (having a Mg^{2+} to Mn^{2+} activity ratio $\simeq$2:1), apparently maintained in either direction. Also, the rate of the Mg^{2+}-catalyzed reversal of glutamine synthesis was found to be approximately proportional to the concentration of unadenylylated subunits in four enzyme preparations.

The regulation of glutamine synthetase activity is of central importance in *Escherichia coli* and multiple mechanisms of control of this activity have been described (see Stadtman *et al.*, 1968). In assigning the role of specific metabolites in regulatory mechanisms, it is important to understand the relationship of substrate binding to the control of the enzymatic activity of allosteric enzymes. This paper is concerned with certain aspects of the binding of substrates to the glutamine synthetase of *E. coli*. Not only is there specific involvement of the substrate binding in the control of enzymatic activity, but the substrates and products of the catalyzed biosynthetic reaction:

$$\text{ATP} + \text{L-glutamate} + \text{NH}_3 \overset{M^{2+}}{\rightleftharpoons} \text{L-glutamine} + \text{ADP} + \text{P}_i$$

are directly related to another mechanism of regulation of this enzyme. This involves enzymatic adenylylation (Kingdon *et al.*, 1967; Wulff *et al.*, 1967; Stadtman *et al.*, 1968) and deadenylylation (Shapiro and Stadtman, 1968a; Battig *et al.*, 1968; Shapiro, 1969), under certain conditions of growth of *E. coli* (Kingdon and Stadtman, 1967b; Heilmeyer *et al.*, 1967; Holzer *et al.*, 1967, 1968). It was shown by Kingdon *et al.* (1967) that adenylylation of glutamine synthetase changes the divalent cation requirement in the biosynthetic reaction from Mg^{2+} to Mn^{2+}, with the Mn^{2+}-dependent biosynthetic activity of adenylylated subunits being intrinsically lower than the Mg^{2+}-dependent activity of unadenylylated subunits. Apparent specific and equivalent activations of adenylylated subunits by Mn^{2+}, and of unadenylylated subunits by Mg^{2+}, were observed during the adenylylation of the native dodecameric enzyme (Stadtman *et al.*, 1968).

The glutamine synthetase from *E. coli* is isolated as a homogeneous protein (Woolfolk *et al.*, 1966) except with respect to the amount of 5′-adenylyl groups attached (Shapiro *et al.*,

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1967). The enzyme has a molecular weight of 600,000 (Shapiro and Ginsburg, 1968), and exists as a molecular aggregate of twelve apparently identical subunits (Woolfolk *et al.*, 1966; Shapiro and Stadtman, 1967) arranged in two hexagonal layers (Valentine *et al.*, 1968). The dodecameric aggregate may be adenylylated to the extent of 12 equiv of 5′-adenylyl groups per enzyme molecule (Kingdon *et al.*, 1967; Wulff *et al.*, 1967). The adenylyl group is covalently bound to the polypeptide structure of glutamine synthetase through a stable phosphodiester bond to a specific tyrosyl hydroxyl group (Shapiro *et al.*, 1967; Shapiro and Stadtman, 1968b).

Since it appears that each subunit may be adenylylated, at least 13 different isoenzyme structures are possible, with 0 to 12 equiv of 5′-adenylyl groups bound per enzyme molecule. In addition, the variation in the molecular distribution of 5′-adenylyl groups within hybrid molecules potentially provides an enormous number of possible isomeric species. Interactions between unadenylylated and adenylylated subunits within hybrid molecules could have a physiological role, and this is considered in conjunction with the substrate binding studies presented here and in an accompanying paper (Ginsburg *et al.*, 1970; see also Stadtman *et al.*, 1968). A previous study of the binding of specific divalent cations to glutamine synthetase (Denton and Ginsburg, 1969) indicated that heterologous subunit interactions within hybrid molecules occur in the binding of the activating divalent cation. In other studies, the removal of Mn^{2+} from native (taut) glutamine synthetase was shown to produce a catalytically inactive (relaxed) enzyme form that could be reactivated (tightened) by the addition of Mn^{2+}, Mg^{2+}, or Ca^{2+} (Kingdon *et al.*, 1968), with a rather large conformational change accompanying the relaxation or tightening processes (Woolfolk and Stadtman, 1967b; Shapiro and Stadtman, 1967; Shapiro and Ginsburg, 1968). Since ATP itself has a high affinity for divalent cations (Perrin and Sharma, 1966), the multiple effects of specific divalent cations on glutamine synthetase obviously are related to the studies reported here on the binding of ATP to this enzyme.

Experimental Section

Enzyme Preparations. The glutamine synthetase preparations used in these studies were of uniform homogeneity with the exception of the amount of adenylyl groups covalently attached to the enzyme.[1] The preparations $E_{\overline{1.2}}$, $E_{\overline{2.3}}$, and $E_{\overline{9}}$ have been described elsewhere (Shapiro *et al.*, 1967; Shapiro and Ginsburg, 1968; Denton and Ginsburg, 1969). The preparation $E_{\overline{2.3}}$ was used to make E_{12} *in vitro*.[2] The relatively unadenylylated $E_{\overline{2.3}}$ enzyme (as the Mg^{2+} enzyme) was incubated at 37° with 1.5 mM glutamine, 2.6 mM [^{14}C]ATP, 50 mM $MgCl_2$, and 20 mM Tris buffer at pH 7.8, and $^1/_{30}$th of the $E_{\overline{2.3}}$ (by weight) of a partially purified (about 75-fold) preparation of ATP: glutamine synthetase adenylyltransferase.[2] The reaction was pulsed with 10 mM KCl, phosphoenolpyruvate, and pyruvate kinase to resynthesize ATP from ADP formed by ATPase activity in the incubation mixture. The extent of adenylylation was determined by the heat-stable, trichloroacetic acid insoluble radioactivity. When adenylylation was complete, the glutamine synthetase was repurified through the acid ammonium sulfate (pH 4.4), acetone, and several more (pH 4.4) ammonium sulfate precipitation steps of the glutamine synthetase purification procedure described by Woolfolk *et al.* (1966). After purification, E_{12} had the spectrum, radioactivity, and Mn^{2+} ($\pm Mg^{2+}$) γ-glutamyl transfer activity (see Stadtman *et al.*, 1968) corresponding to 11.8 ± 0.1 equiv of 5′-adenylyl groups per 600,000 g of enzyme ($E_{\overline{11.8}}$).

Enzyme solutions were dialyzed before use, and protein concentrations were determined from absorbancy measurements at 280 mμ as described previously (Shapiro and Ginsburg, 1968).

The relaxed enzyme was prepared as previously described by incubating the native taut Mn^{2+}–enzyme ($E_{\overline{2.3}}$) with excess EDTA at 25° to chelate the Mn^{2+} and then dialyzing against a buffer at pH 7.4 (4°) containing 10 mM EDTA, 20 mM imidazole-chloride, and 100 mM KCl (Shapiro and Ginsburg, 1968). The tightening process was performed as described by Kingdon *et al.* (1968) in which a stock solution of relaxed enzyme solution was diluted 15–30-fold with buffer to a final concentration of either 5 mM $MnCl_2$ or 50 mM $MgCl_2$ (EDTA <1 mM), and then incubated at 20° for 30 min. The relaxed and tightened glutamine synthetase forms then were used directly in the ATP binding studies. To characterize these enzymes according to the procedure developed by Shapiro and Stadtman (1967), the relaxed enzyme was shown to be completely inactivated by treatment with 10^{-4} M *p*-chloromercuriphenylsulfonate for 2 hr at pH 8 and 0°; whereas the tightened forms correctly were completely resistant to this action of organic mercurials.

Other Materials. [^{14}C]ATP (400 mCi/mmole) was obtained from New England Nuclear Corp. as the tetralithium salt; nonradioactive ATP was purchased as the disodium salt from Sigma Chemical Corp. and was recrystallized three times from 50% ethanol prior to use to remove the small amounts of ADP present. Inorganic [^{32}P]orthophosphate (carrier free) was obtained also from New England Nuclear Corp. Other compounds used were from sources indicated previously (Ginsburg, 1969) or from the usual commercial sources.

Enzyme Assays. Glutamine synthetase activity was measured by the phosphate assay at 37 or 25° (Woolfolk *et al.*, 1966) or by the spectrophotometric assay at ~22–26° described by Kingdon *et al.* (1968) in which ADP formation is coupled to the oxidation of DPNH and resynthesis of ATP from phosphoenolpyruvate by the activities of pyruvate kinase and lactic dehydrogenase. A Cary Model 15 recording spectrophotometer was used to measure velocities in the spec-

[1] For simplicity, $E_{0,\bar{1},\bar{2},\ldots}$ refer to different preparations of purified glutamine synthetase (E) with the same amino acid composition (Shapiro *et al.*, 1967; Denton and Ginsburg, 1969) which contain the average (denoted by the bar) amount of covalently bound 5′-adenylyl groups (designated by the subscript) per molecule of glutamine synthetase of 600,000 molecular weight. The extent of adenylylation in each case is determined by spectral, phosphate, and assay measurements (Kingdon *et al.*, 1967; Shapiro *et al.*, 1967; Stadtman *et al.*, 1968). Consequently, the results reflect the average state of adenylylation of the entire enzyme population since the extent and loci of adenylylation of any one molecule are not known; *i.e.*, there probably exists heterogeneity with respect to the molecular distribution of 5′-adenylyl groups within enzyme preparations $E_{\bar{1}}$ to $E_{\overline{11}}$. The term, $E_{0,1,\ldots}E_{12}$ (without the bar over the subscript), is reserved for the designation of homogeneous preparations in which each molecule is adenylylated to the extent indicated by the subscript. Thus, completely unadenylylated and fully adenylylated glutamine synthetase may be represented as E_0 and E_{12}, respectively.

[2] Unpublished procedure of M. D. Denton and A. Ginsburg.

trophotometric assay system, and the 0.0–0.1 slide-wire was used at low substrate concentrations for the ATP and L-glutamate kinetic data. All velocities were corrected for a very low rate of ADP formation by the different glutamine synthetase preparations in the absence of L-glutamate. The assay conditions are given in the figure legends. Mg^{2+}- or Mn^{2+}-dependent biosynthetic activities could be determined with either the phosphate or spectrophotometric assay systems by using $MgCl_2$ or MnCl, respectively, for the divalent cation requirement.

For studies of the reverse biosynthetic reaction, the following procedure was used: the glutamine synthetase preparation (about 5–10 μg) was incubated at 37° for 10 min in a reaction volume of 0.3 ml containing (for saturating substrate concentrations) 16 mM L-glutamine, 10 mM ADP, and 80–100 mM inorganic [^{32}P]phosphate at pH 7 (specific radioactivity 10^5–10^6 dpm/μmole) with either 50 mM $MgCl_2$ or 1.7 mM $MnCl_2$. When $MnCl_2$ was used as the divalent cation, the pH was adjusted to pH 6 by the addition of 100 mM acetate. The reaction was stopped by the addition of 1 ml of 7% perchloric acid which contained 1 M NaH_2PO_4. Then, 0.5 ml of a water suspension of 40 mg/ml of Norit A (prewashed with 7% phosphoric acid and then water to neutrality) was added. After equilibrating for 10–20 min in ice, the charcoal was collected by filtration on a 0.45 μ Type H A Millipore filter with 4–5 ml of water rinses. The filters were placed on planchets (charcoal side down), held in place by stainless steel rings, and oven dried. Counting was performed with Nuclear-Chicago gas-flow counter. This procedure gives a measurement of the incorporation of [^{32}P]P_i into [γ-^{32}P]ATP (charcoal adsorbable material) for the reverse reaction. Values were corrected for blank values obtained either by the omission of enzyme or glutamine from the reaction mixture. For calculating the μmoles of [γ-^{32}P]ATP formed, an aliquot of the [^{32}P]P_i of known phosphate concentration was counted on a separately prepared charcoal filter. Specific activity of the enzyme is expressed as the micromoles of [γ-^{32}P]ATP formed per minute per milligram of glutamine synthetase.

Binding Measurements. The equilibrium binding of [^{14}C]-ATP to glutamine synthetase was measured by the ultracentrifuge method of Velick *et al.* (1953), as modified by Frieden and Colman (1967). In this procedure, the [^{14}C]ATP is centrifuged with and without protein in a Spinco Model L centrifuge using the 40.3 rotor at 39,000 rpm for 16 hr at 4°. The sedimentation of [^{14}C]ATP itself was determined from four samples centrifuged simultaneous with each group of protein samples, and the average of these was used to correct for the solvent distribution of [^{14}C]ATP. This correction was 5% or less. All supernatants were assayed for enzymatic activity. Usually there was no enzyme present in the supernatant, but occasionally a small amount (<1%) of enzyme remained and if so, the free concentration values of ATP were corrected accordingly. This correction was always minor and therefore did not alter the data. The specific radioactivities of the standard [^{14}C]ATP solutions were determined using the molar extinction coefficient of 15.4×10^3 for ATP at 259 mμ (pH 7), and 1 cm (Boch *et al.*, 1956) and the Nuclear-Chicago Mark 1 scintillation counter with the solvent of Bray (1960). Solvent [^{14}C]ATP concentrations in the centrifuged samples were determined by the same counting procedure, with identical aliquots taken to that of the standard [^{14}C]ATP solution. pH Determinations were made at room temperature (23–25°) with a Radiometer type PHM 25 pH meter equipped with a scale expander and Leeds and Northrup microelectrodes.

Analysis of the Data. The type of information obtained by the centrifuge method of Velick *et al.* (1953) is the same as that obtained by equilibrium dialysis, and therefore is subject to the same mathematical analysis discussed previously (Denton and Ginsburg, 1969; Ginsburg, 1969). The binding parameters are $\bar{\nu}$ (moles of substrate bound per 600,000 g or mole of glutamine synthetase), n' (apparent maximum number of substrate binding sites per enzyme molecule), c (free concentration of substrate), and K_A' (apparent intrinsic association constant). The kinetic data for the Mn^{2+}-activated enzyme forms were found to be best expressed as a saturation function (Frieden, 1967) analogous to the Scatchard plot of binding data (Scatchard, 1949). In the kinetic plot, v' is the observed velocity (or specific activity of the enzyme in micromoles of ADP (or P_i) formed per minute per milligram of enzyme) and S is the molar substrate concentration. In the spectrophotometric assay, initial rates of catalysis (v') are measured, whereas in the phosphate assay, the P_i formed is measured after a timed incubation at 37°. The kinetic data for the Mg^{2+}-activated enzyme forms could be expressed by more familiar saturation functions (see Dixon and Webb, 1964). Hill plots of the data were constructed as outlined by Wyman (1964, 1967).

Results

Studies of ATP Binding. TAUT ENZYME. The binding of ATP–Mn at pH 7.4 (4°) to the Mn^{2+}-activated form of glutamine synthetase is shown in Figure 1. The enzyme preparation used in these studies was adenylylated to the extent of 2.3 equiv of 5′-adenylyl groups per mole of enzyme. Figure 1a illustrates a Scatchard plot of the ATP binding data with 5 mM $MnCl_2$ present. The data extrapolate to an apparent n' of 12 (or one ATP binding site per enzyme subunit), with an apparent association constant of 2×10^5. That is, glutamine synthetase appears to possess a number of potential catalytic sites for the ATP substrate equal to the number of subunits in the enzyme macromolecule. However, it can be seen that at very low ATP concentrations, all of the determinations deviate consistently from the linear extrapolation of the data obtained at higher free ATP levels. At the low free concentrations of ATP, the slope of the plot in Figure 1a indicates that the enzyme has an apparently greater affinity for ATP than at the higher ATP concentrations.

RELAXED ENZYME. In Figure 1b are shown the binding data of Figure 1a plotted differently (see Denton and Ginsburg, 1969) to compare with ATP binding data obtained with relaxed enzyme (EDTA present) or with relaxed enzyme to which either Mn^{2+} or Mg^{2+} has been readded to produce the tightened enzyme form. The theoretical curve of Figure 1b is constructed from the binding parameters obtained at the higher ATP concentrations (Figure 1a; $n' = 12, k_A' = 2 \times 10^5$). The reproducible deviations of the points from the theoretical curve at low ATP concentrations are less obvious in this plot than in that Figure 1a. In the presence of excess EDTA to Mn^{2+}, there was no detectable ATP binding at less than 9×10^{-5} M ATP (Figure 1b). At higher ATP concentrations, the error in the measurements is increased to about the same magnitude as the extent of binding observed at $\sim 10^{-4}$ M ATP with the relaxed enzyme. However, the previous results of Shapiro and Stadtman (1967) and Kingdon *et al.* (1968)

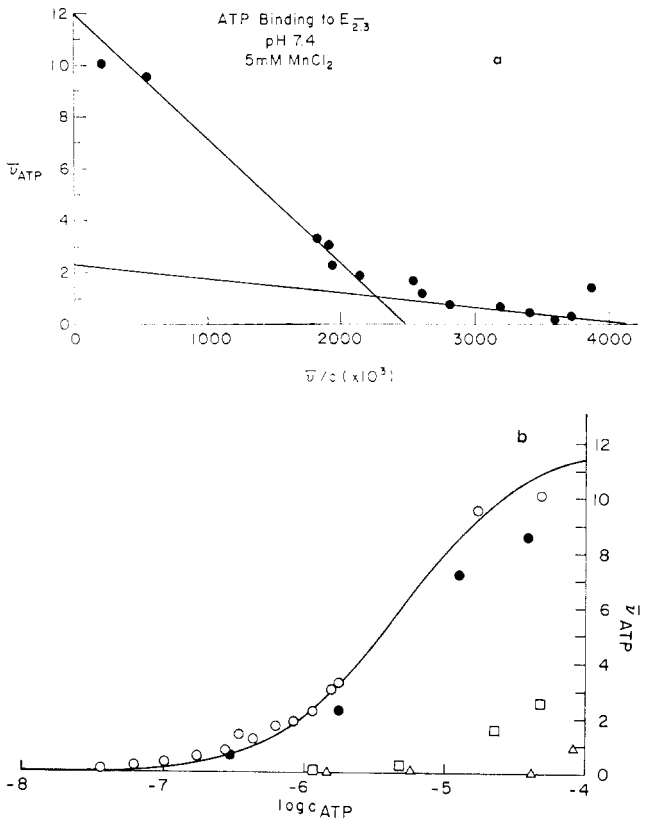

FIGURE 1: The binding of [^{14}C]ATP to $E_{\overline{2.3}}$ at 4°. (a) Scatchard plot of the data for the binding of [^{14}C]ATP to the glutamine synthetase preparation $E_{\overline{2.3}}$ at pH 7.4 in the presence of 5 mM $MnCl_2$, 0.02 M imidazole-chloride, and 0.1 M KCl. (b) (○) Alternate representation of the data in (a) with a theoretical curve constructed from the binding parameters: $n' = 12$, $k_A' = 2 \times 10^5$; (△) binding of [^{14}C]ATP to the relaxed enzyme form with 0.01 M EDTA present in 0.02 M imidazole and 0.1 M KCl (see text); (●, □) binding of [^{14}C]ATP to the tightened enzyme, where relaxed $E_{\overline{2.3}}$ was tightened with 5 mM $MnCl_2$ (●) or 50 mM $MgCl_2$ (□) in the presence of 0.02 M imidazole and 0.1 M KCl (EDTA < 1 mM) at pH 7 and 24° as described in the text.

suggest that concentrations of ATP greater than 1 mM do effect structural changes in the relaxed enzyme form. Thus, it is quite probable that the relaxed enzyme form can bind significant amounts of ATP at much higher ATP concentrations than were used in the binding experiments.

TIGHTENED ENZYME. It is apparent from the data presented in Figure 1a that glutamine synthetase tightened with 5 mM Mn^{2+} has a much higher affinity for ATP than does the enzyme tightened with 50 mM Mg^{2+}. The affinity difference can be estimated to be about 30-fold from the data of Figure 1b, assuming a comparable n' value for the Mn^{2+}- and Mg^{2+}–ATP systems. Kinetic data (see below) indicate that glutamine synthetase has about the same K_m' value for the ATP–Mn complex in the Mn^{2+}-activated assay as for the ATP–Mg complex in the Mg^{2+}-activated biosynthetic assay, in agreement with the previous results of Woolfolk *et al.* (1966). This K_m' value approximates the $1/k_A'$ of 1.6×10^{-4} M^{-1} estimated here from the measurements of the binding of the ATP–Mg complex. Thus, a large shift in affinity occurs when the Mn^{2+}-activated enzyme binds ATP–Mn under assay conditions. The decreased affinity of the Mn–enzyme for ATP–Mn in the presence of other substrates is illustrated below in Figure 2c.

The enzyme tightened with Mn^{2+} consistently bound less ATP at each ATP concentration studied than did the native taut enzyme form. The meaning of the difference between the tightened Mn^{2+}–enzyme and the native forms observed here in the ATP binding is unclear. Two likely possibilities which are not mutually exclusive are that there was an absolute decrease in the total number of binding sites during the relaxation and subsequent tightening process (as indicated by Scatchard plots), and/or there are real differences in the mechanism of the binding of ATP to tightened as compared with taut enzyme. In previous studies, the tightened enzyme form was indistinguishable from the native taut form in enzymatic or most physical–chemical properties (Shapiro and Stadtman, 1967; Kingdon *et al.*, 1968; Shapiro and Ginsburg, 1968). An observed exception was that there is an increased tendency for the tightened form to crystallize out of solutions at low ion strength (Valentine *et al.*, 1968). Although high ionic strengths were employed in these studies, it is possible that the tightened enzyme preparation contained some aggregates with potential ATP binding sites blocked. Alternatively, the tightened enzyme may not regain its full capacity to bind ATP unless other substrates are present, as under assay conditions.

A Kinetic Analysis of ATP Binding to the Mn^{2+}-Activated Enzyme. The high-affinity segment of the Scatchard plot of the ATP binding data shown in Figure 1a extrapolates to a value for n' between 2 and 3. The similarity between this n' value and the number of adenylylated subunits of the $E_{\overline{2.3}}$ preparation[1] initially suggested that the adenylylated subunits bind ATP–Mn with a higher affinity than do unadenylylated subunits. However, this interpretation is not consistent with results of kinetic studies. It was previously shown by Kingdon *et al.* (1967) that when Mn^{2+} is substituted for Mg^{2+} in the biosynthetic assay system the activity of glutamine synthetase is directly proportional to the number of adenylylated subunits. From this and other considerations (Stadtman *et al.*, 1968; see also the accompanying paper, Ginsburg *et al.*, 1970), it is evident that the Mn^{2+}-activated biosynthetic activity is due entirely, or very nearly so, to adenylylated subunits. If the high-affinity binding sites for ATP–Mn (Figure 1a) are solely attributable to the number of adenylylated subunits, the Mn^{2+}-dependent biosynthetic activity should reflect also binding of ATP–Mn to the high-affinity sites; moreover, binding to the low affinity sites (*i.e.*, presumably to the unadenylylated subunits) should lead to catalytically inactive complexes. These predictions are not supported by kinetic studies in which the effect of varying ATP concentrations on the Mn^{2+}-dependent catalytic activity was determined.

In Figure 2a the kinetic data for the ATP saturation function in a Mn^{2+}-dependent biosynthetic assay is plotted in a manner analogous to the Scatchard binding plot (see Methods) so that Figures 1a and 2a may be directly compared. There is a qualitative similarity between the ATP binding and kinetic data obtained with the $E_{\overline{2.3}}$ preparation, with both studies encompassing the same ATP concentration range. Thus, it is evident that the catalytic activity is not restricted to the low ATP concentration range in which apparent high-affinity binding sites are reflected. In fact, in Figure 2a that portion of the substrate concentration–activity curve that should reflect the high-affinity binding sites extrapolates to a low V_{max}',

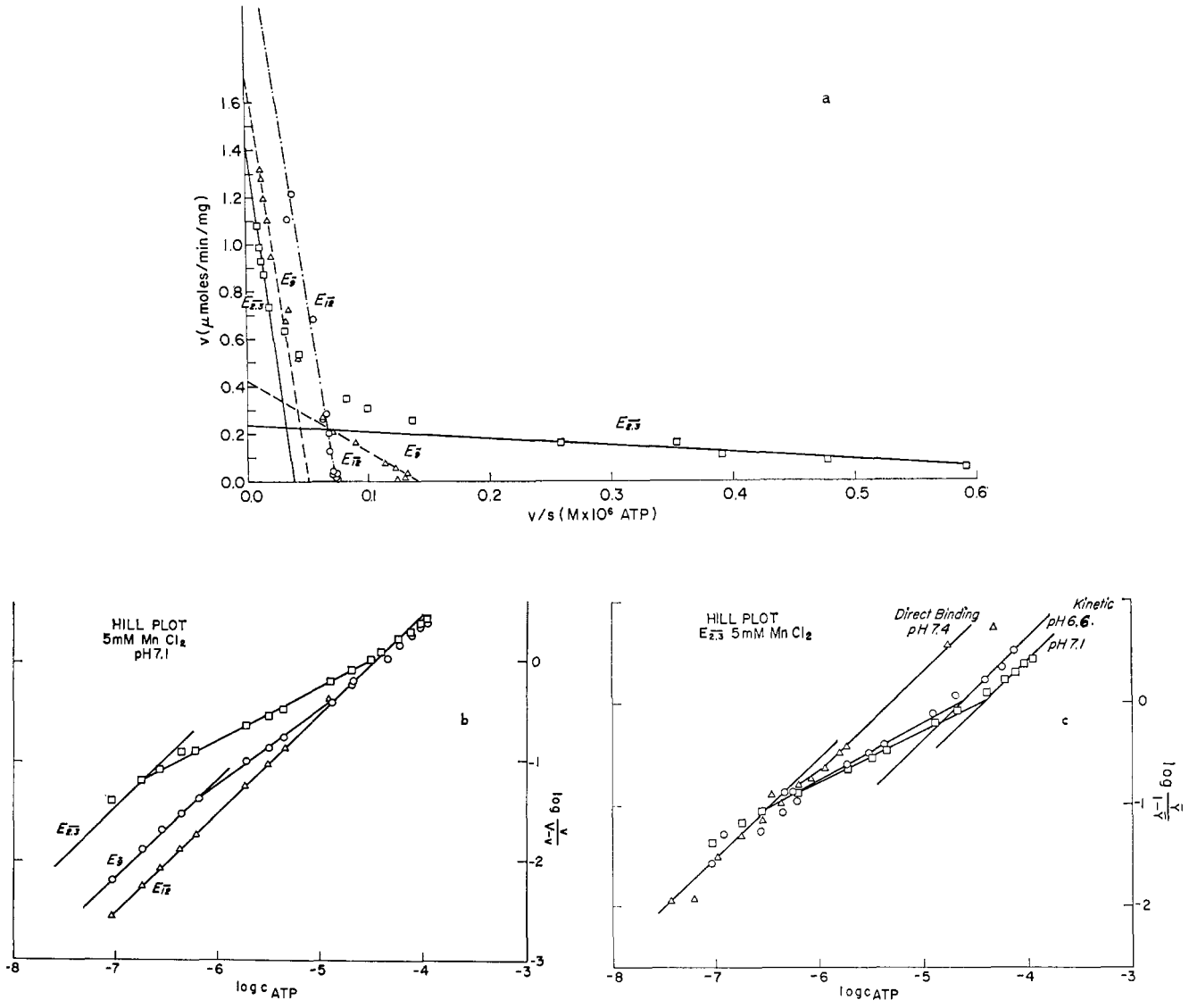

FIGURE 2: Kinetic studies of the binding of ATP to different preparations of glutamine synthetase in the presence of 5 mM $MnCl_2$. (a) The data plotted in a manner analogous to the Scatchard plot, where V is the initial velocity and S is the molar concentration of ATP (see Methods). Three preparations of enzyme were used: (□) $E_{\overline{2,3}}$; (△) $E_{\overline{9}}$; (○) $E_{\overline{11,8}}$. All assays at ~22° contained in addition to 5 mM $MnCl_2$, 30 mM glutamate, 50 mM NH_4Cl, and the other components of the standard spectrophotometric assay at pH 7.1 with 50 mM Tris-maleate buffer. The activities shown are suppressed by the suboptimal assay conditions used (see text) and with $E_{\overline{9}}$ and $E_{\overline{11,8}}$, by the suboptimal concentrations of glutamate and ammonia present (see text). (b) Hill plots of the data in (a): (□) $E_{\overline{2,3}}$; (○) $E_{\overline{9}}$; (△) E_{12} where V is the extrapolated apparent maximum velocity at $v/s = 0$, and v is the observed velocity at the concentration (c) of ATP. (c) Hill plots of: the equilibrium data for the binding of ATP to $E_{\overline{2,3}}$ with 5 mM $MnCl_2$ present (△) given in parts a and b, where $\bar{y} = \bar{v}/12$; the kinetic data for the saturation of $E_{\overline{2,3}}$ with ATP (□) from a and b with $\bar{y} = v/V$; kinetic data for $E_{\overline{2,3}}$ under the same conditions as for a except at pH 6.04 (○).

whereas the low-affinity segment extrapolates to a high V_{max} value. Further, with preparations of glutamine synthetase that are more adenylylated ($E_{\overline{9}}$ and $E_{\overline{11,8}}$ in Figure 2a), it is the apparent high-affinity segment of the plot that may be lost. These results are incompatable therefore with the interpretation that the high-affinity sites of ATP binding (Figure 1a) are specifically attributable to the adenylylated subunits, since there is substantial evidence that only adenylylated subunits are catalytically active in the Mn^{2+} biosynthetic assay system.

In addition to the data for $E_{\overline{2,3}}$, Figure 2a,b also shows the effect of ATP concentration on two other enzyme preparations, $E_{\overline{9,0}}$ and $E_{\overline{11,8}}$.[1] The results of Figure 2a indicate that at the higher ATP concentrations, all three preparations exhibit the same apparent affinity for ATP (identical slopes), but extrapolate to increasing values of V_{max}' with increasing states of adenylylation. The lower affinity sites determined kinetically under these specific assay conditions have slopes (K_m') that are approximately one order of magnitude greater than the slope of the lower affinity site determined by direct binding ($-K_D'$ or $1/-K_A'$) of ATP-Mn in the absence of the other substrates (see Figures 1a and 2c). The slope of the apparent higher affinity segment for $E_{\overline{2,3}}$ in Figure 2a (lower ATP concentrations) is essentially the same as that determined by direct binding, indicating that the kinetic data reflect the binding of ATP at very low concentrations.

With observed deviations from Michaelis–Menten kinetics,

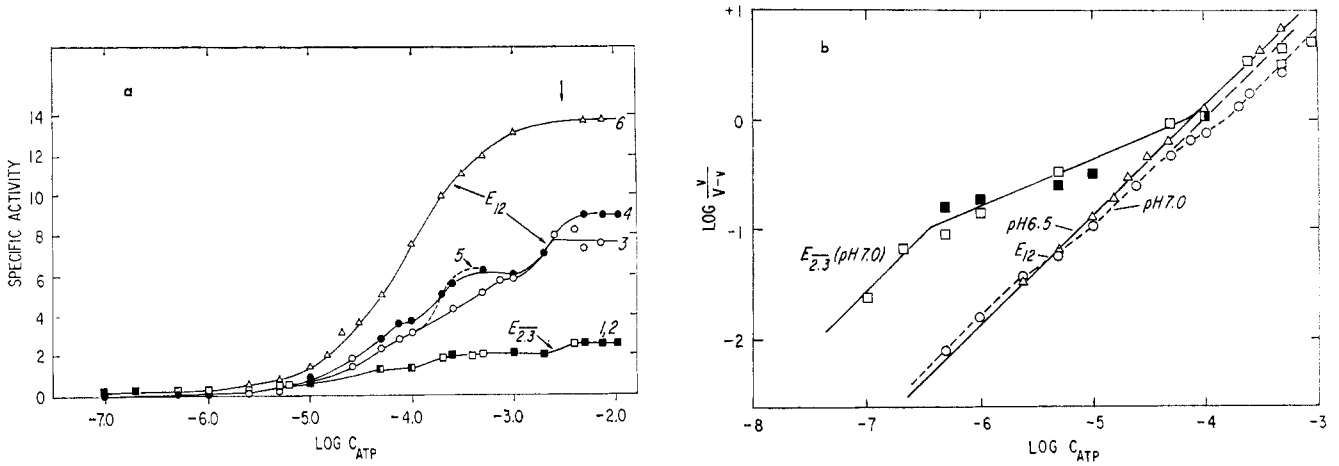

FIGURE 3: The influence of different assay conditions on ATP saturation curves at 25° for Mn^{2+}-activated $E_{2,3}$ and E_{12} preparations. (a) The log of the ATP concentration is plotted against the specific activities of $E_{2,3}$ (□, ■) and E_{12} (○, ●, △). Spectrophotometric assays (see Methods) using a mixed buffer of 50 mM each maleic acid, imidazole, and Tris at a controlled temperature of 25° were used. Besides the normal components, assay mixtures contained: curves 1, 3 (□, ○) at pH 7.03, 5 mM $MnCl_2$, 30 mM L-glutamate, and 50 mM NH_4Cl; (dashed curve 5 shows the only deviation from curve 3 obtained by reducing the $MnCl_2$ concentration tenfold to 0.5 mM $MnCl_2$); curves 2, 4 (■, ●) at pH 7.03, 6 mM $MnCl_2$, 100 mM L-glutamate, and 100 mM NH_4Cl; curve 6 (△) at pH 6.50 with substrate concentrations as for curve 4. At the arrow for the high ATP concentrations, sufficient $MnCl_2$ was added to each assay to provide a 1 mM excess of $MnCl_2$ to ATP. (b) Hill plots of the data from curves 2, 4, and 6 in (a), where v is the observed initial velocity and V is the maximum velocity attained at 5 mM ATP. The data for $E_{2,3}$ (curve 2 in a) are represented by the open symbols, whereas the closed symbols show results obtained with a tightened preparation of $E_{2,3}$ prepared by adding 5 mM $MnCl_2$ to a relaxed preparation of $E_{2,3}$ as in Figure 1b. For clarity, a dashed line is drawn through the results obtained with E_{12} at pH 7 (○), and a single line of unit slope connects the data for E_{12} at pH 6.5 (△).

the data are conveniently expressed in a Hill plot (see Wyman 1964, 1967). Figure 2b shows Hill plots of the kinetic data presented in Figure 2a. At higher ATP concentrations, all three curves approach a slope of one at essentially the same position on the abscissa which is equivalent to the half-saturation point. The data for $E_{11,8}$, which appear to be uncomplicated in Figure 2a, fit a straight line of unit slope that is indicative of this enzyme form having a single apparent affinity constant for ATP–Mn. However, the data for $E_{2,3}$ and E_9 show marked deviations from the single slope for E_{12} with decreasing ATP concentrations. At very low ATP concentrations, the data for the partially adenylylated enzyme preparations again approach unit slopes but these are located at different positions on the abscissa. This reflects the apparent increased affinity of the enzyme for ATP in this ATP concentration range with decreasing extents of adenylylation. The asymptotes at the low and high concentration extremes of the saturation function of the Hill plot with $E_{2,3}$ and E_9 are unity (which indicates that a finite interaction occurs), and are connected by a line with a slope of less than one. The midpoint of the line connecting the asymptotes obtained with $E_{2,3}$ and E_9 is not at zero (half-fractional saturation) on the ordinate. The general features of the Hill plots in Figure 2b strongly suggest that a negative type of interaction occurs between ATP–Mn binding sites of adenylylated subunits when unadenylylated subunits are present in a hybrid enzyme molecule. (These data suggest the existence of hybrid species of glutamine synthetase containing both adenylylated and unadenylylated subunits.)[1]

Figure 2c shows Hill plots of data obtained with the $E_{2,3}$ preparation for the binding of ATP from the direct measurements at pH 7.4 (4°) and from kinetic studies at pH 6.6 and 7.1 (~22°). The ATP binding data are the same as those presented in Figure 1a, and the kinetic data at pH 7.1 are from those in Figure 2b. This figure illustrates the differences between the kinetic and binding data for ATP–Mn with $E_{2,3}$. At low ATP concentrations, all three sets of data are described by a unit slope at the same position on the abscissa. The deviations at higher ATP concentrations is qualitatively, but not quantitatively, the same. The difference between the kinetic determinations at pH 6.6 and 7.1 is small but significant. The difference between the kinetic and direct binding data is much greater. This probably reflects the fact that the other substrates in the assay system (glutamate and ammonia) influence the binding of ATP by glutamine synthetase, rather than an involvement of some unassessed kinetic factor. The fact that the binding and kinetic data only deviate at the high concentrations of ATP indicates that the other substrates are antagonistic toward the saturation, but not toward the initial binding, of the enzyme with ATP–Mn. Although the effect of substrates could be pH dependent, it seems unlikely that the differences observed between the kinetic and binding results in Figure 2c are due to the pH difference between studies. The binding affinity measured directly at pH 7.4 is greater than that deduced from the kinetic studies at pH 7.1, yet in kinetic experiments decreasing the pH appears to increase rather than decrease the apparent affinity for ATP. Possibly temperature difference between the binding (4°) and kinetic (~22°) studies could be of importance. It should be noted finally that throughout these studies $MnCl_2$ was in large excess of the ATP so that in the pH range used, free ATP would be chelated to this metal.

Some Additional Effects of Assay Conditions on the Kinetically Determined ATP Saturation Functions. As our studies were expanded, it was found that the Mn^{2+}-activated enzyme

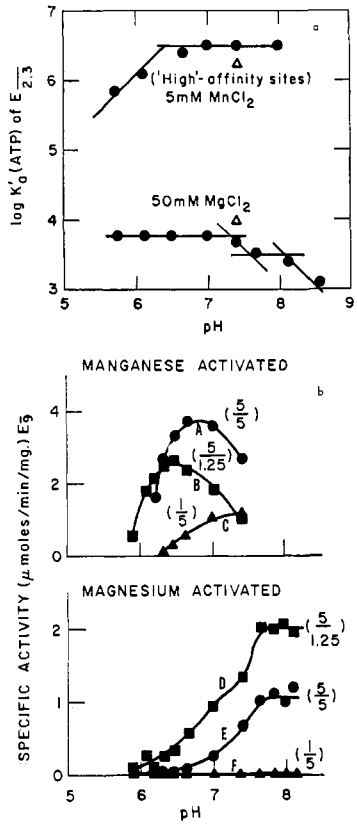

FIGURE 4: (a) Influence of pH on the apparent affinities of $E_{\overline{2.3}}$ for ATP. With 5 mM $MnCl_2$, log K_a' is the logarithm of the reciprocal of the apparent K_m' (●) determined kinetically, as described in the legend for Figure 2a, for the high-affinity sites of $E_{\overline{2.3}}$ at the low concentrations of ATP; (△) plotted from the corresponding ATP binding measurements at the low ATP concentrations shown in Figure 1a. With 50 mM $MgCl_2$, apparent K_m'; (●) determined with $E_{\overline{2.3}}$ in an assay mixture described in the legend to Figure 7 with 30 mM L-glutamate, and plotted as log $(1/K_m')$; (△) plotted from the corresponding ATP binding measurement with the $E_{\overline{2.3}}$ preparation tightened with 50 mM $MgCl_2$ (Figure 1b). These data are plotted according to Dixon and Webb (1964). (b) Influence of pH on the Mn^{2+}-dependent and Mg^{2+}-dependent specific activity of the E_5 preparation of glutamine synthetase at different divalent cation to ATP ratios. The numbers in parentheses are the millimolar concentrations, where the numerator is the divalent cation and the denominator is the ATP concentration. The assay used was the phosphate biosynthetic assay of Woolfolk *et al.* (1966) at 25° using 50 mM Tris–maleic acid at the stated pH values, 100 mM L-glutamate, and 50 mM NH_4Cl. (See text for explanation of curve A–F.)

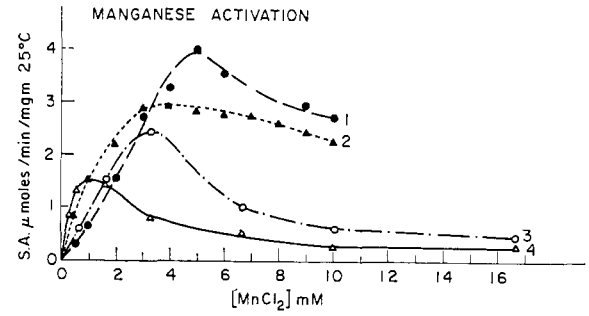

FIGURE 5: Influence of the Mn^{2+} concentration ($MnCl_2$) upon velocity. Velocities were measured with the phosphate biosynthetic assay, using the E_5 preparation and the 50 mM Tris–maleic acid buffer at the stated pH (as in Figure 4b): curve 1, 5 mM ATP at pH 6.64; curve 2, 5 mM ATP + 5 mM $MgCl_2$ at pH 6.64; curve 3, 5 mM ATP at pH 7.56; curve 4, 1.25 mM ATP at pH 7.56.

forms show an apparent decreased affinity for ammonia and glutamate with increasing adenylylation. It was previously found that adenylylation decreased the affinity of glutamine synthetase for Mn^{2+} (Denton and Ginsburg, 1969). A further activation of glutamine synthetase preparations by high concentrations of ATP–Mn was apparent also. As a consequence, ATP saturation curves for $E_{\overline{2.3}}$ and E_{12}[3] were repeated over an expanded ATP concentration range and the results are shown in Figure 3. The ATP curves were determined at 25° for saturating glutamate and ammonia (100 mM L-glutamate and NH_4Cl and 6 mM $MnCl_2$, pH 7.03 and pH 6.55 ± 0.05), as well as for the conditions used previously (30 mM L-glutamate, 50 mM NH_4Cl, and 5 mM $MnCl_2$, pH 7.05). The induced transitions with ATP are shown in Figure 3a, and it is immediately apparent that they extend over an enormous ATP concentration range. Only the E_{12} preparation is affected by the lower glutamate and ammonia levels (curve 3), since $E_{\overline{2.3}}$ is saturated with glutamate and ammonia in both cases (curve 1, 2). Still, the Hill plots of these data shown in Figure 3b for the high levels of substrates, and including the total ATP transition curves for $E_{\overline{2.3}}$ and E_{12} at pH 7, do not differ significantly from those shown in Figure 2b. However, small negative interactions between E_{12} and ATP–Mn at pH 7 do occur under these assay conditions, as shown by the shifting of the points in Figure 3b from a single line of unit slope. The additional transitions induced by concentrations of ATP greater than 10^{-4} M and a horizontal displacement of the pH 7 curves to the right are consequences of including the entire ATP concentration range in Figure 3b. The concentration of ATP required for half-fractional saturation is $1–2 \times 10^{-4}$ M instead of $\sim 3 \times 10^{-5}$ M as shown in Figure 2b.

The final plateaus in the saturation curves of Figure 3a occur at 4 mM ATP. Above this concentration of ATP a 1 mM excess of $MnCl_2$ was added since excess ATP to Mn^{2+} is inhibitory (see Figure 4b below), and pyruvate kinase, which is one of the coupling enzymes in the spectrophotometric assay, also requires divalent cation (Lohmann and Meyerhof, 1934). It was thought that the multiple transitions shown in Figure 3a might arise in part from an inhibitory effect of excess Mn^{2+}, (see Figure 5 below), that is relieved as ATP is added to the system. This cannot account entirely for the activation by increasing levels of ATP, since at the reduced 0.5 mM $MnCl_2$ level (Figure 3a, dashed curve 5) the optimum activity of E_{12} is not attained as ATP is increased to an equimolar concentration. Also, the stepwise nature of the ATP activation and the effect of suboptimal concentrations of L-glutamate and ammonia on the shape of curve 3 suggests that more than one process is occurring.

To test the involvement of Mn^{2+} inhibition in curves 1–4 of Figure 3a, the ATP saturation curve for the fully adenylylated enzyme (E_{12}) was determined at pH 6.5, which is at the pH optimum of the Mn^{2+} assay (curve 6 of Figure 3a; see Ginsburg *et al.*, 1970). As shown in Figure 5 above, the inhibitory

[3] The enzyme ($E_{\overline{11.95}}$), prepared and characterized by the procedures described in Methods, was a generous gift of Dr. Joseph Ciardi in the laboratory of Dr. E. R. Stadtman.

effect of excess $MnCl_2$ is reduced by decreasing the pH. The E_{12} preparation shows a more straightforward response to increasing levels of ATP–Mn at pH ~7 (Figures 2a,b and 3b) than the less adenylylated enzyme forms. It is anticipated from the studies on the binding of divalent cations to glutamine synthetase (Denton and Ginsburg, 1969) that E_{12} also would have the lowest affinity for the activating divalent cation. Figure 2c shows that the $E_{2.3}$ preparation still shows a negative interaction with ATP–Mn at low pH. As shown in Figure 3a,b, a pH decrease from pH 7.0 to 6.5 simplifies the response of E_{12} toward ATP; at pH 6.5, the ATP saturation function of the E_{12} preparation obeys Michaelis–Menten kinetics (see Dixon and Webb, 1964) with a single affinity constant for ATP indicated by the unit slope of the Hill plot (Figure 3b). As found with $E_{2.3}$ (Figure 2c), lowering the pH to pH 6.5 decreases slightly the concentration of ATP required for half-fractional saturation of E_{12} (from $\sim 1.6 \times 10^{-4}$ to $\sim 7.3 \times 10^{-5}$ M ATP). It will be recalled that the corresponding value from binding experiments is about 5×10^{-6} M ATP. Thus, L-glutamate and ammonia appear to be antagonistic toward the binding of ATP under optimal assay conditions also. Possibly, this arises from an influence of these substrates on the binding of specific divalent cations by glutamine synthetase. The tightening experiments of Kingdon *et al.* (1968) suggested that L-glutamate greatly increased the affinity of the enzyme for Mn^{2+}. The results obtained kinetically implicate an intimate relationship between the affinity of the different enzyme forms for Mn^{2+} and the observed interactions with ATP–Mn (see Discussion below).

General Kinetic Effects of pH. In kinetic studies using conditions described in the legend to Figure 2. the apparent association constant ($1/K'_m$) of the high-affinity ATP site of $E_{2.3}$ with 5 mM $MnCl_2$ present showed no large change from pH 6.3–8.0, but from pH 5.7 to 6.3, this constant increased about sixfold (Figure 4a). As shown in Figures 2c and 3b, a pH decrease does produce a slight increase in the overall apparent affinity of Mn^{2+}-activated subunits for ATP. With 50 mM $MgCl_2$ present in the assay instead of $MnCl_2$, when only unadenylylated subunits are presumably active, reciprocal velocity *vs.* 1/ATP plots for $E_{2.3}$ are described by a single line; this is in contrast to the observations with the Mn^{2+}-activated $E_{2.3}$ which are expressions of the adenylylated subunits. With the Mg^{2+}-activated $E_{2.3}$ preparation, the slopes of these reciprocal plots were constant from pH 5.7–7.4 although V_{max} increases in this pH range. The kinetic value of $1/K'_m$ is only slightly less than the value of k'_A estimated from the measurement of ATP binding to the Mg^{2+}–enzyme (Figures 1b). With the Mg^{2+}-activated $E_{2.3}$, a small increase in V_{max} is observed from pH 7.4 to 7.6, over which pH range a slight decrease in $1/K'_m$ was observed. At higher pH (7.4–8.5) the kinetics are more complicated, suggesting possibly that ATP is relieving an inhibition by divalent cations at pH 7.4–8.5, as discussed above for the Mn^{2+}-activated system, or some other complicating factor.

If the results of Figure 4a are interpreted according to Dixon and Webb (1964), the upward slope for the high-affinity, Mn-activated ATP sites reflects the pK of a group situated in the ES complex. The pK's of groups situated in either the free enzyme or free substrate produces a downward bend, as seen at higher ATP levels with 50 mM $MgCl_2$ (Figure 4a) or with 5 mM $MnCl_2$ (indirectly illustrated in the Hill plots of Figures 2c and 3b). The slopes of one observed in Figure 4a should be numerically equal to the change of charge in that pH range. Obviously, different apparent pK's are involved in saturating the high- and low-affinity ATP sites. The major difference at low pH could result from the initial accommodation of the ATP–Mn at the Mn^{2+} activation site (see Discussion) with a net change of charge involved. A subsequent conformational change may cause a net charge change, or pK shift, prior to the combination of ATP–Mn with the partially saturated enzyme. The dissociation of relaxed glutamine synthetase appears to involve the titration of some functional group with $pK' \simeq 7.25$–7.5 (Woolfolk and Stadtman, 1967b), and perhaps the break in the Mg^{2+}-activated enzyme affinity curve for ATP in this pH range reflects an indirect involvement of these groups.

The data in Figure 4b are from experiments with $E_{9.0}$ showing the interrelationship between divalent cations and ATP ratios when velocity is studied as a function of pH. It should be noted in all cases, the assay conditions are suboptimal. When ATP is present in fivefold excess over the Mg^{2+}, no activity could be detected in the pH range studied (curve F). In contrast, when Mn^{2+} was used as the activating cation with the same concentration excess of ATP, significant activity is expressed above pH 6.3 (curve C). When ATP and Mg^{2+} are present in equal concentrations, the activity of the enzyme increases with increasing pH and plateaus at about pH 7.6 (curves E); these velocities are always lower than those observed when Mg^{2+} is present in excess with respect to ATP (curve D), even though the concentration of Mg–ATP complex is greater in the case of curve E. These results indicate that the enzyme requires free Mg^{2+} for activity. The steep activity increase of curve D at about pH 7.4–7.6 has been observed in the spectrophotometric assay–50 mM $MgCl_2$ system with the $E_{2.3}$ preparation also, and appears to represent a small activation. The Mn^{2+}-activated enzyme is inhibited above pH 6.3 by an excess of Mn^{2+} (curve B) so that the optimum pH is lower than that found when Mg^{2+} is the activating ion (curve D). When Mn^{2+} and ATP are present in equal concentrations, the optimum pH is about pH 6.8 (curve A). When Mn^{2+} is in excess to the ATP (curve B), the activity is lower than when a 1:1 ratio of Mn^{2+}–ATP is present (curve A), except at very low pH. These results are consistent with the much greater affinity of the enzyme for Mn^{2+} than for Mg^{2+} and the effect of pH on the affinity of the enzyme for specific divalent cations (Denton and Ginsburg, 1969), together with the inhibitory effect of excess Mn^{2+} at the higher pH values (see also Figure 5).

Figure 5 shows some effects of increasing Mn^{2+} concentrations with different assay conditions. At pH 6.6 (curve 1), the optimum Mn^{2+} to ATP ratio is reached when these concentrations are approximately equal. At higher Mn^{2+} concentrations there is some inhibition. At low Mn^{2+} concentrations, curve 1 is sigmoidal in shape. This is probably because glutamine synthetase requires the presence of both free Mn^{2+} and the Mn^{2+}–ATP complex for catalytic activity; however, at low Mn^{2+} concentration when the ratio of ATP to Mn^{2+} is high, the Mn^{2+} is present mainly as the Mn^{2+}–ATP complex, and relatively little free Mn^{2+} is available to the enzyme. A mathematical model including an activation step requiring free divalent cation for the reaction of an enzyme with the cation–ATP complex has been examined recently by London and Steck (1969), and their analysis predicts sigmoidal curves of the type described by curve 1 of Figure 5 and also an inhibition by high

divalent cation concentrations. At pH 6.6, there is very little activity due to the presence of equal concentrations of Mg^{2+} and ATP (Figure 4b); however, under these conditions the response of the enzyme to low concentrations of Mn^{2+} is much greater than in the absence of Mg^{2+} (compare curves 1 and 2 in Figure 5). Curve 2 shows that the presence of Mg^{2+} lowers the optimum Mn^{2+} to ATP ratio. This increased activity at low Mn^{2+} to ATP ratios is probably due to the higher free Mn^{2+} levels provided by the displacement of Mn^{2+} from the ATP–Mn complex by Mg^{2+}. Although these divalent cations are approximately equally effective in competing for ATP (O'Sullivan and Perrin, 1964), glutamine synthetase has a much higher affinity for Mn^{2+} than for Mg^{2+} (Denton and Ginsburg, 1969). At higher pH (curves 3 and 4), the optimum Mn^{2+} to ATP ratio is lower, as would be predicted from the data shown in Figure 4b, and the increasing affinity of the enzyme for Mn^{2+} with increasing pH (Denton and Ginsburg, 1969). The inhibition by excess Mn^{2+} is also greater at the higher pH.

The pH–activity profiles for some representative glutamine synthetase preparations that are adenylylated to different extents are presented in the accompanying paper (Ginsburg *et al.*, 1970).

Ammonia and L-*Glutamate Saturation Curves in a Mn^{2+}-Activated Biosynthetic Assay at pH 7.* Figure 6a,c shows plots analogous to those of Figure 2a for kinetically measured initial velocities at varying sodium glutamate or ammonium chloride concentrations, respectively, in Mn^{2+}-dependent biosynthetic assay systems. The glutamate saturation curves (Figure 6a) were measured in the Mn^{2+}-dependent spectrophotometric assay for three enzyme preparations adenylylated to different extents ($E_{\overline{2.3}}$, $E_{\overline{9}}$, and $E_{\overline{11.8}}$).[1] The corresponding Hill plots of these data are shown in Figure 6b. The NH_4Cl saturation curve (Figure 6c) was measured with gel-filtered $E_{\overline{9}}$ by the assay method of Woolfolk *et al.* (1966). This assay system utilizes the release of inorganic phosphate at 37° as a measure of biosynthetic activity, and was used instead of the spectrophotometric assay system, from which ammonia could not be completely removed. As in the case of varying ATP concentrations (Figure 2a), biphasic curves of v' *vs.* v'/S were obtained with an apparently higher affinity of the Mn^{2+}-activated enzyme forms for substrate at the lower concentrations of L-glutamate or ammonium chloride.

In contrast to the response of the different Mn^{2+}-activated enzyme forms to ATP (as shown in Figure 2a), the variation as a function of adenylylation occurs also at the high concentrations of L-glutamate rather than at only low concentrations of this substrate (Figure 6a). Thus, the Mn^{2+}–enzyme forms that have different amounts of covalently bound 5′-adenylyl groups require different concentrations of L-glutamate to effect half-fractional saturations with this substrate (Figure 6b). At the high glutamate levels, the apparent K'_m values from the slopes of Figure 6a were approximately 0.8 mM, 3.8 mM, and 5.2 mM L-glutamate for $E_{\overline{2.3}}$, $E_{\overline{9}}$, and $E_{\overline{11.8}}$, respectively. Therefore, adenylylation of glutamine synthetase appears to decrease the affinity of the Mn^{2+}-activated enzymes for glutamate.

The insert to Figure 6a illustrates that the specific activities at saturating glutamate concentrations under these assay conditions are roughly proportional to the number of adenylyl groups ($\bar{n}$)[1] of the enzyme preparation. (Although this result is in agreement with the results of Kingdon *et al.* (1967), it must be stressed that these specific activities are not V_{max} values. In the studies of Figure 6a, NH_4Cl was subsaturating for $E_{\overline{9}}$ and $E_{\overline{11.8}}$; the pH and ATP concentrations were suboptimal for all three enzyme preparations (Ginsburg *et al.*, 1970). Thus, the final activities at saturating glutamate concentrations are not V_{max} values. Further, a small apparent inhibition of the $E_{\overline{2.3}}$ preparation by concentrations of L-glutamate greater than 30 mM was observed.) As a consequence of the Mn^{2+}-activated specific activity increasing with adenylylation, the corresponding contribution at low glutamate concentrations also decreases. Thus, the high-affinity segment of the plots in Figure 6a represent a very small amount of the potential activity at high adenylylation states.

A relaxed preparation of $E_{\overline{2.3}}$ which had been preincubated with $MnCl_2$ as in Figure 1b to produce the tightened enzyme form, had the same response toward glutamate as did the native taut enzyme. These results are shown by the solid symbols in Figure 6a for $E_{\overline{2.3}}$. Also, the ATP saturation curve of the tightened Mn^{2+}-activated $E_{\overline{2.3}}$ preparation in the presence of high glutamate and ammonia concentrations behaved the same as did the native taut form (Figure 3b). Thus, the relaxation and tightening process do not cause a loss in the ability of the $E_{\overline{2.3}}$ enzyme to assume a high-affinity form for substrates under assay conditions in which all substrates are present, with ATP or L-glutamate limiting.

In Figure 6b the data of Figure 6a are plotted in the form of a Hill plot. All three enzyme preparations show an apparent negative interaction with L-glutamate that are described by unit asymptote slopes connected by slopes of less than one. The Hill plots of Figure 6b show that both high- and low-affinity sites for L-glutamate are influenced by the extent of adenylylation of the enzyme preparation. Apparent K'_m values for the high-affinity sites may be estimated by extrapolating the unit slopes at the low concentrations of L-glutamate to zero on the ordinate, corresponding to half-saturation of the different enzyme forms. Using this procedure, 0.4 mM, 1.7 mM, and 3.2 mM are estimated for the K'_m values of the high-affinity sites of $E_{\overline{2.3}}$, $E_{\overline{9}}$, and $E_{\overline{11.8}}$, respectively. The corresponding values for the low-affinity sites determined by the same procedure, are 1.3 mM, 5.0 mM, and 7.6 mM for $E_{\overline{2.3}}$, $E_{\overline{9}}$, and $E_{\overline{11.8}}$, respectively. These latter values are probably more accurate than those estimated from the slopes of Figure 6a.

As shown by the data in Figure 6c, the Mn^{2+}-activated $E_{\overline{9}}$ enzyme preparation responds qualitatively the same to ammonium chloride as to the other substrates in the Mn^{2+}-dependent biosynthetic assay. Only $E_{\overline{9}}$ has been studied with low concentrations of NH_4Cl as a variable; even with extensive precautions some contamination with ammonia still occurred for which suitable corrections were applied to the results shown. The effect of adenylylation on the response of Mn^{2+}-activated glutamine synthetase to NH_4Cl is unknown at this time. All Mn^{2+}-activated enzyme forms could have biphasic NH_4Cl saturation curves (as in the case of glutamate; Figure 5a), or perhaps the bimodal character of the NH_4Cl saturation function is lost with adenylylation (as in the case shown for ATP; Figure 2a). In either case, the results of Figure 6c show that the Mn^{2+}-activated enzyme forms of the $E_{\overline{9}}$ preparation respond in a complex way to the substrate ammonia also.

The Response of Different Enzyme Forms to Substrates in the Mg^{2+}-Activated Biosynthetic Assay. The Mg^{2+}-activated assay specifically measures only the activity of unadenylylated subunits of glutamine synthetase, as discussed in greater detail in the accompanying paper (Ginsburg *et al.*, 1970). It was dem-

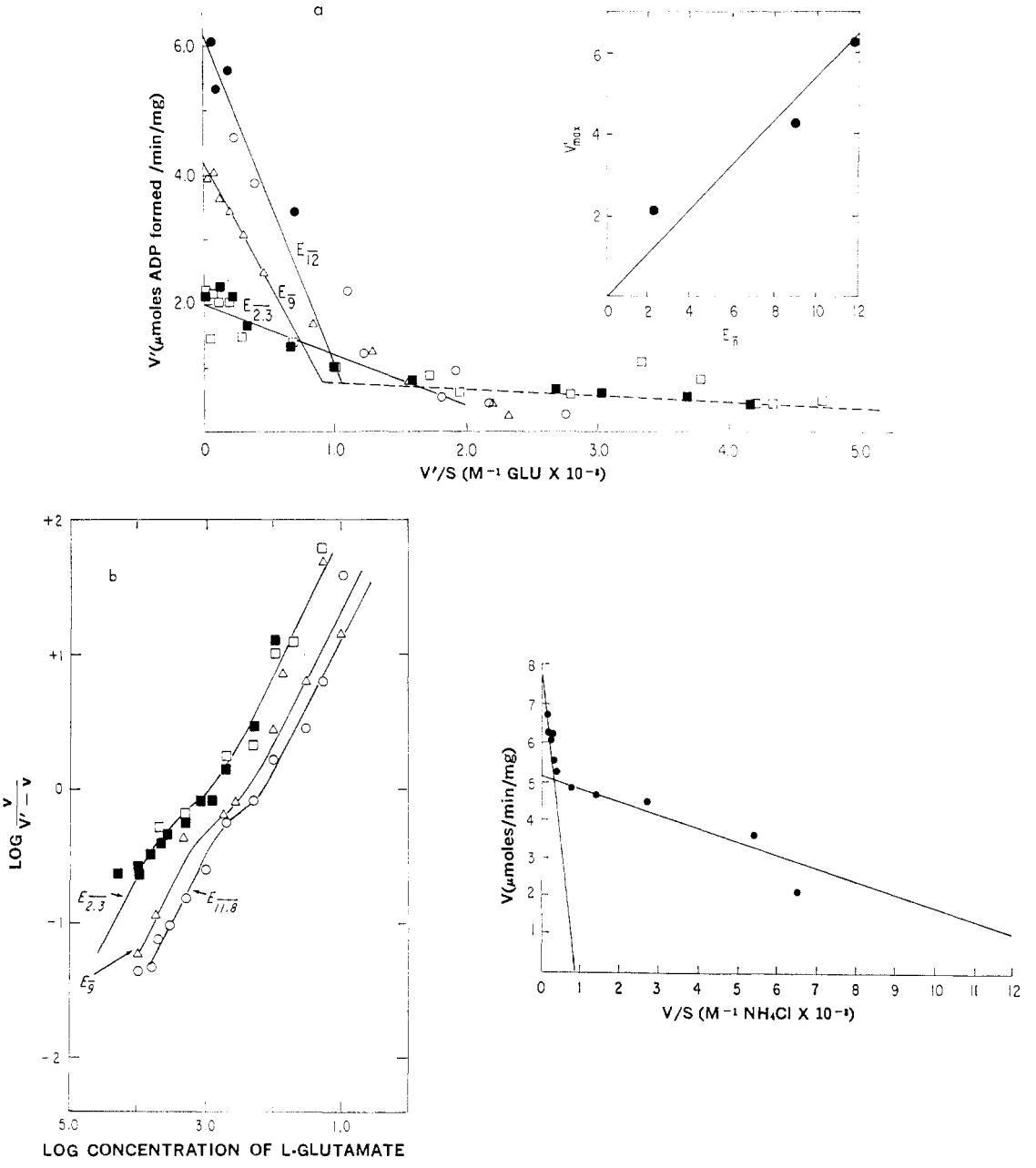

FIGURE 6: Kinetic responses of different glutamine synthetase preparations ($E_{\overline{2.3}}$, $E_{\overline{9}}$, and E_{12}) to varying sodium glutamate (a) or NH_4Cl (c) concentrations in Mn^{2+} biosynthetic assays. (a) The Mn^{2+} spectrophotometric assay at ~25° was used with 0.4 mM ATP–1.4 mM $MnCl_2$, 50 mM NH_4Cl, and 50 mM imidazole buffer at pH 7.1. Initial velocities (V') were measured as described in Methods. The solid symbols for $E_{\overline{2.3}}$ represent results obtained with the tightened enzyme, using for this purpose the addition of 5 mM $MnCl_2$ to a relaxed preparation of $E_{\overline{2.3}}$ as in Figure 1b. The insert shows an approximate linear relationship between the apparent maximum velocities measured under these assay conditions and the extent of adenylylation of the enzyme preparations ($\bar{n}$).[1] (b) Hill plots of the data from (a), where V' is the apparent maximum velocity attained and v is the observed initial velocity at different concentrations of L-glutamate. (c) The saturation of $E_{\overline{9}}$ with NH_4Cl. Ammonia was removed from the $E_{\overline{9}}$ preparation by gel filtration through G-50 Sephadex, and special precautions were taken to avoid contamination of the reagents with atmospheric ammonia. The Mn^{2+}-dependent biosynthetic assay of Woolfolk *et al.* (1966) which measures inorganic phosphate release at 37° was used. The assays contained 100 mM L-glutamate, 7.5 ATP, 5 mM $MnCl_2$, and 50 mM imidazole buffer at pH 7.

onstrated by Kingdon *et al.* (1967) and Wulff *et al.* (1967) that as glutamine synthetase is adenylylated a corresponding loss in Mg^{2+} biosynthetic activity occurs. It could be concluded that the adenylylated form of glutamine synthetase has no Mg^{2+}-activated biosynthetic activity (Stadtman *et al.*, 1968). This was corroborated here by testing the $E_{\overline{11.8}}$ preparation for activity in the Mg^{2+}-dependent spectrophotometric assay system (see Methods). Specific activities of 0.67 and 0.21 were measured for saturating (30 mM L-glutamate, 5 mM ATP, and 100 mM NH_4Cl) and limiting (7.5 mM L-glutamate, 5 mM ATP, and 5–10 mM NH_4Cl) substrate concentrations, respectively. The specific activity of the adenylylated enzyme is 1.6%

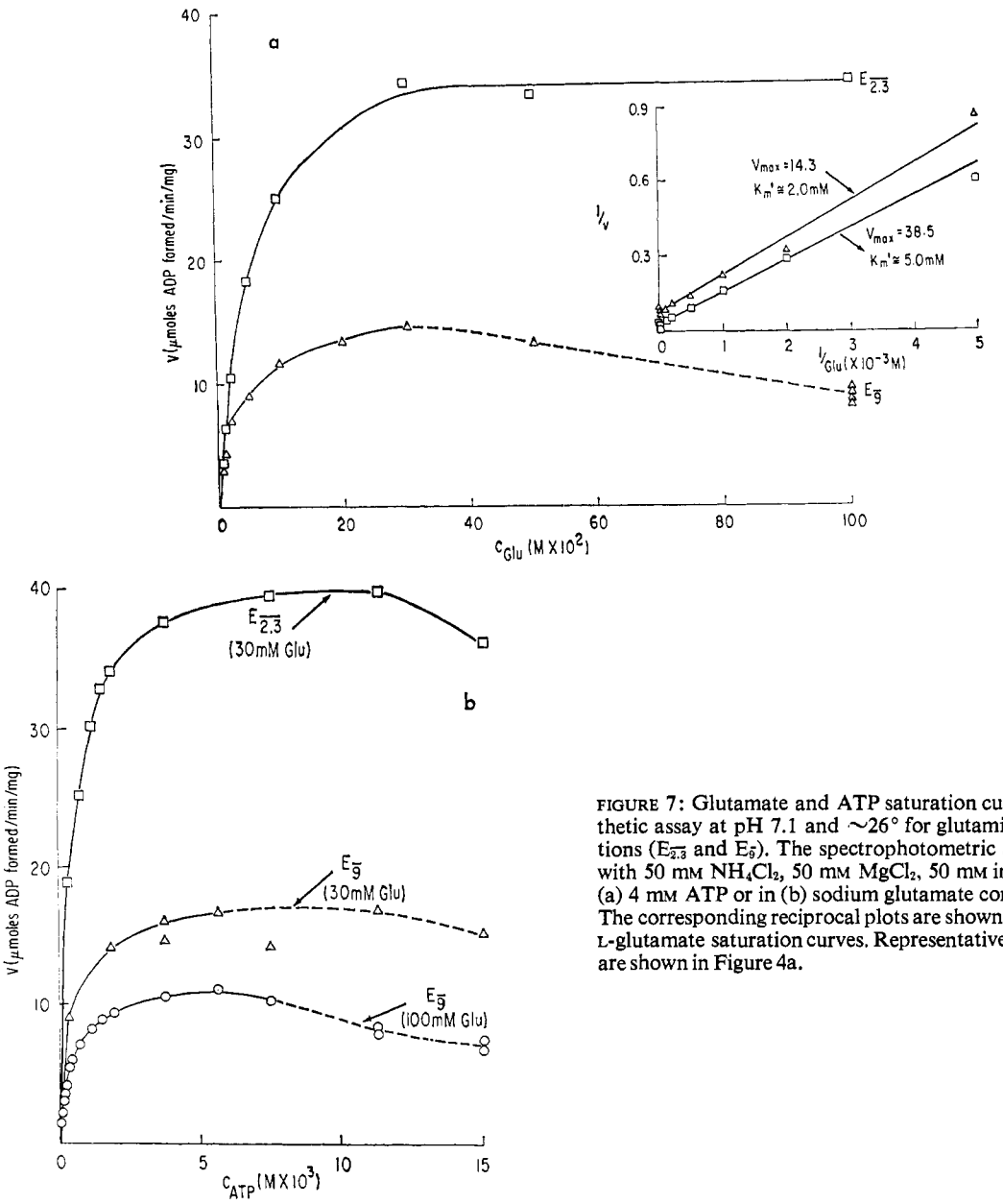

FIGURE 7: Glutamate and ATP saturation curves in a Mg^{2+} biosynthetic assay at pH 7.1 and ~26° for glutamine synthetase preparations ($E_{\overline{2.3}}$ and $E_{\bar{9}}$). The spectrophotometric assay system was used with 50 mM NH_4Cl_2, 50 mM $MgCl_2$, 50 mM imidazole buffer, and in (a) 4 mM ATP or in (b) sodium glutamate concentrations as shown. The corresponding reciprocal plots are shown as an insert to a for the L-glutamate saturation curves. Representative K_m' data for ATP–Mg are shown in Figure 4a.

of that of the $E_{\overline{2.3}}$ preparation measured under the same conditions. If E_{12} is assumed to have a specific activity of zero in the Mg^{2+} assay, these measured values correspond to an enzyme adenylylated to the extent of 11.8 moles of adenylyl groups/mole of glutamine synthetase. This result is in excellent agreement with spectral and radioactivity measurements of the extent of adenylylation of this enzyme preparation (see Methods). Further, the divalent cation specificity is maintained at the limiting substrate levels. This required verification because, as shown below, adenylylated enzyme forms may be inhibited by substrates in the Mg^{2+}-activated assay.

Figure 7a,b shows L-glutamate and ATP curves, respectively, for $E_{\overline{2.3}}$ and $E_{\bar{9}}$ in the Mg^{2+} biosynthetic assay. In contrast to the results obtained with the Mn^{2+}-activated assay (Figures 2a and 6a,c), apparently hyperbolic substrate saturation curves were observed. Also, inhibition by excess substrate was observed with $E_{\bar{9}}$, but not with the $E_{\overline{2.3}}$ preparation at moderate L-glutamate or ATP concentrations. The more adenylylated enzyme forms only are activated by increasing substrate concentrations in the Mn^{2+} assay, whereas Mn^{2+}-activated enzyme forms with few adenylyl groups are inhibited by excess L-glutamate (Ginsburg *et al.*, 1970). The more adenylylated enzyme ($E_{\bar{9}}$) appears to have a lower K_m' for L-glutamate than has $E_{\overline{2.3}}$ in the Mg^{2+} assay (Figure 7a), which is the reverse of that observed in the Mn^{2+} assay also.

Inhibition with excess ATP was observed only when an inhibitory level of L-glutamate was present in the assay of $E_{\bar{9}}$ (Figure 7a,b). The inhibition by high ATP levels with 100 mM glutamate present was observed to increase somewhat during storage at 4° of dilute solutions of the $E_{\bar{9}}$ preparation. Thus, the sensitization to ATP inhibition by inhibitory amounts of glutamate appears to be more complex than the result of a

simple titration of Mg^{2+} from the enzyme by ATP. The $1/K'_m$ values for ATP–Mg (ranging from 2×10^{3} to 3×10^{3} M (which are shown Figure 4a) are not significantly different from the apparent association constant of about 6.3×10^{3} M extrapolated from the binding data for $E_{\overline{2.3}}$ with ATP–Mg (shown in Figure 1b).

A K'_m value of about 5 mM for Mg^{2+} has been measured previously for $E_{\bar{9}}$ in a biosynthetic assay at pH 7.1 with 100 mM L-glutamate and 50 mM NH_4Cl present (Denton and Ginsburg, 1969). Then, the $E_{\bar{9}}$ preparation has about a tenfold greater apparent affinity ($1/K'_m$) for ATP–Mg than for free Mg^{2+}, although as shown in Figure 4b above, free Mg^{2+} ions must be available for activity to be expressed. If the enzyme must first combine with free metal for activation, the K'_m value for ATP–Mg represents that parameter for the Mg^{2+}-activated enzyme form. It is of interest that glutamine synthetase has a much higher affinity for free Mn^{2+} than for the ATP–Mn complex, as measured kinetically or by direct binding. Also, due to the much lower affinity of glutamine synthetase for Mg^{2+} than for Mn^{2+} (Denton and Ginsburg, 1969), inhibition by excess Mg^{2+} only becomes apparent at very high concentrations of Mg^{2+} (>50 mM with 5 mM ATP present).

Some Studies on the Kinetics of the Reverse Biosynthetic Reaction. In studies on the glutamine synthetase from *E. coli* performed by Woolfolk *et al.* (1966) only about 0.001 the rate of the forward reaction could be demonstrated by coupling the formation of ATP from L-glutamine, ADP, and inorganic phosphate at pH 7 and 20° to hexokinase and glucose 6-phosphate dehydrogenase activities, and following the rate of TPN reduction spectrophotometrically. However, the enzyme was shown in the same studies to readily catalyze a γ-glutamyl transfer reaction at 37° in which γ-glutamylhydroxamate is formed from glutamine and hydroxylamine in the presence of Mn^{2+}, arsenate, and ADP.[4]

In the experiments reported here, a sensitive assay system for the reverse reaction was used in which the conversion of radioactive [^{32}P]inorganic phosphate into [γ-^{32}P]ATP was measured by adsorption of the nucleotides on charcoal after incuation of 2–10 μg of glutamine synthetase with glutamine, ADP, and [^{32}P]P_i at 37° (see Methods). The results of some preliminary studies on the kinetics of the reverse biosynthetic reaction using the $E_{\bar{9}}$ preparation are given in Table I. The data show that the enzyme has a high K'_m for phosphate, and slightly lower pH optima than those found for foreward biosynthetic reactions (see the accompanying paper, Ginsburg *et al.*, 1970). The data only include K'_m values for substrates in the Mg^{2+}-activated reverse reaction; the corresponding data for the Mn^{2+}-activated reverse reaction were not obtained because the optimum conditions of phosphate to Mn^{2+} ratios were not determined. However, with 50 mM $MgCl_2$ or 1.7 mM $MnCl_2$ and 100 mM [^{32}P]phosphate the ratio of Mg^{2+}- to Mn^{2+}-dependent activities of $E_{\bar{9}}$ at the pH optima was 1.85:1, which is close to the 1.7:1 ratio measured for this enzyme preparation in the corresponding biosynthetic reactions. The specific activities of the $E_{\bar{9}}$ preparation, and also those of the other enzyme preparations in the Mg^{2+}-dependent reverse reaction (Figure 8), are about $^1/_{50}$th the rates of the corresponding biosynthetic reactions at 37°. This relative rate for the catalyzed reverse reaction is appreciably greater than that measured by Woolfolk *et al.* (1966), and more comparable with the relative rate measured by Levintow and Meister (1954) for the glutamine synthetase from pea seedlings. Possibly, the low rate of the reverse reaction previously observed for the glutamine synthetase from *E. coli* (Woolfolk *et al.*, 1966) was due to a combination of the lower temperature, the low substrate concentrations, and even the assay employed.

The results shown in Figure 8 illustrate that the Mg^{2+}-activated specific activities of four different enzyme preparations ($E_{\overline{1.2}}$, $E_{\overline{2.3}}$, $E_{\bar{9}}$, and $E_{\overline{11.8}}$) in the reverse reaction at saturating substrate concentrations are approximately proportional to the concentration of unadenylylated subunits. The preparation $E_{\overline{11.8}}$ had only 2.9% of the extrapolated value for E_0 (2.72 μmoles of ATP formed/min at 37° per mg of E_0), which gives an extrapolated value for E_{1^2} of zero in the Mg^{2+}-dependent reverse reaction. Therefore, the divalent cation specificity of unadenylylated subunits is maintained in both the forward and reverse reactions of glutamine synthetase. The approximately linear relationship between the Mg^{2+}-dependent specific activity and the average equivalents of adenylyl groups ($\bar{n}$) suggests that the unadenylylated subunits are independent and equivalent in catalyzing the reversal of glutamine synthesis. This result is similar to those obtained previously by Kingdon *et al.* (1967) in the Mg^{2+}- and Mn^{2+}-activated biosynthetic reactions and to results obtained from studies on the γ-glutamyl transfer reaction (Stadtman *et al.*, 1968). More recent studies show that V_{max} is not necessarily a linear function of the extent of adenylylation of the enzyme in either the Mg^{2+}- or Mn^{2+}-dependent biosynthetic reactions (Ginsburg *et al.*, 1970). Apparent linear relationships between specific activity and $\bar{n}$ can result from the variation in the response of different enzyme forms to substrates in the biosynthetic reactions. Thus, more experiments are necessary to determine the optimum substrate for different enzyme forms in the reverse reaction. In particular, very recent experiments have shown that very small amounts of Mn^{2+} added with the enzyme preparation can dramatically inhibit the Mg^{2+}-activated activity in a Mg^{2+} biosynthetic reaction.[5] With the exception of the $E_{\overline{1.2}}$ preparation, the enzyme preparations were predialyzed extensively against 1 mM $MgCl_2$. This treatment, and the subsequent incubation in the assay with 50 mM $MgCl_2$, is known to displace all Mn^{2+} from glutamine synthetase (Ginsburg, 1969). However, the rate of the reverse reaction catalyzed by $E_{\overline{1.2}}$ (Figure 8), in the presence of $\sim 3 \times 10^{-5}$ M $MnCl_2$ in addition to 50 mM $MgCl_2$, is about $^1/_{50}$th of the optimum rate of the biosynthetic reaction, which is the same as that measured for the $E_{\bar{9}}$ enzyme preparation. (The corresponding relative rates for the $E_{\overline{2.3}}$ preparation is about $^1/_{40}$th which is slightly different.) Thus, inhibition by small amounts of $MnCl_2$ in the Mg^{2+}-acti-

[4] More recently, the γ-glutamyl transfer reaction has been systematically investigated by E. R. Stadtman, and from these studies a method of completely inhibiting this activity of adenylylated subunits by the addition of 60 mM $MgCl_2$ to a 0.3 mM $MnCl_2$ assay system at pH 7.15 has been developed (Stadtman *et al.*, 1968). Without added $MgCl_2$, both adenylylated and unadenylylated subunits express equivalent γ-glutamyl transfer activities at pH 7.15, while the activity of unadenylylated subunits is not inhibited by added Mg^{2+}. This procedure incidentally can be used to determine the state of adenylylation of crude or purified glutamine synthetase preparations. The progressive removal of adenylyl groups was shown also to shift the pH optimum from pH 6.8 to 7.9 (Stadtman *et al.*, 1968).

[5] Unpublished data of Dr. Filiberto Cimino, visiting scientist, in the laboratory of Dr. E. R. Stadtman.

TABLE I: Approximate Parameters of the Reverse Biosynthetic Reaction.[a]

Apparent K_m Values[b] (Mg^{2+}-Activated Enzyme at pH 6.8)	
Glutamine	2.6
ADP	0.2
$MgCl_2$	4.3
P_i	7.5
pH Optima	
Mg^{2+} activated	6.8–7.0[c]
Mn^{2+} activated	6.2–6.4[d]

[a] Enzyme preparation ($E_{\bar{5}}$) used; the Mg^{2+}- and Mn^{2+}-dependent specific activities at the pH optima in the reverse reaction at 37° were 0.61 and 0.33, respectively (see Methods). [b] Substrates other than the one varied were at saturating concentrations: 16 mM L-glutamine, 10 mM ADP, and 80–100 mM inorganic [^{32}P]phosphate (10^5–10^6 dpm/μmole) with 50 mM $MgCl_2$. K_m values are expressed as mM. [c] As in *b* with saturating substrate concentrations *d* as in *c* with 1.7 mM $MnCl_2$ instead of 50 mM $MgCl_2$ present, and 100 mM acetate buffer added.

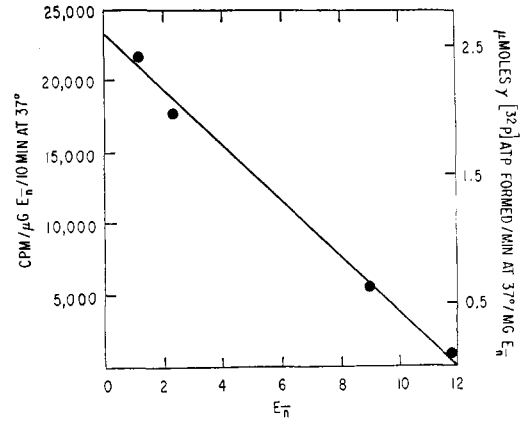

FIGURE 8: The Mg^{2+}-activated reversal of glutamine synthesis at 37° and pH 7 catalyzed by glutamine synthetase preparations, $E_{\overline{1.2}}$, $E_{\overline{2.3}}$, $E_{\bar{5}}$, and $E_{\overline{11.8}}$. The charcoal-adsorbed ^{32}P counts (cpm/μg of enzyme per 10 min at 37°) or the corresponding specific activities on the right ordinate (see Methods) are plotted as a function of the extent of adenylylation of the enzyme, where $\bar{n}$ is the average moles of 5′-adenylyl groups/mole of glutamine synthetase.[1] The assays (0.3 ml) contained 16.7 mM L-glutamine, 10 mM ADP, 50 mM $MgCl_2$, 83 mM [^{32}P]orthophosphate (9×10^5 cpm/μmole of P_i) at pH 7, and 4–10 μg of glutamine synthetase. All values were corrected for a blank of 870 cpm obtained without enzyme or without L-glutamine.

vated reverse reaction very well may not be a factor due to the high concentration of inorganic phosphate in the assay system. If the specific activities of Figure 8 are close to V_{max} values, the reverse reaction corresponds to the γ-glutamyl transfer reaction, in which heterologous interactions between subunits within hybrid enzyme molecules do not appear to be a factor in the catalytic expression of activity (Stadtman *et al.*, 1968).

It is obvious that more detailed studies of the reverse reaction are required in order to interpret the results of Table I and Figure 8 for this complicated regulatory enzyme. Correlations between the reverse biosynthetic and the γ-glutamyl transfer activities would be of particular interest. The requirement for Mn^{2+} and the unique roles of arsenate and hydroxylamine in the γ-glutamyl transfer reaction (Woolfolk *et al.*, 1966) complicate the interpretation of this activity. However, the reverse biosynthetic reaction, which shows the same divalent cation specificity as in the forward direction, should be of value in determining if one or all of the substrates (glutamine, ADP, and phosphate) can be responsible for uncoupling heterologous subunit interactions, present in the biosynthetic reactions, in enzyme molecules containing both adenylylated and unadenylylated subunits.

Discussion

The equilibrium binding studies reported here have shown that a Mn^{2+}–enzyme form of the glutamine synthetase from *E. coli* has the capacity to bind 12 equiv of the substrate, ATP. Since this number is identical with the number of subunits contained by this enzyme (Valentine *et al.*, 1968), each subunit may be thought to contain a catalytic site. Indeed, the enzymatic modification of each subunit by adenylylation changes the catalytic properties of glutamine synthetase (Kingdon *et al.*, 1967; Shapiro *et al.*, 1967; Shapiro and Stadtman, 1968b; Wulff *et al.*, 1967; Stadtman *et al.*, 1968). The catalytic changes observed have been an approximate linear function of adenylylation.

The data presented in Figure 1a showed that the binding of the substrate ATP to an enzyme preparation ($E_{\overline{2.3}}$)[1] that contained relatively few 5′-adenylyl groups does not follow a simple mass action binding curve. Rather, the substrate is bound to the Mn^{2+}–enzyme with a higher apparent intrinsic association constant at low concentrations of ATP than at the higher concentrations of this substrate. There are two likely explanations for this type of binding: (1) There are two independent sets of binding sites for ATP in the enzyme population that have unequal affinities for ATP; (2) the enzyme subunits can undergo conformational changes that affect the binding of ATP and that subunit interactions influence the equilibria between different subunit conformations in a negative or destabilizing manner (Koshland *et al.*, 1966; Wyman, 1967). Although the heterogeneity in the enzyme subunits with respect to adenylylation, together with the binding results (see Results above), initially suggested the first possibility, kinetic studies revealed that the explanation was more complex. That the second possibility is the one to consider here was suggested by kinetic data obtained in a Mn^{2+}-dependent biosynthetic assay that specifically measures the activity of adenylylated subunits.[6] These kinetic studies showed that the activity of adenylylated subunits was expressed at both the low and high ATP concentrations. As illustrated in Figure 2a, the principal features of the ATP binding data are reflected in the kinetic mea-

[6] The reader is referred to the accompanying paper of Ginsburg *et al.* (1970) for a discussion of the absolute divalent cation specificity of adenylylated and unadenylylated subunits in the biosynthetic assay systems, since the interpretations of the combined binding and kinetic data advanced here depend upon this knowledge. Also, a scheme of substrate binding to the Mn^{2+}-activated glutamine synthetase from *E. coli* is presented in the accompanying paper.

surement of the saturation of the $E_{2\text{-}3}$ preparation by ATP. That is, the apparent high-affinity sites of the Mn^{2+}-activated $E_{2\text{-}3}$ preparation for ATP are seen at low ATP concentrations in both studies. At higher ATP concentrations, the affinity of the enzyme for ATP decreases, as the velocity increases. Whereas the binding data show the saturation of all subunits with ATP, the Mn^{2+}-activated velocity corresponds to the fractional saturation of only adenylylated subunits. Therefore, under these assay conditions, the active adenylylated subunits of $E_{2\text{-}3}$ appear to be able to assume both a high- and a low-affinity form. The binding results strongly suggest that inactive enzyme–substrate complexes may be formed. That is, unadenylylated subunits, which are inactive in the Mn^{2+}-dependent biosynthetic assay system, can bind ATP.

The other components of the assay system (L-glutamate and ammonia) appear to influence the apparent affinity of glutamine synthetase for ATP, but these seem to decrease only the affinity of ATP sites evident at high concentrations of ATP with the $E_{2\text{-}3}$ preparation. Thus, the assay components do not appear to affect the high-affinity conformation of the Mn^{2+}-activated $E_{2\text{-}3}$ preparation. In kinetic studies with different enzyme preparations, the high-affinity segment of the kinetic saturation functions with ATP was lost with increasing extents of adenylylation. A fully adenylylated preparation of glutamine synthetase, which is active only in the Mn^{2+} assay, appeared to have only the low-affinity type of ATP binding site. Therefore, it is most likely that subunit interactions are modulated by the state of adenylylation and that the heterologous interactions between the two types of subunits mediate conformational changes that affect the affinity of the catalytic site toward ATP. This type of subunit interaction is unusual in that negative or destabilizing forces must be involved in transforming subunits from a high-affinity to a low-affinity state. A model for this type of subunit interaction involving sequential conformational changes has been proposed by Koshland *et al.* (1966).

Koshland and Neet (1968) have described a negative homotropic or antagonistic effect in the binding of DPN to rabbit muscle glyceraldehyde 3-phosphate dehydrogenase, and this is the first documented example of this type of binding (see also Conway and Koshland, 1968). A negative type of interaction between subunits is difficult to identify because independent binding sites with different affinities for the variable compound under study or an interaction between binding sites also will give Hill plots with interconnecting slopes of less than one (Wyman, 1967). Hill plots with interconnecting slopes of greater than one have been seen with a number of regulatory enzymes, and these are interpreted to indicate a cooperative or stabilizing interaction between subunits (Wyman, 1964; Monod *et al.*, 1965; Rubin and Changeux, 1966). The model for subunit interactions proposed by Monod *et al.* (1965) involves a symmetrical transition. The asymmetrical saturation functions observed here, as well as the negative or destabilizing type of subunit interaction (Figure 2), can be explained in the framework of the general model proposed by Koshland *et al.* (1966). However, there are rather unique additional factors to consider in the binding of substrates to the glutamine synthetase from *E. coli*.

There appears to be an intimate relationship between the binding of ATP and specific divalent cations by glutamine synthetase. The binding studies showed that the relaxed enzyme, prepared by chelating Mn^{2+} with EDTA, does not bind ATP in the same concentration range of ATP as does the native glutamine synthetase (*i.e.*, at $<10^{-4}$ M ATP). The removal of specific divalent cations from the native protein induces a reversible conformational change that results in inactivation, the exposure to solvent of sulfhydryl, tyrosyl, and tryptophanyl groups, and some slight increase in hydrodynamic volume (Kingdom *et al.*, 1968; Shapiro and Stadtman, 1967; Shapiro and Ginsburg, 1968). This conformational change may decrease the affinity of the relaxed enzyme for ATP. In kinetic studies of varying ATP and divalent cation ratios (Figures 4b,5), an obligatory concentration of free (*i.e.*, not complexed to ATP) Mn^{2+} or Mg^{2+} ions for the expression of activity, and an inhibitory effect produced by excess Mn^{2+} are observed. The much lower affinity of the enzyme or Mg^{2+} (Denton and Ginsburg, 1969) can account for the lack of inhibition produced by excess Mg^{2+}. Complicated relationships are involved in these studies, and for further details refer to the recent paper of London and Steck (1969) who examine a model for the reaction of an enzyme with ATP in which a metal activates the enzyme and also forms an ATP–metal complex. Earlier studies showed that glutamine synthetase has 12 sites, or one site per subunit, whith a very high affinity for Mn^{2+} ions that are associated with the activation or tightening process (Denton and Ginsburg, 1969). Adenylylation decreased only the affinity of the high-affinity sites for Mn^{2+}, and it appeared to produce this effect by modulating a conformational change per subunit that is associated with the independent binding of the first 12 Mn^{2+} ions. If there is a correlation between the affinity constants for activating divalent cations and conformational variations among the different taut enzyme forms, an enzyme with few adenylyl groups may be *overtightened* by Mn^{2+} to an extent that requires some structural loosening by substrate for optimal catalysis. It is therefore possible that the observed negative interaction between ATP–Mn and the $E_{2\text{-}3}$ preparation arises from antagonistic conformational changes induced by the activating Mn^{2+} ion and by the accommodation of the ATP–Mn complex at all of the catalytic sites. Several considerations make this an attractive idea. The negative interaction between the Mn–enzyme and ATP–Mn is lost with increasing adenylylation which also decreases the affinity of the enzyme for the activating Mn^{2+} ions. These same effects are not observed in the interaction of the Mg–enzyme with ATP–Mg where the apparent association constant for Mg^{2+} is similar to that estimated kinetically here for ATP–Mg. Finally, the K'_m values for ATP–Mn in the Mn^{2+}-activated biosynthetic assay at half-fractional saturation are the same for different enzyme preparations, and for ATP–Mg in the Mg^{2+}-activated assay system (see also Woolfolk *et al.*, 1966). The constant of $1/k'_\lambda$ of 2×10^{-4} M^{-1} estimated here for the binding of ATP–Mg to the Mg^{2+} form of the enzyme approximates the corresponding kinetic parameter. Thus, the large shift in affinity occurs when a Mn^{2+} form of the enzyme containing few 5′-adenylyl groups combines with ATP–Mn under assay conditions. Whatever the mechanism, a Mn^{2+}-activated enzyme that is adenylylated to a low extent must be able to assume a conformation that has a higher affinity for ATP–Mn at subsaturating than at saturating concentrations of ATP–Mn.

The inhibition by excess Mn^{2+} with the glutamine synthetase–ATP system probably arises from a second set of 12 Mn^{2+} binding sites of intermediate affinity that the enzyme has for Mn^{2+}, with an apparent association constant of $\sim 10^4$

(Denton and Ginsburg, 1969). The presence of Mn^{2+} at this site perhaps blocks the approach of an ATP–Mn complex, particularly if a structural change is induced in the combination of ATP with the Mn–enzyme and if this structural change is antagonistic to that induced by the subunit combining with the second Mn^{2+}. The inhibitory sites for divalent cations are not necessarily located in the immediate vicinity of the catalytic sites.

Preliminary studies have indicated that the response of glutamine synthetase preparations to the other substrates in either a Mn^{2+}- or a Mg^{2+}-activated biosynthetic assay[6] is complex. In a Mn^{2+}-dependent assay, apparently higher affinities for L-glutamate and NH_4Cl are observed at low than at high concentrations of these substrates. This type of negative interaction was observed between L-glutamate and Mn^{2+}-activated enzyme forms that contain different amounts of covalently bound 5′-adenylyl groups, and these observations are extended in the accompanying paper (Ginsburg *et al.*, 1970). Apparent K'_m differences at the high concentrations of L-glutamate were observed for the different Mn–enzyme forms, with adenylylation producing an apparent decrease in the affinity of the Mn–enzyme for L-glutamate. Then, a qualitative difference between the ATP and L-glutamate saturation curves exists in that high concentrations of L-glutamate also effects the variation in response of the different Mn^{2+}–enzyme forms. Substrate saturation curves in a Mg^{2+}-dependent biosynthetic assay, although apparently hyperbolic, revealed that enzymes adenylylated to different extents have slightly different K'_m values for L-glutamate.

In the Mg^{2+} assay, increasing adenylylation appeared to increase the affinity of the Mg^{2+}–enzyme for L-glutamate, although in these cases adenylylation sensitized glutamine synthetase to inhibition by high concentrations of this substrate. These observations imply a converse relationship between the behavior of the different enzyme forms in the Mg^{2+} and Mn^{2+} assay systems. It is therefore quite possible that the interaction of substrates with the Mg^{2+}-activated enzyme also involves negative or destabilizing forces, but that intermediates in this assay system have affinities not sufficiently different for kinetic resolution.

Several different mechanisms for regulating the activity of the glutamine synthetase of *E. coli* have been described, and these have been reviewed by Holzer and Stadtman (Holzer *et al.*, 1967; Stadtman *et al.*, 1968). The results of the preliminary studies presented here suggest that a further fine control of glutamine synthetase activity resides in the cellular concentrations of the substrates of this enzyme (L-glutamate, ammonia, ATP, and specific divalent cations). At high adenylylation states, the Mg^{2+}-activated enzyme has a higher affinity than at low adenylylation states for substrate so that the depletion of ammonia and L-glutamate (favoring the deadenylylation reaction) allows the glutamine synthetase to express activity that ultimately will be increased markedly by deadenylylation. The accumulation of L-glutamine at high L-glutamate and ammonia levels (favoring the adenylylation reaction) will inhibit the Mg^{2+}-dependent activity which adenylylation will accomplish more effectively. The Mn^{2+}-dependent activity is affected in a converse manner; *i.e.*, at high adenylylation states, the enzyme has a relatively poor affinity for substrate when the requirement for glutamine synthetase activity is the lowest; at low adenylylation states, the enzyme has the capacity to react with very low concentrations of substrates and is sensitized to inhibition by corresponding high substrate concentrations. The substrate effects are subtle, since the profound changes in glutamine synthetase activity are produced by adenylylation (activated by ATP, Mg^{2+}, and L-glutamine, inhibited by α-ketoglutarate, L-glutamate, and UTP (Kingdon *et al.*, 1967; Wulff *et al.*, 1967; Stadtman *et al.*, 1968)) and deadenylylation (activated by α-ketoglutarate, ATP, and UTP with Mg^{2+} or Mn^{2+}, and inhibited by L-glutamate and L-glutamine (Shapiro and Stadtman, 1968a; Battig *et al.*, 1968; Shapiro, 1969)). The important relationship between glutamine synthetase activity and the nucleotide:divalent cation ratio has been discussed elsewhere (Stadtman *et al.*, 1968). The interactions of the glutamine synthetase with substrates also affect the response of this enzyme to inhibitors (Woolfolk and Stadtman, 1967a; Ginsburg, 1969), the feedback inhibition of glutamine synthetase by the end products of glutamine metabolism being another important regulatory mechanism (Woolfolk and Stadtman, 1964, 1967a). At low states of adenylylation, a depletion of substrates makes the Mg^{2+}-dependent activity more sensitive to feedback inhibition, but the Mn^{2+}-activity much less responsive to inhibitors. At high states of adenylylation, a depletion of substrates makes both the Mg^{2+} and Mn^{2+}-dependent activities most responsive to feedback inhibitors. As these compounds are utilized in synthetic mechanisms, the adenylylated glutamine synthetase will maximally respond to low levels of substrates. As deadenylylation occurs and the enzyme becomes much more active with Mg^{2+} activation, it could be advantageous for glutamine synthetase to have a lower affinity for substrates if these (L-glutamate, NH_3, and ATP) are utilized in other pathways. Although the exact physiological function of the subunit interactions of glutamine synthetase are speculative, it is certain that the response of the different enzyme forms to substrates is an important feature of the regulatory mechanisms.

In summary, the results presented here have shown that glutamine synthetase preparations have affinities for substrates in the biosynthetic reaction that are determined by the adenylylation state of the enzyme. Destabilizing forces apparently are involved in heterologous subunit interactions during the saturation of a hybrid dodecameric enzyme molecule, containing both adenylylated and unadenylylated subunits. This type of negative interaction is more marked between the Mn^{2+}-activated enzyme and substrates than when Mg^{2+} is the activating divalent cation. The involvement of three substrates, either of two activating divalent cations, environmental effects, and an enormous number of possible enzyme forms have complicated these studies.

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