REGULATION OF GLUTAMINE SYNTHETASE, VIII. ATP : GLUTAMINE SYNTHETASE ADENYLYLTRANSFERASE, AN ENZYME THAT CATALYZES ALTERATIONS IN THE REGULATORY PROPERTIES OF GLUTAMINE SYNTHETASE

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Escherichia coli produces two distinct forms of glutamine synthetase, depending on the manner in which the organism is grown;[1, 2] these forms have been designated synthetases I and II for convenience. A preparation containing mostly synthetase I was obtained from log-phase cells grown on glucose and NH_4Cl, and a preparation containing mostly synthetase II from stationary-phase cells grown on glycerol and glutamate.[3] The two enzymes are identical in amino acid composition and in sedimentation behavior,[4] but synthetase II contains covalently bound AMP which synthetase I lacks.[5] This paper describes an enzyme (ATP:glutamine synthetase adenylyltransferase) derived from *E. coli* W that catalyzes the conversion of synthetase I to synthetase II in the presence of ATP and Mg^{++}. During this conversion the labeled moiety of C^{14}-ATP or α-P^{32}-ATP is converted into a protein-bound form. The enzyme was partially purified by a procedure identical to that used by Mecke *et al.* for the isolation of a "glutamine synthetase-inactivating enzyme" from *E. coli* B,[6] and it is possible that the adenylyltransferase is analogous to, if not identical with, their "inactivating" enzyme. However, contrary to their results, the biosynthetic activity of the glutamine synthetase from *E. coli* W is not completely lost as a result of the adenylylation reaction, but its specificity for divalent cation activation is greatly altered. These and other properties of the adenylylation reaction and its relationship to the reaction described by Mecke *et al.*[6] are described.

Materials and Methods.—Materials: α-P^{32}-ATP and 8-C^{14}-ATP were obtained from Schwartz BioResearch, Inc., Orangeburg, New York. Preparations of glutamine synthetases I and II were obtained by the procedure of Woolfolk *et al.*[7] from *E. coli* W, grown as previously described.[2] The adenylyltransferase was prepared from *E. coli* W by the procedure which Mecke *et al.* used in preparing their *E. coli* B inactivating enzyme.[6] The *E. coli* W cells used for this purpose were grown on glycerol and glutamate in the same manner used for the production of glutamine synthetase II.[2]

Enzyme assays: Glutamine synthetase was assayed by three methods: (*a*) the Mg^{++} biosynthetic assay which involves measuring the release of phosphate from ATP in the presence of glutamate, ammonia, and 50 mM $MgCl_2$;[7] (*b*) the Mn^{++} biosynthetic assay which is the same as (*a*), except that 5 mM $MnCl_2$ is substituted for the $MgCl_2$; and (*c*) the γ-glutamyl transfer assay which was previously described.[2]

Radioactivity: Protein-bound radioactivity was determined by precipitation of the radioactive protein on filter-paper disks as described by Mans and Novelli,[8] except that the washes with hot trichloracetic acid and hot ethanol-ether were omitted.

TABLE 1

CONVERSION OF GLUTAMINE SYNTHETASE I TO SYNTHETASE II

Effector	Untreated Enzymes: Synthetase I	Untreated Enzymes: Synthetase II	Treated Synthetase I: Complete	Treated Synthetase I: −ATP	Treated Synthetase I: −Mg	De-adenylylated synthetase II
AMP, 40 mM	110	26	14	90	94	98
Glycine, 40 mM	29	41	48	34	34	15
Tryptophan, 20 mM	98	55	48	96	101	102
Histidine, 40 mM	107	50	46	110	108	113

γ-Glutamyl transfer assays were performed as described in *Methods*, in the presence and absence of effector. The results are expressed as relative specific activity; i.e., $\left(\frac{\text{activity with effector}}{\text{activity without effector}}\right) \times 100$. Numbers less than 100 represent inhibition, and numbers greater than 100, stimulation. Untreated synthetases I and II were assayed as isolated, and are included for comparison; these data have been published elsewhere.[2] Treated synthetase I had been reacted 15 min at 37° in an adenylylating medium. The complete adenylylation reaction mixture contained 10 mM Tris, pH 7.6; 50 mM $MgCl_2$; 7 mM ATP; 24 μg synthetase I; and 0.57 mg adenylyltransferase, in a volume of 60 μl. De-adenylylated synthetase II was synthetase II treated with snake venom diesterase as previously described.[5]

Results.—Conversion of synthetase I to synthetase II: Table 1 summarizes previous data[1, 2] showing that the γ-glutamyl transferase activity of synthetase II is less inhibited by glycine and is more strongly inhibited by either AMP, tryptophan, or histidine than is the transferase activity of synthetase I. In addition, Table 1 shows that when synthetase I is incubated with the adenylyltransferase, ATP, and Mg^{++}, it is converted to a form which resembles synthetase II. For this conversion, 5 mM $MnCl_2$ could not replace $MgCl_2$ nor could 4.2 mM CTP, UTP, or GTP substitute for ATP. Maximal conversion occurred at 2.5 mM ATP; some was detected at 0.25 mM ATP.

Since synthetase II contains covalently bound AMP whereas synthetase I does not,[5] it is assumed that the conversion of synthetase I to a form with kinetic properties similar to those of synthetase II is accompanied by adenylylation of the enzyme. The fact that covalently bound AMP is responsible for the unique kinetic characteristics of synthetase II is evinced by the demonstration that removal of AMP from synthetase II, by digestion with snake venom phosphodiesterase,[5] results in its conversion to a form (de-adenylylated synthetase II) that responds to effectors in a manner similar to synthetase I (see last column in Table 1).

Table 2 shows the specific activities of the four enzymes discussed in Table 1, in the three different assays. Note that adenylylated synthetase I has a lower activity in the Mg^{++} biosynthetic assay, and a higher activity in the Mn^{++} biosynthetic assay and in the γ-glutamyl transfer assay, than does either synthetase I or II, whereas de-adenylylated synthetase II has properties similar to those of synthetase I. The intermediate position of synthetase II suggests that it is slightly contaminated with synthetase I. Preparation I contains only 1.2 equivalents of adenylyl groups per mole.[5] However, de-adenylylated enzyme probably contains even less adenylyl groups since the ratio of absorbancy at 260 mμ to that at 280 mμ for preparation I (0.545) is higher than that for the de-adenylylated enzyme

TABLE 2

SPECIFIC ACTIVITIES OF SYNTHETASES I AND II AND THEIR DERIVATIVES

Assay	Specific Activity: Synthetase I	De-adenylylated synthetase II	Synthetase II	Adenylylated synthetase
Mg^{++} biosynthetic	60.1	32.3	12.8	2.2
Mn^{++} biosynthetic	2.0	1.9	8.9	25.0
γ-Glutamyl transfer	24.8	23.4	48.8	114.0

Assays performed as described in *Methods*. Results are expressed as μmoles per min per mg enzyme. Enzyme preparations are described in the legend of Table 1.

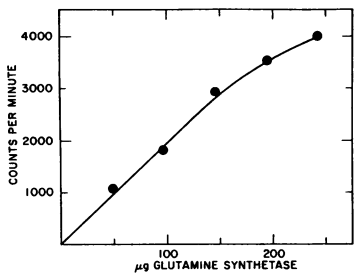

FIG. 1.—Incorporation of C^{14} from ATP into glutamine synthetase. Reaction mixtures contained 10 mM Tris buffer, pH 7.6; 50 mM $MgCl_2$; 11 mM C^{14}-ATP (specific activity 1170 cpm/mμmole); 0.38 mg of adenylyltransferase; and the indicated amounts of glutamine synthetase I in a volume of 60 μl. At the end of a 2-hr incubation at 37°, protein-bound radioactivity was determined on a 50-μl sample as described in *Methods*. The value obtained with no added glutamine synthetase (440 cpm) was subtracted from the values obtained for the other samples.

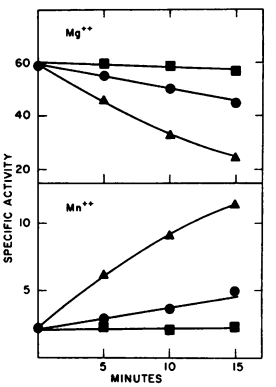

FIG. 2.—Effect of glutamate and glutamine on the rate of the adenylylation reaction. Reaction mixtures contained 10 mM Tris, pH 7.6; 50 mM $MgCl_2$; 7 mM ATP; 24 μg glutamine synthetase I; and 95 μg adenylyltransferase in a volume of 0.1 ml. The reaction was started by the addition of ATP, and was run at 37°. At the indicated times, samples were diluted with cold water and assayed immediately in the Mg^{++} and Mn^{++} biosynthetic assays as described in *Methods*. ●——●, Control; ▲——▲, plus 1 mM glutamine; ■——■, plus 0.1 *M* glutamate.

(0.525). This may account for the relatively lower specific activity of de-adenylylated enzyme in the Mg biosynthetic assay (Table 2).

Covalent binding of the AMP moiety of 8-C^{14}-ATP and α-P^{32}-ATP to synthetase I: Proof that adenylylation of the enzyme accompanies the conversion of synthetase I to a form with kinetic properties of synthetase II was obtained by showing that when synthetase I is incubated with adenylyltransferase, Mg^{++}, and 8-C^{14}-ATP, labeled carbon from ATP is incorporated into TCA-precipitable material (see Fig. 1); the amount of the labeled moiety incorporated is proportional to the concentration of synthetase I added, over a severalfold range. Using data from the linear portion of the curve in Figure 1, it was calculated that 10.4 equivalents of the labeled moiety of C^{14}-ATP were bound per mole of synthetase I. Since *E. coli* glutamine synthetase contains 12 identical subunits,[7] this result approaches closely the value of one equivalent of labeled moiety bound per subunit of synthetase I. In similar experiments with α-P^{32}-ATP, 8.9 equivalents of P^{32}-labeled phosphate were incorporated per mole of enzyme. Control experiments indicated that no protein-bound radioactivity was formed if $MgCl_2$ or adenylyltransferase was omitted from the reaction mixtures.

Effect of glutamine and glutamate on the rate of adenylylation: The data of Table 2 suggest that the conversion of synthetase I to synthetase II can be followed by measuring either an increase in activity in the Mn^{++} biosynthetic assay or a decrease in activity in the Mg^{++} biosynthetic assay. This is illustrated by the data in Figure 2, which show that reciprocal changes in the two assay systems occur with time when preparation I is incubated with ATP, Mg^{++}, and the adenylyltransferase. The rates of change are greatly accelerated in the presence of gluta-

mine whereas glutamate inhibits these changes. The top portion of Figure 2, showing decrease in activity in the Mg^{++} biosynthetic assay, is reminiscent of the time course of "inactivation" described by Mecke *et al.*[6] However, the lower portion of the figure shows that concomitant activation occurs when the enzyme is assayed in the Mn^{++} biosynthetic assay. For convenience, further adenylylation experiments were performed in the presence of 1 mM glutamine.

Time course of the adenylylation reaction: Further evidence that adenylylation of synthetase I is responsible for the changes in kinetic properties was obtained from an experiment in which the time course of adenylylation, as measured by the incorporation of labeled carbon from C^{14}-ATP was compared with the time courses of the changes in activities in the Mg^{++} and Mn^{++} biosynthetic assays, and also of the susceptibility of the glutamyl transferase activity to inhibition by AMP. The results are shown in Figure 3. It is evident that the increase in protein-bound C^{14}-AMP is accompanied by an increase in activity in the Mn^{++} biosynthetic assay and a decrease in activity in the Mg^{++} biosynthetic assay. It is noteworthy that the change in susceptibility of the transferase activity to inhibition by AMP occurs more rapidly than do changes in the other parameters. This suggests that the change in effector response may occur with less extensive adenylylation. The possibility that the changes in AMP sensitivity do not involve adenylylation but are caused by other enzymes in the impure adenylyltransferase preparation is contraindicated by the data of Table 1 showing that de-adenylylation of synthetase II by highly purified snake venom phosphodiesterase[5] results in its conversion to a form that is insensitive to AMP inhibition.

Kinetic properties of adenylylated enzyme: As noted above, the synthetase II

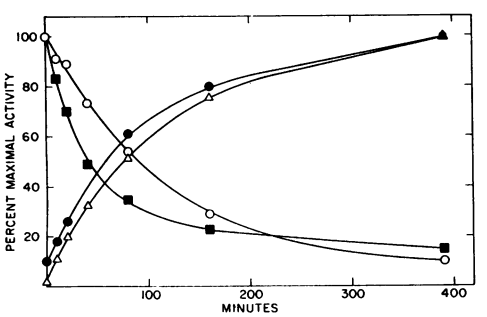

FIG. 3

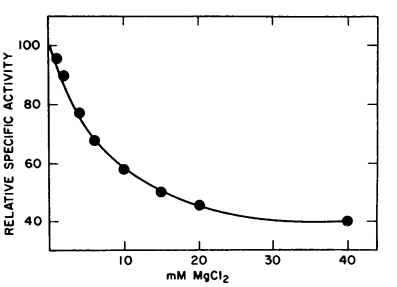

FIG. 4

FIG. 3.—Time course of the adenylylation reaction. Reaction mixtures contained 10 mM Tris, (pH 7.6), 50 mM $MgCl_2$, 1 mM glutamine, 6.9 mM C^{14}-ATP, 0.48 mg glutamine synthetase I, and 0.19 mg adenylyltransferase in a volume of 0.2 ml. At the indicated times, 2-μl samples were diluted into 0.1 ml of cold water for enzyme assays, and 20-μl samples were assayed for protein-bound radioactivity. To obtain a common ordinate, Mg^{++} and Mn^{++} biosynthetic assays have been expressed as per cent of maximal activity, as has the protein-bound radioactivity. The value given for response to AMP in the γ-glutamyl transfer assay is the relative specific activity in the presence of 40 mM AMP as compared to a paired control without AMP. O——O, Mg^{++} biosynthetic assay; ●——●, Mn^{++} biosynthetic assay; Δ——Δ, protein-bound radioactivity; ■——■, relative specific activity in the presence of AMP in the transfer assay.

FIG. 4.—Inhibition of the adenylylated enzyme by $MgCl_2$. The Mn^{++} biosynthetic assay was used. Reaction mixtures contained 1.2 μg adenylylated enzyme and the indicated amounts of $MgCl_2$; incubation time 8 min.

preparation is only partly adenylylated and may be contaminated with synthetase I. To obtain data on more nearly completely adenylylated enzyme, a large amount of synthetase I was adenylylated under the conditions employed in Figure 3, and was reisolated by acid ammonium sulfate precipitation.[7] On the basis of its absorbancy at 280 and 260 mμ the adenylylated enzyme thus obtained contained *ca.* 11.8 equivalents of AMP per mole. It was 11.5 times more active in the Mn^{++} biosynthetic assay than in the Mg^{++} assay; thus it behaves like the adenylylated enzyme described in Tables 1 and 2.

Inhibition by Mg^{++}: Although the adenylylated enzyme is relatively specific for Mn^{++}, its activity in the Mn^{++} biosynthetic assay is partially inhibited by Mg^{++} (see Fig. 4). Since only partial inhibition (60%) is obtained with saturating concentrations of Mg^{++} (40 mM), this inhibition probably does not involve direct competition with Mn^{++} at the activating sites on the enzyme. The possibility that Mg^{++} causes a shift in the pH optimum of the enzyme in the presence of Mn^{++}, as is true for the mammalian enzyme,[9] was not determined.

Response of adenylylated enzyme to effectors: The influence of various effectors on the γ-glutamyl transferase activity has already been discussed (see Table 1). Since the biosynthetic activity of adenylylated enzyme is nearly specific for Mn^{++} whereas synthetase I is nearly specific for Mg^{++}, the responses of each enzyme to various effectors in the appropriate biosynthetic assay system were examined. Thus, the adenylylated enzyme was studied in the Mn^{++} biosynthetic assay and synthetase I was tested in the Mg^{++} assay. Since some of the effectors are competitive with respect to glutamate and others are competitive with NH_4^+,[10, 11] the concentrations of glutamate and NH_4^+ were both made limiting in the biosynthetic assay to accentuate the responses to competitive inhibitors. The data in Figure 5 show that under these conditions the adenylylated enzyme is more sensitive to inhibition by histidine, CTP, and tryptophan than is synthetase I, and is less sensitive to inhibition by glycine and alanine (cf. refs. 1 and 2). The effects of these ligands on the two enzymes are therefore qualitatively the same as their effects on the glutamyl transferase activity.[1, 2] With AMP, however, responses in the

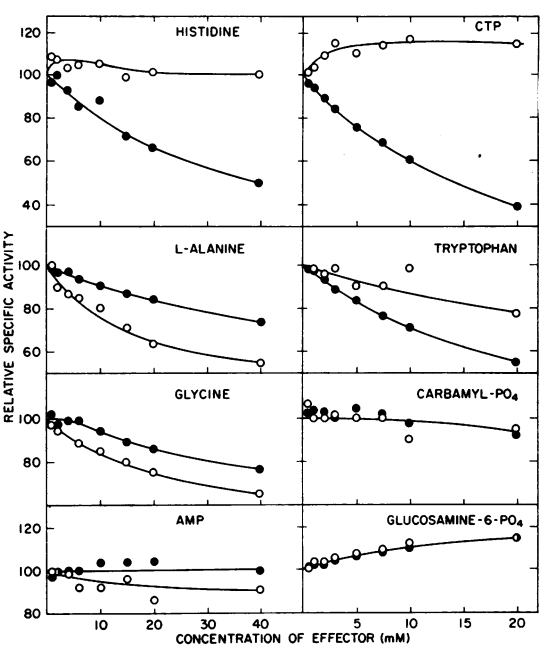

FIG. 5.—Effector responses of adenylylated and unmodified synthetase I preparations. Activity was measured in the biosynthetic assays except that concentrations of NH_4Cl and glutamate were reduced to 1 mM and 7.5 mM, respectively; 10 min at 37°. Effector concentrations were as shown. ●——●, 4.8 μg of adenylylated enzyme, Mn^{++} biosynthetic assay; O——O, 0.4 μg of unmodified synthetase I, Mg^{++} biosynthetic assay.

biosynthetic assays were exactly the reverse of those observed in the transfer assay[1, 2] (see also Table 1). Thus 40 mM AMP causes 80 per cent inhibition of the transferase activity of the adenylylated enzyme but it stimulates (10%) the transferase activity of synthetase I. On the other hand, AMP slightly inhibits the biosynthetic activity of synthetase I, but is without effect on the adenylylated enzyme. Lack of inhibition by glucosamine-6-phosphate or carbamyl phosphate is not explained (cf. ref. 11).

Apart from the AMP response, the effector alterations of biosynthetic activity were qualitatively as expected from previous transfer assay experiments.[1] However, the extent of inhibition with AMP and alanine was much less than is usually observed.[11] This is because nonsaturating concentrations of NH_4^+ and glutamate were used in the present study. With saturating levels of substrates, synthetase I was inhibited 40 per cent by 40 mM AMP, and 75 per cent by 40 mM L-alanine; inhibitions of 15 and 45 per cent, respectively, were obtained with nonsaturating substrates. These results are compatible with the noncompetitive nature of the inhibition by alanine and AMP,[10, 11] and suggest that glutamate, NH_4^+, or both may increase the affinity of the enzyme for these inhibitors under assay conditions. Nevertheless, there is a reciprocal relationship in the behavior of synthetase I and the adenylylated enzyme toward AMP in the biosynthetic assay, as compared to that in the glutamyl transfer assay. Hence, the synthetase I preparation is inhibited by AMP in the biosynthetic reaction, whereas it is activated by AMP in the transfer assay.

Standard curve for estimating the extent of adenylylation: The experimental points in Figure 6 describe the relationship between the amount of covalently bound AMP in various pure glutamine synthetase preparations and the ratio of absorbance at 260 mμ to that at 280 mμ. The line represents a theoretical curve calculated for the addition of free AMP to a solution of the completely unadenylylated enzyme. The experimental points agree well with the calculated curve and indicate that there is no significant alteration in the absorbancy characteristics of AMP when it is bound to the enzyme. The observed relationship provides the basis for a simple spectrophotometric method for estimating the number of equivalents of AMP bound to the enzyme. Note that one preparation seems to have

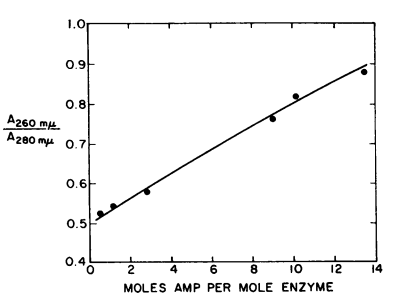

FIG. 6.—Spectral correlates of the extent of adenylylation of different glutamine synthetase preparations. The line is a theoretical curve for the addition of AMP ($\epsilon_{260\,m\mu} = 1.5 \times 10^4\ M^{-1}\ cm^{-1}$; $\epsilon_{280\,m\mu} = 0.225 \times 10^4\ M^{-1}\ cm^{-1}$; pH 7.0) to unadenylylated glutamine synthetase ($\epsilon_{280\,m\mu} = 4.4 \times 10^5\ M^{-1}\ cm^{-1}$; pH 7.0; $A_{260}/A_{280} = 0.5$); these values are obtained by extrapolation from the value for the extinction coefficient for synthetase II, obtained previously.[5,7] The points represent experimental values determined on several pure glutamine synthetase preparations after exhaustive dialysis against 0.01 *M* imidazole buffer, pH 7.0. Spectra were recorded in a Cary 15 spectrophotometer with correction for light-scattering by extrapolation of the absorbancy at 340 mμ. Total organic phosphate was used to estimate the number of moles of AMP per mole of enzyme, as previously described. All points represent the average of two determinations. The two highest values are for adenylylated preparations, made by utilizing the enzyme described in this paper; the lowest value is for an unadenylylated preparation, made by digesting glutamine synthetase II with snake venom phosphodiesterase.[5] The three intermediate points are for homogeneous glutamine synthetase preparations as purified from *E. coli*.

more than one mole of organic phosphate per subunit, or 13.4 per mole of enzyme. This may represent non-AMP organic phosphate bound to the enzyme, or may indicate that the adenylylating enzyme is capable of placing more than 1 AMP on each subunit.

Discussion.—Glutamine synthetase activity in *E. coli* is controlled by repression of enzyme synthesis[7, 10, 12] and by cumulative feedback inhibition of catalytic activity.[10, 11] It is now evident that adenylylation of glutamine synthetase represents a third mechanism for the cellular regulation of this enzyme.

The fact that the rate of adenylylation is stimulated by glutamine and is retarded by glutamate provides a mechanism by which both substrate and product can influence glutamine metabolism. With adenylylation, the enzyme is converted from a biosynthetically more active species, specific for Mg^{++}, to a less active form that is specific for Mn^{++}. In addition, the pH optimum for the enzyme is shifted from 8.0 to 7.0.[2] Moreover, other studies[13] suggest that the catalytic activity of the Mn^{++}-dependent enzyme (i.e., the adenylylated enzyme) is greatly influenced by the ratio of ATP to Mn^{++}, and, at any given ratio, is further influenced by the total concentrations of nucleotide di- and triphosphates. Since one state of nitrogen nutrition favors the almost exclusive production of adenylylated enzyme whereas a different state of nitrogen nutrition favors production of the unadenylylated form,[2, 5] the physiological importance of the adenylylation reaction seems assured. Nevertheless, in view of the appalling complexity of the regulatory mechanism, as evinced by the above-mentioned pH and divalent ion interrelationships and by the multiple effector responses, an attempt to rationalize the present observations with specific aspects of glutamine metabolism is premature. A better understanding of the factors controlling the levels of the adenylylated versus unadenylylated enzyme must await a greater knowledge of intracellular levels of various nitrogen compounds and of the factors that influence the adenylyltransferase and the de-adenylylating enzyme proposed on the basis of *in vivo* observations.[2]

Since glutamine synthetase is composed of 12 apparently identical subunits, and since about 12 equivalents of AMP are contained in the fully adenylylated enzyme, each subunit is probably able to bind one adenylyl group. It remains to be determined whether preparations with an intermediate number of covalently bound AMP residues represent mixtures of completely adenylylated and unadenylylated enzyme molecules or if they represent populations of enzymes in which only part of the subunits are adenylylated.

However, the data in Figure 2 indicate that only a limited degree of adenylylation is required to achieve complete sensitivity of the γ-glutamyl transferase activity to inhibition by AMP, whereas more extensive adenylylation is required to effect the change in divalent cation specificity. This indicates that enzyme molecules with intermediate levels of adenylylation do exist and suggests also the possibility that the responsiveness to different effectors may independently be affected by different degrees of adenylylation.

The adenylylation reaction probably proceeds according to the equation:

$$\text{Glutamine synthetase} + \text{ATP} \xrightarrow[Mg^{++}]{\text{adenylyltransferase}} \text{adenylyl glutamine synthetase} + \text{pyrophosphate.}$$

To date, no attempt has been made to identify pyrophosphate as a product. The adenylyltransferase is either identical to or analogous to the "inactivating enzyme" isolated from *E. coli* B by Mecke *et al.*[6] However, since adenylylation is accompanied by an increase in biosynthetic activity in the presence of Mn^{++}, as well as a decrease in the presence of Mg^{++}, the name "ATP:glutamine synthetase adenylyltransferase" is preferred to the name "inactivating enzyme" proposed by Mecke *et al.*[6]

Summary.—*Escherichia coli* contains an enzyme, ATP:glutamine synthetase adenylyltransferase, that catalyzes the covalent attachment of the adenylyl moiety of ATP to glutamine synthetase I. The adenylylation reaction requires Mg^{++}, is activated by glutamine, is inhibited by glutamate, and is accompanied by conversion of the Mg^{++}-dependent glutamine synthetase I to a less active form (synthetase II) that is specific for Mn^{++} and is relatively more sensitive to several feedback effectors. Completely adenylylated synthetase contains about 12 adenylyl groups per mole, i.e., 1 per subunit.

Abbreviations used: AMP, adenosine 5′-phosphate; ATP, adenosine 5′-triphosphate; CTP, cytidine 5′-triphosphate; UTP, uridine triphosphate; GTP, guanosine 5′-triphosphate; TCA, trichloroacetic acid.

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[3] Strictly speaking, pure synthetase I probably contains no adenylyl groups and synthetase II probably contains 12 equivalents of covalently bound AMP per mole. However, for purposes of the present discussion synthetases I and II are identical, respectively, with the glutamine synthetase preparations I and II described previously.[5] Synthetase I is probably contaminated with a small amount of synthetase II, and vice versa.

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