The Glutamine Synthetase Deadenylylating Enzyme System from *Escherichia coli*. Resolution into Two Components, Specific Nucleotide Stimulation, and Cofactor Requirements*

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ABSTRACT: Glutamine synthetase from *Escherichia coli* exists as a dodecameric aggregate which has varying amounts of 5'-adenylyl groups bound to it; there may be from 0 to 12 equiv of adenylyl groups bound per mole of enzyme. The adenylyl residues are hydrolyzed from glutamine synthetase in a reaction catalyzed by the glutamine synthetase deadenylylating enzyme, which has been purified 20-fold by a combination of protamine sulfate fractionation, ammonium sulfate fractionation, and adsorption to and elution from alumina C-γ gel. The enzyme may be resolved into two components, both of which are required for activity, by gel filtration on Bio-Gel A-0.5, in the presence of phosphate buffer and dithiothreitol.

The enzyme requires either Mn^{2+} or Mg^{2+} for activity, with the former being effective at one-tenth the concentration of the latter. Ca^{2+}, Cd^{2+}, Zn^{2+}, and Ba^{2+} are not activators of the reaction. The pH optimum for deadenylylation is rather sharp at pH 7.3. Deadenylylating enzyme activity is stimulated more than twofold by inorganic phosphate or arsenate; sulfate has little effect on the reaction, and pyrophosphate completely inhibits it. The deadenylylation reaction is stimulated considerably by specific nucleotides. Thus, more than 45-fold activation is afforded by a combination of uridine triphosphate (0.8 mM) and adenosine triphosphate (40 μM). Adenosine triphosphate is most effective when added to subsaturating concentrations of uridine triphosphate; under these conditions, uridine triphosphate and adenosine triphosphate behave in a synergistic fashion in stimulating deadenylylation. A ribosomal ribonucleic acid preparation from *E. coli* may replace adenosine triphosphate in the synergistic stimulation of the reaction, as may adenosine diphosphate. A precursor of glutamine, α-ketoglutarate, stimulates the deadenylylation reaction 15-fold; glutamine itself causes almost complete inhibition. The concentration of α-ketoglutarate which effects half-maximal activation is 1.0 mM. The concentration of glutamine which inhibits the reaction 50% is 0.25 mM. A model is presented which suggests that the relative concentrations of α-ketoglutarate, glutamate, glutamine, and the products of glutamine metabolism are the decisive factors in the control of nitrogen metabolism in *E. coli*.

During the past 2 years, investigations in this laboratory and that of H. Holzer, in Freiburg, have led to the discovery of a novel form of metabolic control modifications in enzyme activity induced by the covalent attachment of adenylyl residues. Thus, the glutamine synthetase from *Escherichia coli* has been shown to exist in several forms, which differ from one another in the number of covalently bound 5'-adenylyl groups (Shapiro *et al.*, 1967; Wulff *et al.*, 1967). *E. coli* contains an enzyme ATP–glutamine synthetase adenylyltransferase (Kingdon *et al.*, 1967; Wulff *et al.*, 1967) that catalyzes the transfer of the adenylyl moiety of ATP to glutamine synthetase in a reaction that is stimulated by glutamine and inhibited by glutamate. The adenylyl group is joined in a stable phosphodiester bond with the hydroxyl group of a specific tyrosine residue of glutamine synthetase (Shapiro and Stadtman, 1968c). Glutamine synthetase may be adenylylated to the extent of one adenylyl group per subunit of 50,000 molecular weight, or 12 equiv/mole of enzyme (Kingdon *et al.*, 1967).

Adenylylated glutamine synthetase has markedly different properties from unadenylylated. The adenylylated enzyme is intrinsically less active in catalyzing glutamine biosynthesis than is unadenylylated enzyme (Kingdon *et al.*, 1967; Wulff *et al.*, 1967). In addition, unadenylylated enzyme is specifically activated by Mg^{2+}, whereas the adenylylated preparation is dependent upon Mn^{2+} (Kingdon *et al.*, 1967). The adenylylated enzyme form is also much more susceptible to inhibition by some products of glutamine metabolism than is the unadenylylated preparation, when either the biosynthetic activity or the γ-glutamyl transfer activity of glutamine synthetase is examined (Kingdon *et al.*, 1967). The pH optimum for catalysis of γ-glutamyl transfer differs markedly in the two enzyme forms, being 6.8 in the adenylylated preparation and 7.9 in the unadenylylated one; both have the same activity when studied at pH 7.15 (Stadtman *et al.*, 1968a). In addition, the γ-glutamyl transfer activity of the adenylylated enzyme may be specifically inhibited in the presence of Mg^{2+}; this has served as a convenient assay for the extent[1] of adenyl-

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[1] The extent of adenylylation refers to the average number of equivalents of 5'-adenylyl residues bound to glutamine synthetase per mole (600,000 g) of enzyme. $E_{\bar{n}}$ refers to enzyme preparations containing $\bar{n}$ equivalents of adenylylate bound per mole. Since the enzyme can be adenylylated to the extent of one adenylyl residue per subunit (50,000 g), the $\bar{n}$ values vary from 0 to 12.

ylation of crude preparations of glutamine synthetase (Stadtman *et al.*, 1968a).

The extent of adenylylation is dependent upon the growth conditions of *E. coli* (Kingdon and Stadtman, 1967b; Holzer *et al.*, 1968). Deadenylylation of glutamine synthetase occurs *in vivo* upon switching from a nitrogen-rich to a nitrogen-poor growth medium (Kingdon and Stadtman, 1967b; Heilmeyer *et al.*, 1967) and is detected by alterations in the kinetic characteristics of the enzyme. *E. coli* cell extracts contain an enzyme which catalyzes the removal of adenylyl residues from glutamine synthetase (Shapiro and Stadtman, 1968a; Battig *et al.*, 1968). This glutamine synthetase deadenylylating enzyme is activated by α-ketoglutarate and inhibited by glutamine. The present communication describes a partial purification of this enzyme and the resolution of it into two components, both of which are required for deadenylylating activity. In addition, the requirement for specific nucleotide fractions and other cofactors in the deadenylylation reaction is presented. Finally, a scheme by which the nitrogen metabolism of *E. coli* may be regulated during growth by adenylylation and deadenylylation of glutamine synthetase is discussed.

Experimental Procedure

Materials. The labeled nucleotides used in these experiments ([^{14}C]ATP, uniformly labeled; [^{3}H]ADP, uniformly labeled; and [^{3}H]AMP, uniformly labeled) were from New England Nuclear; dithiothreitol and α-ketoglutarate were from Calbiochem, as was protamine sulfate (Lot No. 8000007); Bio-Gel A 0.5 200-400 mesh was from Bio-Rad (Lot 4805); poly U, poly A, poly G, poly C, poly I, 5S RNA, and 16S plus 23S RNA were from Miles Laboratories. The tRNA preparations and several other RNA preparations examined in this study were kindly provided by Dr. G. D. Novelli; alumina C-γ was prepared according to Wilstatter, as described by Colowick (1955), and was a generous gift of Mr. J. Davis.

Methods. The glutamine synthetase used in this study was purified as described previously (Woolfolk *et al.*, 1966) and as purified had 2.3 equiv of adenylylate bound per mole of enzyme ($E_{\overline{2.3}}$). The adenylyltransferase used to adenylylate this preparation of glutamine synthetase with [^{14}C]ATP was purified ~75-fold. Both preparations were generous gifts of Drs. M. D. Denton and A. Ginsburg, who provided a great deal of aid in adjusting the conditions of adenylylation to yield radioactively labeled glutamine synthetase of relatively high specific radioactivity. Conditions for the adenylylation reaction were as previously reported (Kingdon *et al.*, 1967), with the following modifications. The glutamine synthetase was predialyzed against 1 mM $MgCl_2$–20 mM imidazole chloride (pH 7.1) and was used at 10 mg/ml; the adenylyltransferase was 0.2 mg/ml; ATP was 2.6 mM, with a specific radioactivity of 12,000 dpm/mμmole. In order to regenerate the ATP which had been converted into ADP by the significant ATPase activity of the substrate quantities of glutamine synthetase used in the adenylylation reaction, 8 mM phosphoenolpyruvate, 100 μg of pyruvate kinase, and 0.1 M KCl were added to the adenylylation reaction mixture.[2] When a total of 12 equiv of adenylate/mole of enzyme was bound, as determined by measuring the amount of radioactivity which was insoluble in boiling trichloroacetic acid,[2] the adenylylated glutamine synthetase was purified from the reaction mixture using the acetone and ammonium sulfate procedures previously described (Woolfolk *et al.*, 1966). The purified glutamine synthetase was then dialyzed against 1000 volumes of buffer (10 mM imidazole chloride–1 mM $MnCl_2$, pH 7.1) for four 12-hr dialyses, each using fresh dialysate. The resultant glutamine synthetase contained 11.8 equiv of AMP/mole of enzyme ($E_{\overline{11.8}}$) with a specific radioactivity of 232,000 dpm/mg of enzyme. This preparation was used as the substrate for all of the deadenylylation experiments reported below. About 2.6% of the radioactivity was not acid precipitable, *i.e.*, was not covalently bound; all of the data were corrected for this free radioactivity.

The standard assay procedure for the deadenylylating enzyme at 37° was as follows. The reaction mixture contained 0.1 M imidazole chloride (pH 7.2), 1.25 mM dithiothreitol, 15 mM potassium phosphate, 0.8 mM UTP, 40 μM ATP, 1.0 mM $MnCl_2$, 15 mM α-ketoglutarate, and 21 μg of adenylylated glutamine synthetase ($E_{\overline{11.8}}$), in a final volume of 0.2 ml (pH 7.3). Enough deadenylylating enzyme was added to permit up to 40% deadenylylation of the glutamine synthetase, under which conditions the reaction is linear for up to 90 min. The reaction was terminated by the addition of 50 μl of 12% perchloric acid, then 10 μl of 10% bovine serum albumin was added, to ensure precipitation of the glutamine synthetase. After centrifuging at 1500*g* for 15 min, a 150-μl aliquot of the supernatant solution was transferred to a counting vial and neutralized with 30 μl of 2 N KOH. Radioactivity was measured in the solution of Bray (1960) at 75% efficiency, using a Nuclear-Chicago Mark I scintillation counter. Sufficient time periods of counting were used to ensure a statistical counting accuracy of 2% or greater. A unit of enzyme is defined as that required to remove 1 $\mu\mu$mole of adenylyl residue/min. at 37° from adenylylated glutamine synthetase.

Protein was routinely estimated using a micromodification of the biuret reaction described by Layne (1957), in that the protein solution was first precipitated with 8% trichloroacetic acid, then the precipitated protein was resuspended in 0.2 ml of water, and 0.8 ml of biuret reagent was added. The color thus developed was compared with that obtained in a similar fashion with a bovine serum albumin standard. The concentration of glutamine synthetase was determined by using the specific absorption coefficient for glutamine synthetase at 290 mμ (in 10 mM imidazole chloride–1 mM $MnCl_2$, pH 7.1), which is 0.385 for a 0.1% protein solution in a 1-cm light path. This value was obtained from dry weight measurements made previously upon glutamine synthetase (Shapiro and Stadtman, 1967) and has the advantage over the 280-mμ absorption coefficient that it is independent of the extent of adenylylation.

[2] M. D. Denton and A. Ginsburg, unpublished data.

In order to compare the effectiveness of the polynucleotide preparations examined, their concentrations were normalized to that of the component bases by assuming that the molar nucleotide base extinction coefficient was 10,000 M^{-1} cm^{-1} (pH 7). All polynucleotide preparations were examined at similar nucleotide equivalent concentrations. All mononucleotide and nucleoside concentrations were estimated from their reported extinction coefficients.

Results

Purification of the Deadenylylating Enzyme. GROWTH OF *E. coli.* The organism used in these studies was *E. coli* strain W, grown to late logarithmic phase in an ammonium chloride–glucose growth medium as described by Kingdon and Stadtman (1967b). After they were harvested, the cells were frozen in liquid nitrogen and kept frozen until used. There was no loss in deadenylylating activity for up to 6-months storage of the cells.

PREPARATION OF CELL EXTRACTS. Cells were suspended in a Waring Blendor, in two times their volume of a buffer composed of 50 mM imidazole chloride and 20 mM 2-mercaptoethanol (pH 7.0). This and all subsequent steps were performed at 4°. The cells were then disrupted by sonication, as follows: 200 ml of cell suspension was stirred in a 250-ml beaker placed in an ice–water–NaCl cooling bath; a sonicator probe (Branson S-75) was placed in the cell suspension, and four 3-min bursts of sonication (9 A) were employed to break the cells. With careful stirring, the temperature of the cell extract never went above 12°. The resulting particulate suspension was centrifuged at 20,000*g* for 1 hr and the supernatant solution was taken, diluted with water to a protein concentration of 35 mg/ml, and used as the cell extract.

PROTAMINE SULFATE FRACTIONATION. Protamine sulfate (25 mg/ml) was added to the cell extract, to a final concentration of 3.8 mg/ml. The optimal concentration of protamine depends upon the batch of reagent used; consequently, the optimal concentration was selected as the highest one which did not precipitate any deadenylylating enzyme, and was determined for each new batch of protamine sulfate. The mixture was equilibrated for 30 min, then centrifuged at 20,000*g* for 30 min.

30–45% $(NH_4)_2SO_4$ FRACTIONATION. Solid ammonium sulfate was added to the supernatant solution from the previous step (176 mg/ml of supernatant); the mixture was equilibrated with stirring for 25 min, then centrifuged at 20,000*g* for 30 min. After centrifugation, ammonium sulfate was added to the supernatant solution (95 mg/ml) and the suspension was equilibrated and centrifuged as before. The resultant precipitate was resuspended in buffer (50 mM imidazole chloride–20 mM 2-mercaptoethanol) equal to $^1/_{20}$ the volume of the crude cell extract, then dialyzed overnight against the same buffer.

ALUMINA C-γ ADSORPTION AND ELUTION. The ammonium sulfate fraction from the previous step was diluted to a protein concentration of 12 mg/ml. Then alumina C-γ gel, 14 mg/ml in water (see Experimental Procedure), was added with stirring until a gel:protein ratio of 1.5 (w/w) was reached. The suspension was equilibrated for 20 min, then centrifuged (10,000*g*, 20 min). The supernatant solution was discarded, and the precipitate was washed with phosphate buffer (50 mM potassium phosphate (pH 7.4)–20 mM 2-mercaptoethanol) equal to the volume of the protein solution at the beginning of this step. The mixture was stirred for 20 min, then centrifuged as before. The supernatant solution was again discarded, and the precipitate was washed with phosphate buffer (100 mM potassium phosphate (pH 7.4)–20 mM 2-mercaptoethanol) of the same volume as the previous wash. The mixture was equilibrated and centrifuged as before, then the supernatant solution was retained as the alumina C-γ eluate.

0–50% $(NH_4)_2SO_4$ FRACTIONATION. Solid ammonium sulfate was added slowly with stirring to the alumina C-γ eluate (313 mg/ml of eluate); the resulting suspension was stirred for 25 min, then centrifuged (20,000*g*, 30 min). The precipitate was resuspended in buffer (50 mM potassium phosphate–10 mM dithiothreitol) equal in volume to one-tenth the alumina C-γ eluate.

This purification procedure leads, in 2 days, to a preparation of enzyme which is 20-fold purified (Table I).

TABLE I: Purification of the Deadenylylating Enzyme System.[a]

Preparation	Total Protein (mg)	Sp Act. (units/mg)	Yield (%)
Cell extract	10,200	0.75	100
Protamine sulfate supernatant	6,350	1.3	104
30–45% $(NH_4)_2SO_4$ fraction	1,440	3.2	59
Alumina C-γ eluate	334	8.8	38
0–50% $(NH_4)_2SO_4$ fraction	217	15.2	42

[a] The purification procedures are described in Results. One unit of enzyme activity is the amount required to deadenylylate 1 μμmole of glutamine synthetase subunit/min. The enzyme activity was determined under the conditions described in Methods, and protein was measured by the biuret reaction.

The enzyme thus obtained is still quite impure, as evidenced by disc gel electrophoresis. Attempts at organic solvent fractionation and heat fractionation led to inactivation of the enzyme preparation. Storage of this fraction is best at 4° in the phosphate–dithiothreitol buffer of the last step, at protein concentrations between 20 and 40 mg per ml. Under these conditions, the enzyme loses about 50% of its activity in 2 weeks. Freezing in liquid nitrogen did not enhance the stability.

RESOLUTION OF THE DEADENYLYLATING ENZYME INTO TWO COMPONENTS. When the 20-fold purified enzyme

preparation from Table I was chromatographed on Bio-Gel A-0.5, in the presence of 50 mM potassium phosphate and 10 mM dithiothreitol (pH 7.4), there was almost no deadenylylating activity found when the column fractions were assayed (Figure 1, triangles). However, when one of the inactive fractions from either side of this small peak of activity was used to assay the inactive fractions of the other side of the peak, quite large peaks of deadenylylating activity were discovered (Figure 1). Thus, by mixing the column fractions in this manner, two peaks of enzyme activity were located, and labeled P_I and P_{II} in Figure 1. By combining fractions, 100% of the activity could be reconstituted.

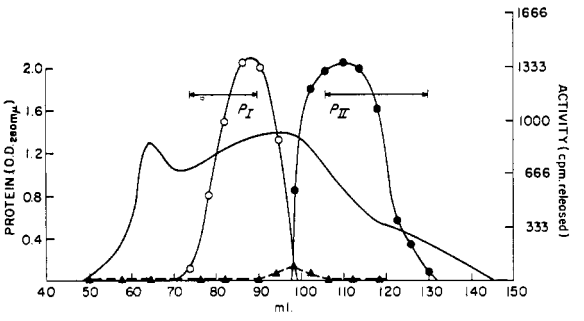


FIGURE 1: Resolution of the deadenylylating enzyme system into two required components. The figure illustrates the results of chromatography on a 2 × 44 cm column of agarose (Bio-Gel A-0.5). The column was equilibrated overnight with 50 mM potassium phosphate and 10 mM dithiothreitol (pH 7.4) then 1 ml of the 20-fold-purified deadenylylating enzyme preparation from Table I (46.5 mg/ml) was carefully placed on the column and the column was eluted with the same buffer at 22 ml/hr. Fractions (2 ml) were collected and used for the measurement of activity and protein concentration. Protein was estimated by the absorbancy of the fractions at 280 mμ and is indicated by the solid line. Activity was determined using the standard assay (see Methods) and is reported as counts per minute released in 30-min incubation. For the activity determinations, 50 μl of each (2.0 ml) fraction was assessed by itself (▲–▲), or in the presence of 50 μl of the inactive 109-ml fraction (peak labeled P_I; O–O) or in the presence of 50 μl of the 90-ml fraction (peak labled P_{II}; ●–●). Each group of fractions indicated by the arrows was then pooled, made 50% saturated with $(NH_4)_2SO_4$, then was resuspended in 1.0 ml of 50 mM potassium phosphate and 10 mM dithiothreitol (pH 7.4) and saved for futher study, as P_I and P_{II}, respectively.

The fractions indicated by the arrows in Figure 1 were pooled and used for further experiments. Each was selected to be free of the other component. From their separation by chromatography on Bio-Gel A-0.5, the two components appear to differ significantly in size. It is also of some interest that better resolution of the two components was achieved when dithiothreitol was used in the buffer instead of 2-mercaptoethanol.

Each of the two fractions, P_I and P_{II}, is inactive by itself. The two components interact reciprocally to effect deadenylylation of glutamine synthetase, as shown in Figure 2. In the presence of both components, the extent of deadenylylation is proportional to the amount of each component. The results of some initial experiments designed to test the nature of the interaction of the two components are described below.

TABLE II: Requirements for Deadenylylation of Glutamine Synthetase.

Reaction Mixture	Act. (units)
Complete system[a]	4.6
−α-Ketoglutarate	0.3
−UTP	0.7
−ATP	3.5
−UTP and ATP	0.1
$-MnCl_2$	0.0
−Phosphate[b]	2.0
−Dithiothreitol[b]	4.1

[a] The complete system consisted of 0.1 M imidazole chloride, 2.4 mM dithiothreitol, 22 mM potassium phosphate, 20 mM α-ketoglutarate, 1.0 mM UTP, 20 μM ATP, 1.0 mM $MnCl_2$, 21.6 μg of adenylylated glutamine synthetase, 83 μg of P_I, and 42 μg of P_{II}, in a final volume of 0.2 ml, pH 7.3. Activity was measured as described in Methods. [b] Since P_I and P_{II} were each stored in 50 mM potassium phosphate (pH 7.4) and 10 mM dithiothreitol, the final concentrations of these two reactants were actually 2 and 0.4 mM, respectively, when they were not added to the reaction mixture.

Requirements for Deadenylylation. Table II lists the requirements for the deadenylylation reaction and shows to what extent omission of any component of the reaction mixture affects the activity. Maximal activity occurs when the complete mixture contains, in addition to adenylylated glutamine synthetase and both components of the deadenylylating enzyme system (P_I and P_{II}), a divalent cation, UTP and ATP, α-ketoglutarate, phosphate, and dithiothreitol. The previously reported stimulation of the reaction (Shapiro and Stadtman, 1968a) by albumin and NaF, seen in the crude cell extract, was no longer evident with the more highly purified enzyme preparation examined here. Albumin was no longer required when either 2-mercaptoethanol or dithiothreitol was added to the deadenylylating enzyme during both purification and assay. The loss of the requirement for NaF in the reaction is discussed below.

Divalent Cations. The enzyme is activated by either $MnCl_2$ or $MgCl_2$ (Figure 3), but not at all by Ca^{2+}, Cd^{2+}, Zn^{2+}, or Ba^{2+}. Mg^{2+} is required at ten times the concentration of Mn^{2+} and is only 75% as effective as Mn^{2+} at saturation. The activation by these cations is cooperative, with a sigmoidal curve being generated as a function of effector concentration. Both Mg^{2+} and Mn^{2+} affect the structure of glutamine synthetase (Shapiro and Ginsburg, 1968; Kingdon *et al.*, 1968), and may in addition affect the interaction of glutamine synthetase with components P_I and P_{II}. In this rather complex system, involving three macromolecular components and several small molecules which may interact, such a sigmoidal response is not surprising. At the present state of purity of the deadenylylating enzyme system, a more thorough kinetic or structural evaluation of the observed sigmoidal kinetics is premature.

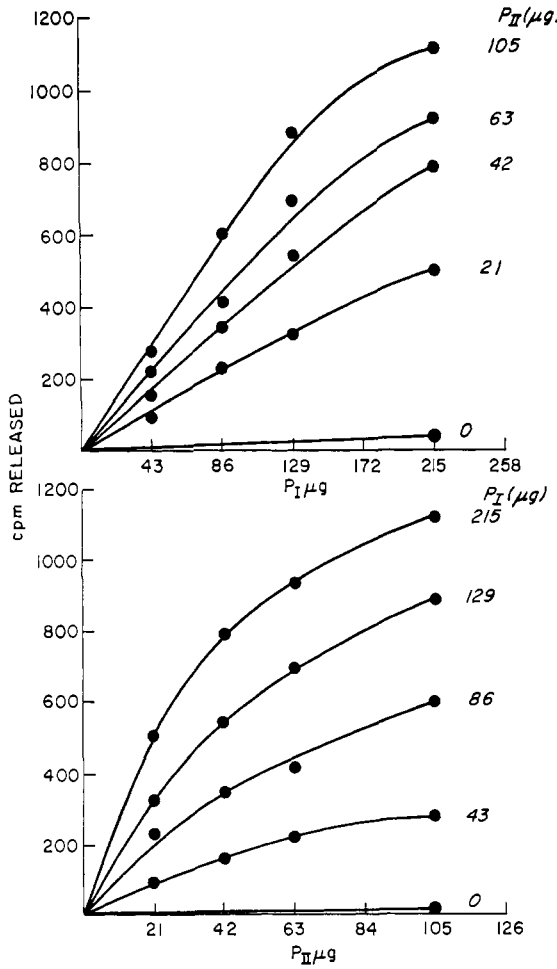


FIGURE 2: Interaction of components P_I and P_{II}. Activity was determined in the standard assay mixture (see Methods) and is reported as counts per minute released in 20-min incubation. The upper figure demonstrates the deadenylylating activity of increasing amounts of P_I in the presence of increasing levels of P_{II}. Conversely, the lower figure illustrates the activity of P_{II}, in the presence of increasing quantities of P_I.

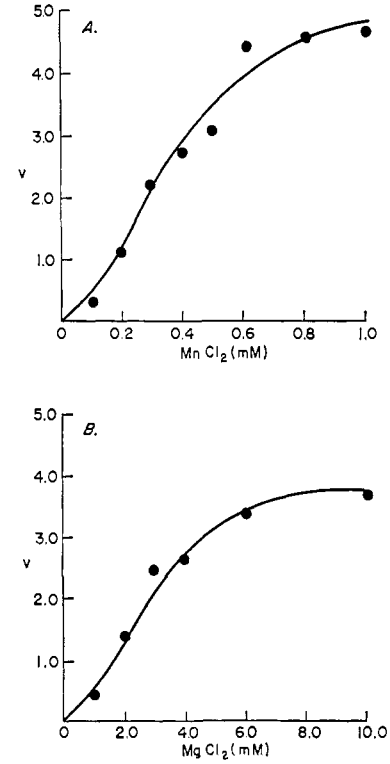


FIGURE 3: Divalent cation requirement for deadenylylation. Activity was determined with the system described in Table II, except that the concentration of divalent cation was varied in the reaction mixture, as noted. (A) Activity in the presence of increasing amounts of $MnCl_2$. (B) Activity in the presence of increasing amounts of $MgCl_2$. The reaction velocity is reported in units of enzyme activity (see Methods).

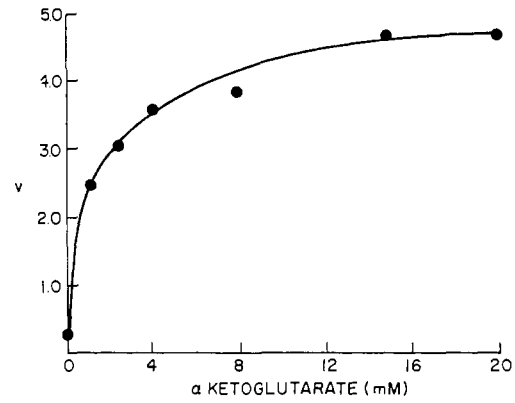


FIGURE 4: α-Ketoglutarate stimulation of the deadenylylating reaction. Activity was determined as in Table II, except that the concentration of α-ketoglutarate was varied as indicated in the figure. Velocity is in units of enzyme activity.

α-Ketoglutarate Stimulation. It was previously reported that α-ketoglutarate stimulated the deadenylylating enzyme considerably (Shapiro and Stadtman, 1967a). Neither glutamate nor α-keto acids other than α-ketoglutarate were effective. In Table II and Figure 4 it is shown that the deadenylylating enzyme system is almost totally inactive in the absence of α-ketoglutarate; the half-maximal stimulatory effect is at 1 mM. The significance of this dependence upon α-ketoglutarate is discussed below.

Anion Effects on the Deadenylylation Reaction. Figure 5 illustrates the effect of increasing concentrations of potassium phosphate on the deadenylylation reaction. It is not clear from this study what the enzyme activity would be in the total absence of phosphate, since it was present in the buffers used to store the deadenylylating enzyme, to enhance its stability. However, phosphate does stimulate the reaction more than twofold under the conditions studied. The requirement for phosphate was not detected until the 30–45% ammonium sulfate fraction of deadenylylating enzyme was assayed, when it was noted that there was an apparent loss in activity that could be restored by adding potassium phosphate to the reaction mixture. All enzyme fractions after this step were stabilized by phosphate.

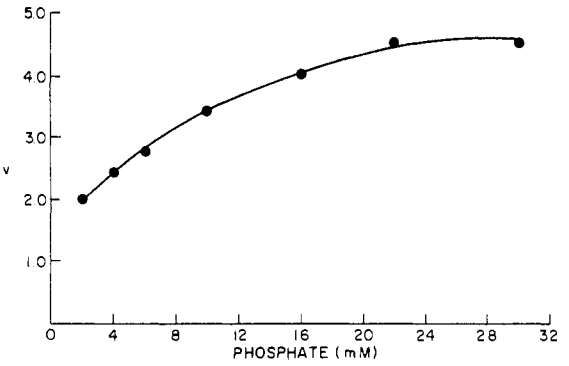


FIGURE 5: Phosphate stimulation of the deadenylylating reaction. Activity was determined as in Table II, except that the concentration of phosphate was varied as indicated. Velocity is in units of enzyme activity.

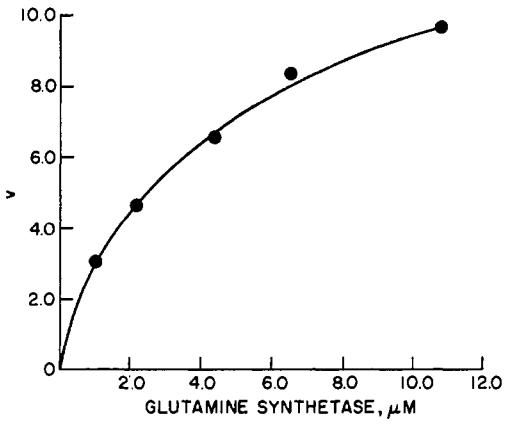


FIGURE 6: Substrate concentration effects upon deadenylylation. Activity was determined as in Table II, except that the concentration of glutamine synthetase concentration is expressed in micromolar subunits, which in this case ($E_{11.8}$) is approximately equivalent to micromoles of adenylyl groups.[1] Velocity is in units of enzyme activity.

Table III illustrates some effects of anions on the deadenylylation reaction. Potassium arsenate is as effective an activator of the reaction as is potassium phosphate. Sulfate is somewhat inhibitory. Sodium chloride and potassium chloride each slightly inhibit the reaction, making it clear that the stimulatory effects of potassium phosphate and arsenate are indeed due to specific effects of the anions. Pyrophosphate has a marked inhibitory effect on the deadenylylation reaction, giving complete inhibition at 20 mM concentration, perhaps due to chelation of Mn^{2+}.

Substrate Concentration Effects. Figure 6 shows the effect on the deadenylylation reaction of increasing the concentration of adenylyl groups. The saturation curve appears hyperbolic, yielding a linear double-reciprocal plot. The apparent K_m for adenylyl groups from such an analysis was 4 μM. Most of the experiments reported in this paper were performed at 2.2 μM adenylyl groups.

pH Optimum of the Deadenylylation Reaction. As shown in Figure 7, the optimal pH for activity is 7.3. The reaction rate has a rather sharp maximum at this pH, decreasing to 75% of its optimal value within 0.5 pH above the maximum or 0.8 pH below it. The specific activity in the mixed buffer system was higher than that observed in the plain imidazole buffer used for all the other studies; the reason for this is not presently clear.

TABLE III: Effects of Anions on the Deadenylylation Reaction.[a]

Compound Added	Act. (units)
None	2.2
Potassium phosphate	4.6
Potassium arsenate	4.7
Potassium sulfate	1.5
Sodium pyrophosphate	0.0
NaCl	1.7
KCl	1.9

[a] The reaction conditions were as in the legend to Table II, except that potassium phosphate was either omitted from the reaction mixture or was replaced by one of the compounds mentioned above. All compounds were tested at 20 mM.

Inhibition of Deadenylylation by Glutamine and Glutamate. Glutamine inhibits the deadenylylation reaction almost completely, with 50% inhibition occurring at 0.25 mM glutamine (Figure 8). Glutamate is also an inhibitor; 0.5 mM gives about 60% inhibition of deadenylylation (W. Anderson, unpublished data). The possible physiologic significance of these inhibitions, as well as the activation of deadenylylation seen with α-ketoglutarate, is discussed below.

Nucleotide Activation of Deadenylylation. One of the most interesting features of the deadenylylating reaction is that it is specifically activated by nucleotide fractions, the most active of which are a combination of UTP and ATP. This requirement was discovered during purification of the enzyme, when, after adding protamine sulfate to the crude extract and centrifuging to remove the precipitated material, the supernatant fraction was found to have lost activity. Activity could be restored by readdition of the inactive precipitate (Shapiro and Stadtman, 1968b). Several polyribonucleotide fractions could replace the protamine precipitate as activators of the supernatant fraction; the most active of these were rRNA and mixed whole cell RNA preparations. Purified tRNA preparations had no ability to restore activity, nor did any of the homoribopolymers, aside from poly U. UTP, ATP, and CTP were also active in stimulating deadenylylation, but their monophosphate and diphosphate analogs were less effective.

With further purification of the deadenylylating enzyme, by ammonium sulfate fractionation, it became obvious that a combination of either UTP and CTP or UTP and ATP was the most effective activator of the reaction, with either combination increasing the deadenylylating activity some 15–20-fold (Stadtman *et al.*, 1968a). The only polynucleotide fractions effective in

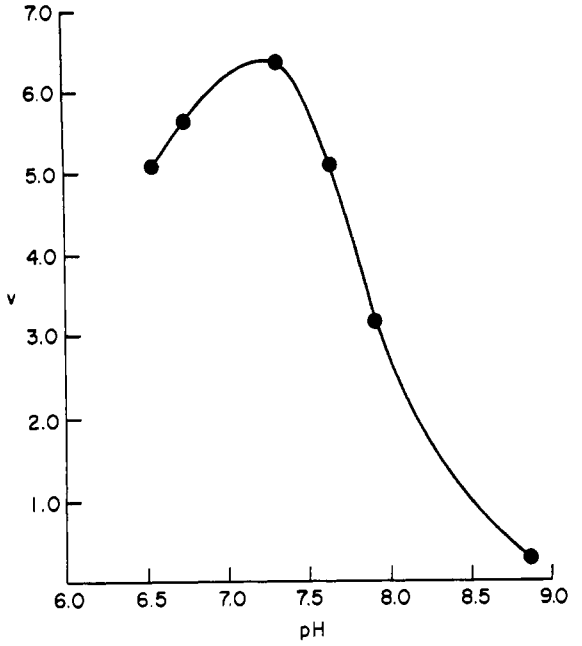


FIGURE 7: Effect of pH on the activity of the deadenylylating enzyme system. Activity was determined as in Table II, except that a mixed buffer (imidazole, 2-methylimidazole, and 2,4-dimethylimidazole) was used instead of imidazole alone. The concentration of each buffer component was 0.1 M. The pH of the reaction mixture is that measured at 25°, before the addition of the protein components to initiate the deadenylylation reaction. Velocity is expressed in units of enzyme activity.

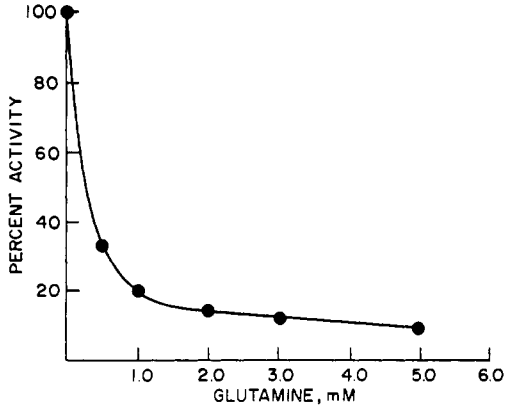


FIGURE 8: Glutamine inhibition of the deadenylylating reaction. The assay conditions were as in Table II, with the addition of glutamine at the concentrations indicated above.

promoting deadenylylation catalyzed by the more highly purified enzyme were poly U and rRNA preparations.

The nucleotide stimulatory effects were evaluated using fractions P_I and P_{II}, which are more than 20-fold purified. Figure 9 illustrates the stimulation of the reaction by UTP and ATP, alone or in concert. In Figure 9A (closed circles) is shown the effect of UTP added to the reaction in the absence of ATP; the saturation curve

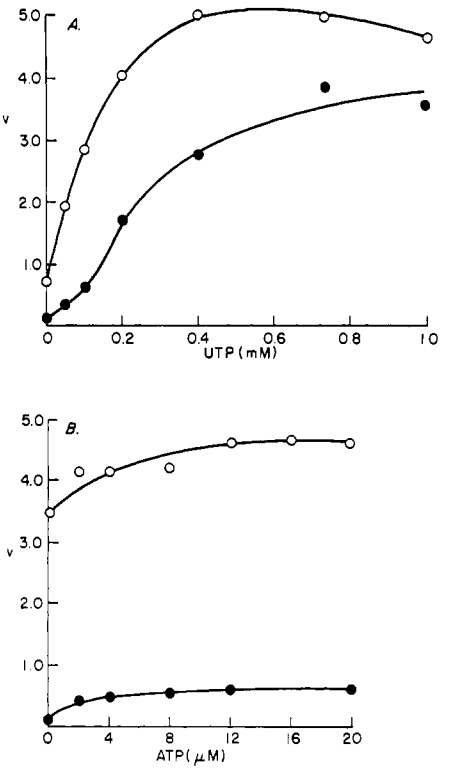


FIGURE 9: Stimulation of deadenylylation by UTP and ATP. The assay conditions were as in Table II, except that the concentrations of nucleotides were varied as shown. (A) Effect of increasing concentrations of UTP in the absence (●——●) or in the presence (○——○) of 20 μM ATP. (B) Effect of increasing concentrations of ATP in the absence (●——●) or in the presence (○——○) of 1.0 mM UTP.

is sigmoidal. When a saturating concentration of ATP is present, however, the shape of the saturation curve for UTP changes significantly (Figure 9A, open circles). Under these conditions, UTP is effective in activating the reaction at lower concentrations. Figure 9B shows the effects of increasing concentrations of ATP, either by itself (closed circles), or in the presence of a saturating amount of UTP (open circles). The data in this figure clearly illustrate that ATP is rather ineffective either by itself or in the presence of saturating concentrations of UTP. The most marked effect of ATP is seen when UTP is present at intermediate levels (Figure 9A), and the response of the deadenylylating enzyme to the combination of nucleotides is synergistic under these conditions. This fact is also made apparent in Figure 10 where a saturation curve for ATP is shown at an intermediate concentration of UTP (0.25 mM). It is also of interest that the ATP effect is seen at about two orders of magnitude lower concentration than is the UTP effect, as is evident from Figures 9 and 10.

The capacity of various nucleotides, when tested individually, to stimulate the deadenylylation reaction is shown in Table IV. All of the compounds were examined at concentrations where UTP had about half of its maximal effect. UTP is the only uridine derivative

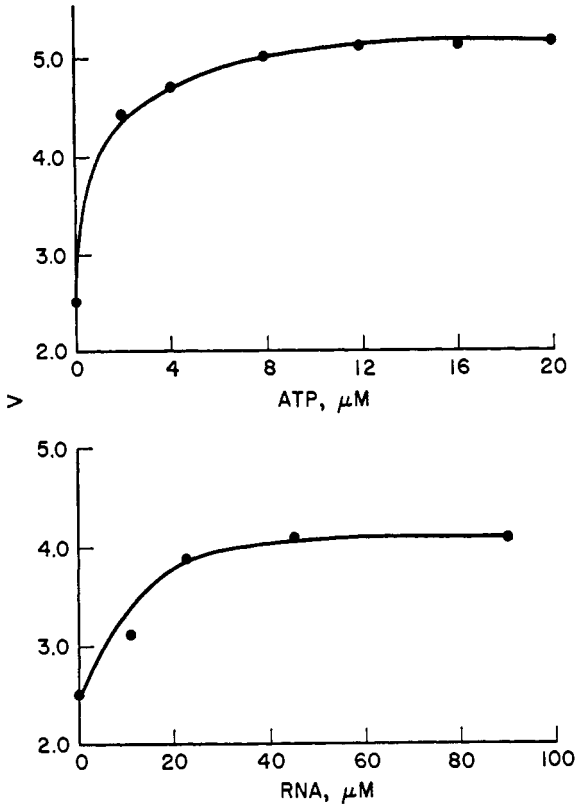


FIGURE 10: Synergistic effects of UTP and either ATP or RNA. The assay conditions were as in Table II, except that the UTP concentration was 0.25 mM, and ATP was varied in concentration as shown (upper curve); or ATP was omitted, and the concentration of *E. coli* 16S + 23S rRNA was varied as shown (lower curve).

which activates the reaction, ATP is much less effective, and AMP is almost as active as ATP. Other nucleoside triphosphates have low activity, as do the polynucleotide fractions which had been effective in activating less highly purified deadenylylating enzyme.

The ability of various nucleotides to replace ATP as an activator of deadenylylation in the presence of a fixed level of UTP (0.4 mM) is shown in Table V. All of the mononucleotides thus studied were used at low concentrations, equivalent to those used for ATP, for two reasons: (1) the nucleotide preparations might have had trace contamination by ATP, and any effect seen might have been due to the ATP present; (2) high levels of some nucleotides have been shown to inhibit deadenylylation when added in addition to UTP. As seen in Table V, ATP was the most effective nucleotide studied as the synergistic effector with UTP; ADP was somewhat less effective. Noteworthy is the fact that a mixed 16S plus 23S RNA preparation from *E. coli* was almost as effective as ATP in the synergistic activation of the reaction. The RNA was present at ten times the individual base concentration of ATP (see Methods), so its stimulatory effects could well have been due to contaminating ATP. To test this possibility, the rate of deadenylylation as a function of the concentrations of either ATP or the RNA preparation was examined in the presence of an intermediate concentration of UTP (Figure 10). The synergistic activation of the reaction by ATP and UTP under these conditions is half-maximal at 1 μM ATP; the reaction rate is doubled at saturation. The 16S plus

TABLE IV: Effects of Individual Nucleotides on the Deadenylylation Reaction.[a]

Compound Added	Act. (units)
None	0.0
UTP	2.5
UDP	0.0
UMP	0.0
Uridine	0.0
ATP	0.5
ADP	0.1
AMP	0.4
Adenosine	0.1
ITP	0.2
GTP	0.2
CTP	0.3
E. coli 5S rRNA	0.2
E. coli 16S plus 23S rRNA	0.2
Polyuridylic acid	0.0

[a] The reaction conditions were as in Table II, but both UTP and ATP were omitted from the reaction mixture and replaced by one of the compounds mentioned above. The concentrations of all mononucleotide and nucleosides were 0.4 mM; the polynucleotide preparations were 0.2 mM.

TABLE V: Stimulatory Effects of Various Nucleotides in the Presence of UTP.[a]

Compound Added	Act. (units)	R^b
UTP alone	2.2	1.0
+ATP	3.6	1.6
+ADP	2.8	1.3
+AMP	2.4	1.1
+adenosine	2.1	1.0
+ITP	2.2	1.0
+GTP	2.0	0.9
+CTP	2.2	1.0
+*E. coli* 5S rRNA	2.5	1.1
+*E. coli* 16S plus 23S rRNA	2.9	1.3
+Polyuridylic acid	1.9	0.9

[a] The reaction conditions were as in Table II, except that 0.4 mM UTP was present in all samples; other mononucleotides and nucleosides were 20 μM; polynucleotide preparations were 0.2 mM in individual base equivalent (see Methods). [b] R is defined as the ratio of the activity in the mixture containing UTP to that in the mixture containing UTP and the other mentioned nucleotide.

23S RNA preparation behaves synergistically with UTP, with a half-maximal effect seen at 10 μM nucleotide equivalents. In order for the RNA preparation to be effective only as a result of contaminating ATP, 10% of the nucleotide equivalents of the preparation would have to be ATP, which is unlikely. Other evidence making such contamination unlikely is the observation that stimulation of deadenylylation under these conditions by the RNA preparation is only 65% at saturation, compared with 100% for ATP. Thus, even under saturating conditions there are differences between the two effects. When the RNA effect is considered in terms of molecular equivalents, rather than nucleotide equivalents, the 16S plus 23S RNA preparation is much more active than ATP as a synergist with UTP.

Table VI shows the stimulatory effects of other nucleotide preparations when examined in the presence of 10 μM ATP, at a saturating concentration of this effector. Under these conditions, UTP is the most effective activator of the deadenylylation reaction. None of the other uridine nucleotides has an effect, but uridine itself is somewhat stimulatory. In addition, CTP is the only other nucleoside triphosphate which shows stimulation of the reaction, and the polynucleotide preparations were ineffective.

TABLE VI: Stimulatory Effects of Various Nucleotides in the Presence of ATP.[a]

Compound Added	Act. (units)	R[b]
ATP alone	0.9	1.0
+UTP	5.0	5.5
+UDP	0.4	0.4
+UMP	0.7	0.8
+Uridine	1.5	1.7
+ITP	0.5	0.6
+GTP	0.6	0.7
+CTP	1.7	1.9
+*E. coli* 5S rRNA	0.6	0.7
+*E. coli* 16S plus 23S rRNA	0.6	0.7
+Polyuridylic acid	1.0	1.1

[a] The reaction conditions were as in Table II, except that ATP was 10 μM and the concentrations of the nucleotides and nucleosides were 0.4 mM; the polynucleotide preparations were each 0.2 mM in individual base equivalent (see Methods). [b] R is defined as the ratio of the activity in the mixture containing ATP to that in the mixture containing ATP and the other mentioned nucleotide.

Products of the Deadenylylation Reaction. In the initial studies demonstrating the existence of adenylylated and unadenylylated preparations of glutamine synthetase, it was shown that snake venom phosphodiesterase released 5′-adenylic acid from the adenylylated enzyme preparation (Shapiro *et al.*, 1967). Similar attempts have been made to identify the nucleotide product of the re-

TABLE VII: Product of the Deadenylylation Reaction.

Separation System	Products Found[a] Adenosine	AMP	ADP
Thin-layer chromatography[b]			
D_0 (cm)	9.0	6.3	3.9
Per cent of total counts per minute applied	12	50	38
Thin-layer electrophoresis[c]			
D_0 (cm)	3.7	7.3	1.5
Per cent of total counts per minute applied	10	49	41

[a] Deadenylylation was performed using the conditions described in Methods but 0.32 mg of adenylylated glutamine synthetase and 0.5 mg of the most highly purified fraction of deadenylylating enzyme from Table I were employed, in a final volume of 0.25 ml. The reaction proceeded for 6 hr at which time it was stopped by the addition of 3% perchloric acid and an excess (*ca.* 3 μmoles) of unlabeled adenosine, AMP, ADP, and ATP. The pH of the supernatant solution was brought to 5.0 with 2 N KOH; the solution was cooled to 0°, and the resultant supernatant solution removed from the insoluble $KClO_4$. Sufficient charcoal was added to just adsorb all of the labeled nucleotide, as judged by removal of radioactivity from the supernatant solution after the charcoal was removed by centrifugation. The charcoal containing the adsorbed nucleotides was then washed with water and eluted with three successive washes of 40% acetone containing 0.3% NH_3. The eluate was evaporated to dryness and resuspended in water for the following separation procedures. D_0 is the distance that the nucleotide or nucleoside moved from the origin in each case. Per cent of total refers to the per cent of the counts recovered from the analysis present as each of the three compounds. [b] Thin-layer chromatography was performed on cellulose-coated film, in a solvent system composed of *t*-amyl alcohol–formic acid–water (3:2:1). The solvent front had moved 13 cm at the end of the chromatography. [c] Thin-layer electrophoresis was performed on polyethylenimine-coated film which was washed with water and dried before use. Samples were placed 5 cm from one end of the polyethylenimine sheets (20 × 20 cm), then 4% formic acid was applied with a brush to the sheet, care being taken not to touch the origin with the brush; the origin is moistened by capillarity. The sheet was then placed in an electrophoresis tank, contact was made with 4% formic acid placed in the anodal and cathodal buffer chambers, by means of filter paper wicks, and the electrophoresis begun. Electrophoresis was for 3 hr at 475 V, 20 mA. The nucleotides all migrated toward the anode.

action catalyzed by the *E. coli* deadenylylating enzyme system. Table VII shows the results of such an analysis. The only radioactive products found were adenosine, AMP, and ADP, and these accounted for more than 90% of the radioactive preparation placed on the thin-layer sheets for subsequent separation. Adenosine accounted for only 10% of the radioactive material, whereas AMP and ADP were present in approximately equal amounts. In other experiments, that will not be reported in detail, it was found that if the deadenylylating reaction was carried out in the presence of free tritium-labeled AMP, the ADP formed contained both ^{14}C and ^{3}H. Similarly, in the presence of added tritium-labeled ADP, the AMP produced contained both isotopes. These results indicate that the adenine nucleotides are interconvertible, probably through the action of a contaminating adenylylate kinase. Thus, with the interconversion of AMP and ADP which is seen, it is impossible to state unequivocally that AMP is the primary product of deadenylylation catalyzed by the *E. coli* enzyme; the possibility that ADP is the primary product which is then degraded to AMP has not been excluded.

The protein product of the deadenylylation reaction has been characterized previously (Shapiro and Stadtman, 1968a) as behaving like unadenylylated glutamine synthetase in that it is relatively inactive with Mn^{2+}, much more active with Mg^{2+}, and less susceptible to inhibition by products of glutamine metabolism than is the adenylylated enzyme.

Role of P_I and P_{II} in the Deadenylylation Reaction. The UTP, ATP, or α-ketoglutarate which is required to deadenylylate glutamine synthetase could be activating an enzyme component in fractions P_I, P_{II}, or both. To examine this possibility, these fractions were incubated together, in the presence of the complete reaction mixture, but without any adenylylated glutamine synthetase. After this preincubation period, the mixture was diluted into a reaction mixture which was either complete, or lacking ATP, UTP, or α-ketoglutarate. If the two proteins could interact to form an activated species, then the extent of deadenylylation seen with the activated fractions would be expected to be greater than in an unactivated preparation. Likewise, if this activation required a component of the reaction mixture which was no longer needed by activated enzyme, there should be no requirement for this component with activated material. A control was run with preincubation in water alone with no components of the reaction mixture other than the phosphate and dithiothreitol of the enzyme buffer. As shown in Table VIII there was no significant difference seen in the activity of the two preincubated samples in the presence of the complete reaction mixture. The only differences seen in the presence of reaction mixtures which lacked a component was that caused by the fraction of that component carried over by the enzyme when it was diluted from the preincubation mixture into the assay mixture. Thus, from these experiments it does not appear that there is a stable activated enzyme form that is responsible for deadenylylation. The possibility has not been excluded that activation of a fraction lasts for only relatively few enzymatic events, with subsequent decay to an unactivated state.

TABLE VIII: The Effect of Preincubating Fractions P_I and P_{II} with the Deadenylylating Reaction Mixture.[a]

	Assay			
Preincubation	−α-KG	−UTP	−ATP	Complete
Reaction mixture	32	15	69	81
Control	7	10	64	76

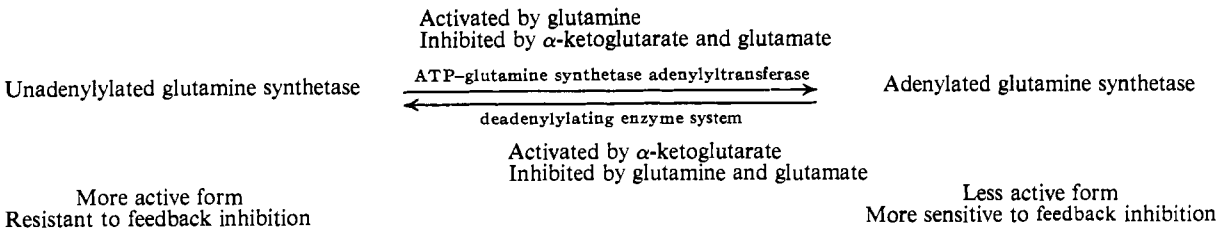
[a] The reaction mixture used for preincubation of fractions P_I and P_{II} had the composition of the complete assay mixture described in Methods, except that it lacked adenylylated glutamine synthetase. Fractions P_I (0.21 mg) and P_{II} (0.1 mg) were incubated in 30 μl of this mixture for 50 min at 37°; a control had the same quantities of enzyme fractions preincubated in water (30 μl). At the end of the preliminary incubation, 6 μl of each fraction was taken for assay under each of the conditions listed in the columns above; that is, either in the complete assay mixture (as in Methods), or in an assay mixture lacking one of the components shown. In each case, the assay mixtures contained the substrate, adenylylated glutamine synthetase. Activity is reported as units per 70 min. α-KG = α-ketoglutarate.

Discussion

Hydrolysis of the 5′-adenylyl-*O*-tyrosyl residue of glutamine synthetase to release AMP may be effected by a phosphodiesterase preparation from snake venom (Shapiro *et al.*, 1967), which requires only divalent ion for activity. Yet, to achieve the same end, *E. coli* has elaborated a complex deadenylylation system, requiring at least two macromolecular components (aside from adenylylated glutamine synthetase) and several cofactors, which include α-ketoglutarate, phosphate, and nucleotides, in addition to a divalent cation. With this system, the microorganism has many degrees of freedom by which it may control the deadenylylation reaction, and thereby the nature of the glutamine synthetase preparation present in its cell.

The discovery of two enzyme components required for the deadenylylation of glutamine synthetase helps to explain some of the difficulties that were found during attempts to establish a purification procedure. Hopefully, by reexamining some of the previously unsuccessful procedures, and using either component to assay for the other, a purification scheme leading to homogeneous preparations of P_I and P_{II} can be devised. Such preparations will be needed to ascertain the mechanism of the interaction of P_I and P_{II} with glutamine synthetase, as well as to study the relationship of the cofactors to the various macromolecular components involved in the deadenylylation reaction. For example, the relationship of glutamine, ATP, divalent cation, and sulfhydryl reagents to the structure and function of glutamine synthetase has been well documented (Stadtman *et al.*, 1968a,b); thus, it is impossible to tell at present whether these effectors interact with glutamine synthetase or

SCHEME I



with the components of the deadenylylating enzyme. For similar reasons, as discussed above, it is impossible to make any statement about the possible structural correlates of the sigmoidal curves which were found with several of the cofactors of the reaction.

At this stage of purification, however, certain features of the reaction are clear. There is a definite synergistic stimulation of deadenylylation by UTP and ATP, or by UTP and a rRNA fraction. Whether the rRNA effect is specific for the 16S plus 23S preparation examined must be more thoroughly investigated with RNA preparations of known purity. The existence of a specific RNA species capable of interacting with an enzyme not directly concerned with its metabolism would be of fundamental significance in any consideration of cellular organization and control systems. For this reason, the RNA stimulatory effect will be pursued by using more highly purified preparations of both the enzyme components and the polynucleotide fractions.

When ATP and UTP are provided in the deadenylylating reaction mixture, NaF fails to stimulate deadenylylation in any fractions of enzyme beyond the crude extract stage. The previously observed stimulation by fluoride was therefore probably only due to its inhibition of nucleoside triphosphatase activities, thereby maintaining the endogenous concentrations of nucleoside triphosphates needed for the activation of the deadenylylation system. The finding that poly U and some other polyribonucleotide fractions could stimulate deadenylylation in less purified material, but were unable to stimulate the reaction using more purified enzyme preparations, suggests that the activity of these polynucleotide preparations was dependent upon their conversion into nucleoside triphosphates by contaminating enzymes in the crude deadenylylating enzyme preparations. On the other hand, the fact that 16S plus 23S rRNA was also active in stimulating the more highly purified enzyme preparations studied here, as a synergist with UTP, indicates that the RNA may also have a specific effect.

Nitrogen starvation and nitrogen excess represent the two extremes of the total spectrum of nitrogen nutrition. The maintenance of optimal cellular growth and development depends upon effective utilization of nitrogenous metabolites in both cases. Glutamine is an important intermediate in the biosyntheses of several critical nitrogen metabolites, among which are histidine, tryptophan, carbamyl phosphate, glucosamine 6-phosphate, AMP, and CTP (Stadtman *et al.*, 1968b). In nitrogen starvation, it is obvious that there would be a relative decrease in the intracellular concentrations of glutamine and its metabolic products, with a probable corresponding increase in the relative concentration of α-ketoglutarate, the carbohydrate skeleton of glutamine. It can thus be postulated that the nitrogen saturation ratio, defined as (glutamine + glutamine product)/α-ketoglutarate, would be low. In the contrasting situation, with nitrogen excess, there should be a plethora of nitrogenous metabolites. If these are present in excess, they would decrease their own production, by means of the many feedback controls effective in biosynthetic reactions (Stadtman, 1966). This decrease in synthesis of products of glutamine would lead to a corresponding increase in the concentration of glutamine, and a high nitrogen saturation ratio. The concept of a nitrogen saturation ratio is formally analagous to the adenylate charge ratio of Atkinson and Walton (1967). Like the adenylate charge concept, it stresses the importance of *relative* concentrations of intermediary metabolites as effectors of cellular control, rather than *absolute* concentrations of one or several compounds.

E. coli has evolved a system for the regulation of its nitrogen metabolism that is very sensitive to the nitrogen saturation ratio, the existence of adenylylated and unadenylylated forms of glutamine synthetase, with markedly different properties, which are enzymatically interconvertible. Scheme I shows the two forms of the enzyme, characteristic properties of each, and the enzymes which change one to the other, with their respective metabolic effectors. It is clear from this scheme that conditions of nitrogen privation, where the nitrogen saturation ratio is low, lead to activation of the deadenylylating enzyme and inhibition of the adenylyltransferase. This results in the formation of relatively unadenylylated glutamine synthetase. Since this form of the enzyme is intrinsically more active, as well as being less susceptible to feedback inhibition, ammonia or other nitrogenous compounds should be converted into glutamine, and thus into other nitrogen-containing metabolites, with high efficiency. On the other hand, with high levels of the nitrogen saturation ratio, as might be expected with growth under conditions of nitrogen excess, the deadenylylating enzyme would be inhibited, and the adenylylating enzyme activated. This would result in glutamine synthetase being present in its adenylylated form, which is less active and more susceptible to feedback inhibition by glutamine products (Kingdon *et al.*, 1967). Thus, accumulated nitrogenous metabolites can decrease the synthesis of glutamine even further, by

feedback inhibition, thereby preventing an accumulation of glutamine when it is no longer needed.

The role of glutamate in the regulation of glutamine synthetase activity is enigmatic. As shown in Scheme I, glutamate inhibits both the adenylyltransferase and the deadenylylating enzyme. Thus, relative increases in glutamate would tend to preserve an existing form of glutamine synthetase. Although one can imagine metabolic conditions where such regulation might be beneficial for the cell, a detailed discussion of such specific conditions would not really be germane to the general model presented here. This model suggests that shifts away from nitrogen metabolite homeostasis are reflected in an alteration in the nitrogen saturation ratio, thereby leading to either adenylylation or deadenylylation of glutamine synthetase, in order to return the nitrogen metabolism of *E. coli* to a condition of homeostasis.

Acknowledgments

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