Kinetic Studies of the Mechanism of *S*-Adenosylmethionine Synthetase from Yeast*

Ronald C. Greene

ABSTRACT: Optimal conditions for adenosylmethionine synthesis by yeast adenosylmethionine synthetase are pH 9, 0.1 M K^+, and an excess of 0.005 M Mg^{2+} over that bound as magnesium adenosine triphosphate. At high pH or low ATP, higher concentrations of magnesium are inhibitory but the inhibition is partially overcome by increasing the potassium concentration. Tripolyphosphate hydrolysis is about twice as fast as adenosylmethionine synthesis and its pH *vs.* activity curve is flat between pH 6 and 8.5. The rate of adenosylmethionine synthesis from selenomethionine and its pH *vs.* activity curve resemble those of the tripolyphosphatase activity rather than those of adenosylmethionine synthesis from methionine. The kinetic results of adenosylmethionine synthesis and tripolyphosphate hydrolysis including product inhibition patterns and both stimulation and inhibition of the tripolyphosphatase by adenosylmethionine can be rationalized by a branched model with a single binding site for each substrate or product.

The formation of *S*-adenosylmethionine by transfer of the adenosyl moiety of ATP from tripolyphosphate to the sulfur of methionine is the only known *de novo* biosynthesis of a sulfonium group. The reaction was first discovered in rabbit liver and the products of the reaction were shown to be adenosylmethionine, PP_i, and P_i (Cantoni, 1953). Yeast adenosylmethionine synthetase has been partially purified and its properties have been studied (Mudd and Cantoni, 1958; Mudd, 1963). The enzyme from yeast was reported to have an absolute requirement for a divalent cation which is most effectively filled by Mg^{2+} or Mn^{2+} and for a monovalent cation, with K^+, Rb^+, or NH_4^+ being much more active than Na^+, Li^+, or Cs^+. The concentrations of both cations required for maximal activity were found to be unusually high and incubation solutions were routinely made 0.3 M in each. The enzyme was also said to exhibit a requirement for glutathione. Maximal enzyme activity was attained at pH 7.6 although pH values above 8 were not tested because of the instability of adenosylmethionine in alkaline media. Mudd (1962, 1963) has presented evidence that implicates inorganic tripolyphosphate as an intermediate in the reaction and showed that the enzyme has tripolyphosphatase activity which is stimulated by adenosylmethionine. Mudd and Mann (1963) have calculated several thermodynamic constants of the reaction based on published thermodynamic parameters of related reactions and some kinetic measurements of their own. These workers have postulated a mechanism in which there is a random order of addition of substrates and release of products. This paper reports the results of kinetic measurements of adenosylmethionine synthesis and tripolyphosphate hydrolysis and their implications on possible reaction mechanisms. The terminology used is that of Cleland (1963a). As a prelude to the kinetic work, the effects of various reaction parameters such as pH and cation concentrations were evaluated. It was found that under certain conditions high concentrations of magnesium are inhibitory and that both the pH optimum and the potassium requirement are influenced by the magnesium concentration. Although selection of optimal incubation conditions is somewhat arbitrary under these circumstances, most kinetic experiments have been done at pH 9 in media containing 0.1 M K^+ and a 0.005 M excess of Mg^{2+} over the ATP concentration.

Experimental Section

Pentasodium tripolyphosphate hexahydrate was prepared and recrystallized as described by Van Wazer (1961). Paper electrophoresis showed the tripolyphosphate to be free of other phosphorus-containing compounds. Adenosylmethionine chloride was either obtained from Mycofarm Delft and was repurified by precipitation and regeneration of the reineckate using a procedure similar to that described by Cantoni (1957) or was prepared by a modification of the procedure of Schlenk *et al.* (1959). On paper electrophoresis, the adenosylmethionine gave one spot which was ultraviolet absorbing, ninhydrin reactive, and which also bound phosphomolybdate. ATP-8-^{14}C was obtained from Schwarz BioResearch, Inc., and was 98% pure as judged by chromatography on Dowex 1; Na_2 ATP was obtained from the Sigma Chemical Co. L-Methionine was either Mann Analyzed grade from the Mann Chemical Co. or N.R.C. grade from Calbiochem. All other reagents were the purest available grades from standard commercial sources.

Enzyme Purification. Adenosylmethionine synthetase was prepared by a modification of the method of Mudd (1963). The two procedures are not significantly different through the calcium phosphate gel step. In the modified procedure the dialyzed calcium phosphate gel eluate (pH 6.3, 0.02 M potassium phosphate; protein content 6–8 mg/ml) is placed in an ice bath on a magnetic stirrer and solid bentonite (30 mg/ml) is added with rapid stirring. If necessary, the suspension is homogenized to remove lumps and the mixture is stirred for 20 min. The bentonite is removed by centrifugation at 20,000*g*

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for 30 min. The bentonite is discarded and the supernatant liquid is adjusted to pH 5.2 (4°) by the dropwise addition of 0.25 N acetic acid. The enzyme is unstable at this stage, so as rapidly as possible bentonite (4 mg/ml) is added with stirring and homogenization if necessary to remove lumps. Stirring is continued for 20 min and the bentonite is recovered by centrifugation for 30 min at 20,000*g*. The supernatant liquid is discarded and the bentonite is suspended in 0.02 M Tris free base–0.001 M Na_2EDTA (one-tenth of the volume of the pH 5.2 solution treated with bentonite). The suspension is stirred for 30 min in an ice bath and the bentonite is removed by centrifugation at 40,000 rpm for 30 min in a Spinco Model L centrifuge, a procedure which is necessary to sediment colloidal particles of bentonite. The eluate is adjusted to pH 7.6 with 0.25 N acetic acid and the protein is precipitated by addition of solid ammonium sulfate (0.58 g/ml). The ammonium sulfate precipitate is dissolved in a minimal volume of 0.02 M Tris-HCl–0.001 M EDTA (pH 7.6) and chromatographed on a 1.9 × 50 cm column of Sephadex G-100. The active fractions can be stored frozen at −15° without appreciable loss of activity for several months. The specific activity of the enzyme at this state varies between 6 and 11 units per mg of protein. The purer preparations (~11 units/mg) showed a single symmetrical peak on sedimentation in the analytical ultracentrifuge and chromatography on DEAE-Sephadex or hydroxylapatite did not result in significant increase in specific activity. When tested by disc gel electrophoresis, the enzyme showed one major band and, in various preparations, two to four much smaller bands. The enzyme activity has been shown to be associated with the major band.

Enzyme Assays. ROUTINE ADENOSYLMETHIONINE SYNTHETASE ASSAY. Determination of enzyme activity is performed by measurement of adenosylmethionine as 260-mμ-absorbing material which is not bound to Dowex 1 at pH 8. The original procedure of Cantoni and Durell (1957) was modified to remove ATP by passing the neutralized samples through 1 × 3 cm columns of Dowex 1 chloride (1 × 4 cm columns of freshly washed Dowex 1 are used when very low blanks are necessary) instead of shaking the samples with a suspension of Dowex 1, since the columns are much more effective for removing the last traces of ATP. The procedure used is as follows: after incubation of reaction mixtures (which vary in composition according to the particular experiment) for the desired length of time, the reactions are stopped by the addition of 1 ml of 6% $HClO_4$ to each tube. The precipitated protein and $KClO_4$ are removed by centrifugation and a 1-ml aliquot of each supernatant is added to 10 ml of 0.015 M Tris (pH 7.4) containing 2 drops of bromothymol blue, which is then adjusted to pH 7.5–8 with 1 N NaOH. The neutralized solution is poured through a Dowex 1 column, and the tube and column are then successively washed with two 5-ml aliquots of water and the combined column effluent and washes are made up to 25 ml with water and mixed. The absorbancy of the effluent is measured at 260 mμ using a cuvet with a 1-cm light path or one with a 10-cm light path, depending upon the sensitivity required. An activity determination includes one or more blanks incubated under exactly the same conditions as the experimentals but without the addition of methionine. After purification of the enzyme through the bentonite step, blanks are essentially zero and their ultraviolet absorption is that of the reagents (primarily material shed from the Dowex column). In some more complex experiments, when enzyme of sufficient purity is used, reaction mixtures containing methionine to which $HClO_4$ is added before enzyme, are used as blanks.

Incubation Procedure for Kinetics of Adenosylmethionine Synthesis. Incubation mixtures containing appropriate quantities of reactants in a volume of 0.9 ml are preincubated for 5-min at 37°, then at 30-sec intervals 0.1-ml aliquots of enzyme are added to each tube. After 10-min incubation, 1-ml aliquots of 6% $HClO_4$ are added to each tube also at 30-sec intervals. The adenosylmethionine content of the $HClO_4$ supernatants was measured as described above using 10-cm light-path cuvets. When adenosylmethionine inhibition was measured, this procedure could not be used since the amount of adenosylmethionine synthesized was small compared with the amount added as an inhibitor. In these experiments, ATP-8-^{14}C was used as the substrate and the assay and chromatography procedure was carried out in the same manner as above except that instead of measuring the absorbance of the column effluent 10-ml aliquots were counted by emulsification with Triton X-100 and scintillation solution (Patterson and Greene, 1965).

TRIPOLYPHOSPHATASE ASSAY. For the kinetic experiments incubations were carried out for 10 min at 37° in 2 ml of medium containing 0.09 M Tris–0.09 M histidine (pH 9), 0.1 M KCl, 0.005 M mercaptoethanol, and 0.005 M $MgCl_2$ in excess of the polyphosphate concentration. Except for the substrates this is the same medium defined as optimal in the kinetic studies of adenosylmethionine synthesis. Other incubation conditions were used as indicated. Phosphate determinations were carried out by a procedure similar to that of Martin and Doty (1949), as modified by Gibbs *et al.* (1965). In the kinetic experiments, where high sensitivity is required in order to measure reaction rates without depletion of substrate, 1 ml of molybdate reagent (4 N H_2SO_4, 4% ammonium molybdate, and 0.02 M silicotungstate) is added to the entire incubation mixture (2 ml), the solution is immediately mixed, extracted with 2 ml of isobutyl alcohol, and stored in ice. As soon as possible after extraction (within 20 min), the phases are separated by centrifugation in the cold and a 1-ml aliquot of the isobutyl alcohol phase is taken for color development; 1 ml of 1 N H_2SO_4 in absolute ethanol is added to the aliquot of isobutyl alcohol and mixed, then 0.2 ml of 0.125% $SnCl_2$ in 1 N H_2SO_4 is added; the solution is mixed, and its absorbance is measured at 725 mμ (100 mμmoles of P_i gives an absorbance of 0.58). In experiments where less sensitivity is required, smaller aliquots are analyzed. All assay procedures used in the kinetic experiments were linear for 15 min and some showed slight deviation from linearity at 20 min. Thus the 10-min incubation periods yield reasonable approximations of initial rates for computation of kinetic parameters.

Calculation of Data. Kinetic constants were computed from experimental data with the IBM 7072 computer of the Duke University Computing Laboratory using programs written by Cleland (1963b) of the University of Wisconsin and kindly supplied by him to Dr. B. Bulos of the Biochemistry Department at Duke University. In the plots of the kinetic data shown in this paper, the points are averages of experimental data and the lines are computed from the data using the programs mentioned above.

Results

pH Optimum. In earlier studies of the pH optimum of yeast

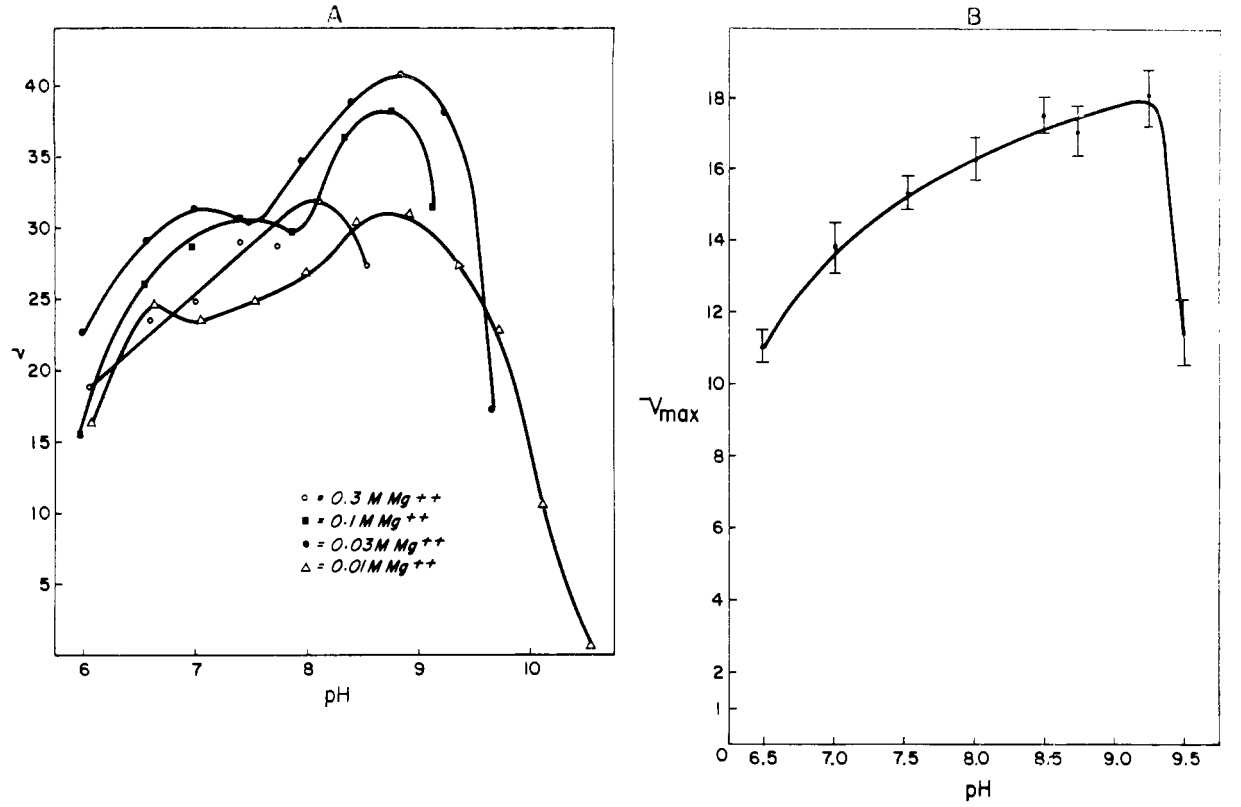

FIGURE 1: Adenosylmethionine synthesis. (A) Rate of adenosylmethionine synthesis, in millimicromoles per minute *vs.* pH, at several magnesium concentrations. Incubation mixtures contain 0.02 M ATP, 0.02 M methionine, 0.3 M KCl, 0.005 M mercaptoethanol, $MgCl_2$ as indicated, 0.09 M Tris, 0.09 M histidine, and 0.09 M triethylamine; buffer was adjusted with HCl or KOH. pH measurements were made on aliquots of reaction mixtures at 37°. (B) Maximal velocities of adenosylmethionine synthesis in millimicromoles per minute *vs.* pH. Incubation mixtures contained Tris–histidine buffer (0.09 M each) adjusted with NaOH or HCl, 0.005 M excess $MgCl_2$, 0.1 M KCl, and 0.005 M mercaptoethanol; indicated pH values were measured on aliquots of reaction mixtures at 37°. Maximal velocities computed by the sequential initial velocity program obtained from Cleland. The vertical bars indicate the standard error of each point.

adenosylmethionine synthetase (Mudd and Cantoni, 1958) activity measurements above pH 8 were not made because of the instability of adenosylmethionine in alkaline media. Although adenosylmethionine is degraded to adenine and ribosylmethionine under mild alkaline conditions (Parks and Schlenk, 1958), the assay procedure used here would measure the adenine as adenosylmethionine with relatively little error due to differences in molar absorbancy. (In Tris at pH 7.5 the absorption of adenine is approximately 85% as great as that of adenosylmethionine.) Experiments in which adenosylmethionine is separated from adenine and adenine nucleotides by use of Dowex 50 have shown that incubation for 30 min at 37° and pH 9 does not result in significant degradation of adenosylmethionine. Figure 1A shows the effects of pH on adenosylmethionine synthetase activity at various magnesium concentrations. In those media where the magnesium concentration exceeds that of ATP, pH values higher than those shown in Figure 1A cannot be tested due to precipitation of magnesium hydroxide–ATP complexes. At the highest magnesium concentration, the same as that used by Mudd and Cantoni (1958), a broad curve with a peak near pH 8 is obtained. However, at lower magnesium concentrations where activity may be measured at higher pH values an activity peak at pH 9 is revealed. Figure 1B is a plot of V_{max} *vs.* pH with a magnesium concentration 0.005 M greater than the ATP concentration. The maximal velocity of adenosylmethionine formation increases slowly from pH 6.5 to 9.2 and then decreases precipitously at pH 9.5. Attempts to measure reaction rates in media of comparable composition at pH 9.8 did not yield reproducible results because of precipitate formation, but the velocity of the reaction was considerably lower than at pH 9.5.

As illustrated in Figure 2 the rate of tripolyphosphate hydrolysis is insensitive to changes in pH between pH 6 and 8.5 with activity falling to about half-maximal value at pH 5 and 9.5. This curve shows no indication of a peak at pH 9 such as those seen in the curves of Figure 1A. If selenomethionine is used as a substrate in the adenosylmethionine synthetic reaction, the pH *vs.* activity curve is virtually identical with that for the tripolyphosphatase activity. The rates of tripolyphosphate hydrolysis and adenosylmethionine synthetase from selenomethionine are the same and both are approximately 1.8 times faster than the rate of adenosylmethionine formation from methionine at pH 7.6. Mudd and Mann (1963) have reported a similar difference in the rates of the tripolyphosphatase and adenosylmethionine synthetase reactions.

Cation Requirements. Since the pH *vs.* activity curves of Figure 1A suggested that excess magnesium may be inhibitory at pH 9, the cation requirements of the adenosylmethionine synthetase activity at this pH were evaluated. As shown in Figure 3A with increasing concentrations of magnesium the enzyme activity rises, passes through a maximum and then falls. Changing the potassium concentrations from 0.001 to 0.005 M causes an increase in enzyme activity at all magnesium concentrations. Further increase in potassium has little effect

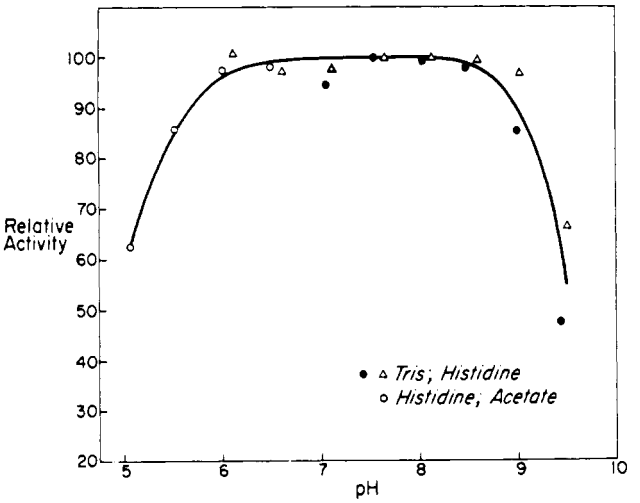

FIGURE 2: Effect of pH on tripolyphosphatase activity. Except for the buffers, incubation media had the standard composition plus 0.001 M PPP_i, 0.00001 M adenosylmethionine, and 0.1 unit of enzyme. Buffers were 0.09 M histidine acetate or 0.09 M histidine,–0.09 M Tris (adjusted with NaOH or HCl). pH measurements were made on aliquots of incubation mixtures at 37°; 100% activity was between 4.2 and 4.4 mμmoles per min in the different experiments.

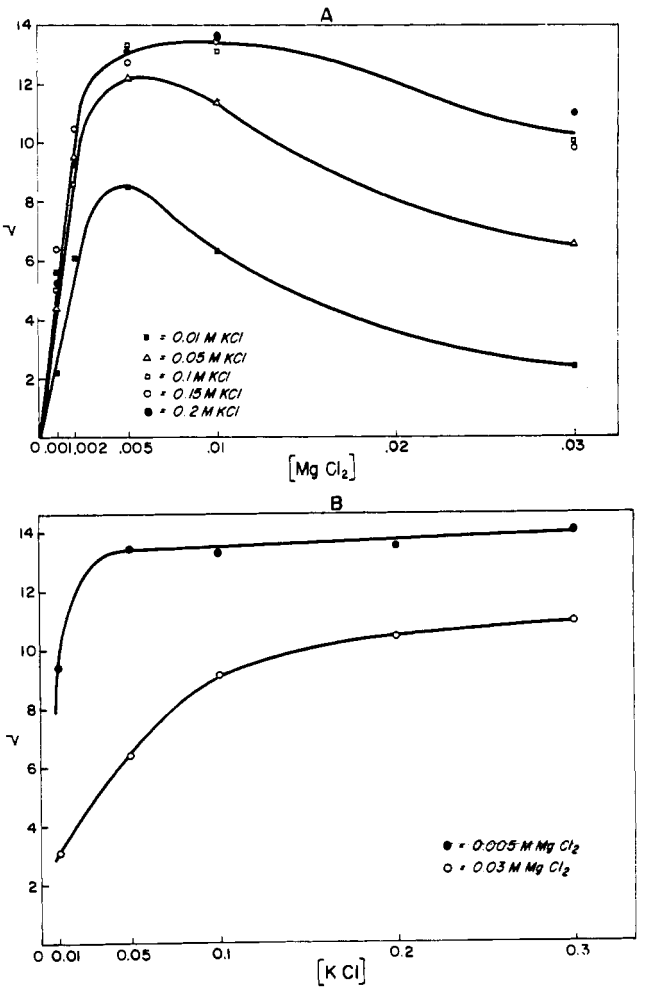

FIGURE 3: Cation requirements. (A) Effect of $MgCl_2$ concentration on the rate (millimicromoles per minute) of adenosylmethionine synthesis at several concentrations of KCl. Incubation mixtures contain pH 9 buffer (0.09 M Tris and 0.09 M histidine adjusted with NaOH), 0.002 M ATP, 0.01 M methionine, 0.005 M mercaptoethanol, and KCl and $MgCl_2$ as indicated. (B) Effect of KCl concentration on the rate (millimicromoles per minute) of adenosylmethionine synthesis at two $MgCl_2$ concentrations. Incubation conditions are the same as those given above.

on the activity at the optimal magnesium concentration, but reduces the inhibition by excess magnesium. Figure 3B shows the effect of potassium concentration on enzyme activity at two concentrations of magnesium. While maximum activity is obtained with 0.005 M KCl in the media with the lower magnesium concentration, higher concentrations of potassium partially reverse, but fail to overcome, the inhibition due to excess magnesium in those reaction media with the higher magnesium concentration. Under the incubation conditions used by Mudd and Cantoni (1958) in their evaluation of the magnesium requirement of adenosylmethionine synthetase (0.15 M Tris, pH 7.6, 0.3 M KCl, 0.002 M ATP, 0.002 M L-methionine, and 0.008 M GSH), magnesium concentrations as high as 0.3 M do not show significant inhibition. However, if the concentration of ATP is lowered, high concentrations of magnesium are inhibitory even in the presence of 0.3 M KCl.

In the lower pH range manganese is almost as effective as magnesium, for example, at pH 7.8 the rate of adenosylmethionine synthesis in media containing manganous ion is 80% as great as that obtained in media with magnesium. At pH 9 manganese is considerably less effective and depending upon the ATP concentration replacement of magnesium with manganese causes a 50–70% reduction in activity. Although manganese alone is relatively inactive at pH 9 in media with a low ATP concentration, addition of small amounts of manganese to such media containing an optimal concentration of magnesium causes a marked increase in enzyme activity (approximately 50%) indicating a synergistic action of these two ions. The enzyme is completely inactive when incubated at pH 9 in media containing beryllium instead of magnesium and addition of beryllium to a series of media containing different concentrations of magnesium neither stimulates nor inhibits enzyme activity.

As has been previously reported (Mudd and Cantoni, 1958) the monovalent cation requirement can be partially fulfilled by sodium or lithium (10–20%); when tested in the presence of limiting potassium (0.005 M), 0.05 M sodium has no effect on the rate of reaction while the same concentration of lithium causes about 50% inhibition. Thus, as long as an adequate amount of potassium is present, sodium compounds (such as Na_2ATP or NaOH for buffer pH adjustment) can be used without affecting the rate of the enzyme reaction.

The tripolyphosphatase activity has a magnesium requirement which is similar to that of adenosylmethionine synthetase activity. At pH 9 in the presence of 0.1 M KCl a magnesium concentration greater than the tripolyphosphate concentration is required for maximum activity and excess magnesium is inhibitory. The tripolyphosphatase activity is less dependent upon potassium than is the adenosylmethionine synthetase activity. In incubation media containing 0.005 M Mg^{2+} in excess of ATP or PPP_i at pH 9 adenosylmethionine synthetase activity in the absence of potassium is only 10% of that obtained with 0.1 M potassium, while the omission of potassium causes only a 40% reduction in the tripolyphosphatase activity.

Although the selection of optimal ionic conditions is somewhat arbitrary, a potassium concentration greater than 0.1 M and sufficient magnesium to form a 1:1 complex with the ATP or PPP_i and leave a 0.005 M excess are within the optimal range.

Sulfhydryl Requirement. Omission of a sulfhydryl compound from the incubation medium has no effect on the rate of adenosylmethionine synthetase. This result is in contrast to that of Mudd and Cantoni (1958) who report a requirement for glutathione, but is consistent with the observation that adenosylmethionine synthetase is insensitive to prolonged treatment with sulfhydryl reagents (*p*-mercuribenzoate, iodoacetate).[1] Although they do not appear to be required, sulfhydryl compounds are routinely added to incubation media to avoid formation of methionine sulfoxide.

Affect of Fluoride on Tripolyphosphatase. After the experiments on the kinetics of adenosylmethionine synthetase were partially completed, it was found that the enzyme preparation had a small amount of pyrophosphatase activity (P_i release from 0.001 M PP_i is 0.19 mμmole/min as compared with 4.3 mμmoles/min from 0.001 M PPP_i in the presence of 0.0001 M adenosylmethionine). Since the concentration of pyrophosphate produced during the kinetic incubation is quite low, this amount of contamination does not interfere with the experiments done in the presence of adenosylmethionine. In those experiments without adenosylmethionine, where the tripolyphosphatase activity is greatly reduced, and in the pyrophosphate inhibition experiments, it is necessary to add 0.01 M sodium fluoride which completely inhibits the pyrophosphatase. In an enzyme preparation free of pyrophosphatase, prepared by chromatography on Sephadex G-200, 0.01 M sodium fluoride was shown to have no significant effect on the tripolyphosphatase activity, either in the presence or absence of adenosylmethionine, thus confirming the results of Mudd and Cantoni (1958).

Kinetics of Adenosylmethionine Synthetase. As shown in Figure 4, the lines obtained by plotting the reciprocal of the concentration of one substrate at several concentrations of the other substrate are convergent. The results presented in this figure were obtained at pH 7.6 with 0.3 M $MgCl_2$. Similar plots were also obtained at pH 9 in the presence of 0.03 M Mg^{2+} and at pH 9 with a Mg^{2+} concentration of 0.005 M in excess of the ATP. Such kinetic behavior can be interpreted as evidence for a mechanism where both substrates must bind to the enzyme before any products can be formed (Alberty, 1953), but they give no information about the order of substrate addition or product release. Cleland (1963a) has pointed out that the total rate equation for each sequence of association of substrates and dissociation of products predicts a characteristic product inhibition pattern. The total rate equation for an enzyme reaction with two substrates and three products, which is the inverse of one presented by Cleland (1963a) for a reaction with three substrates and two products, has been derived by the method of King and Altman (1956). As will be shown below the pattern of inhibition by the products can be rationalized by a model in which the binding of ATP precedes that of methionine and the order of product release is orthophosphate, adenosylmethionine, pyrophosphate (ATP, Me: P_i, adenosylmethionine and PP_i). If this sequence of substrate addition is valid it is possible to evaluate the dissociation constant of the enzyme ATP complex ($K_{i\,ATP}$).[2] The kinetic constants of the initial velocity equation have been computed on the assumption that this proposed sequence does prevail. These constants

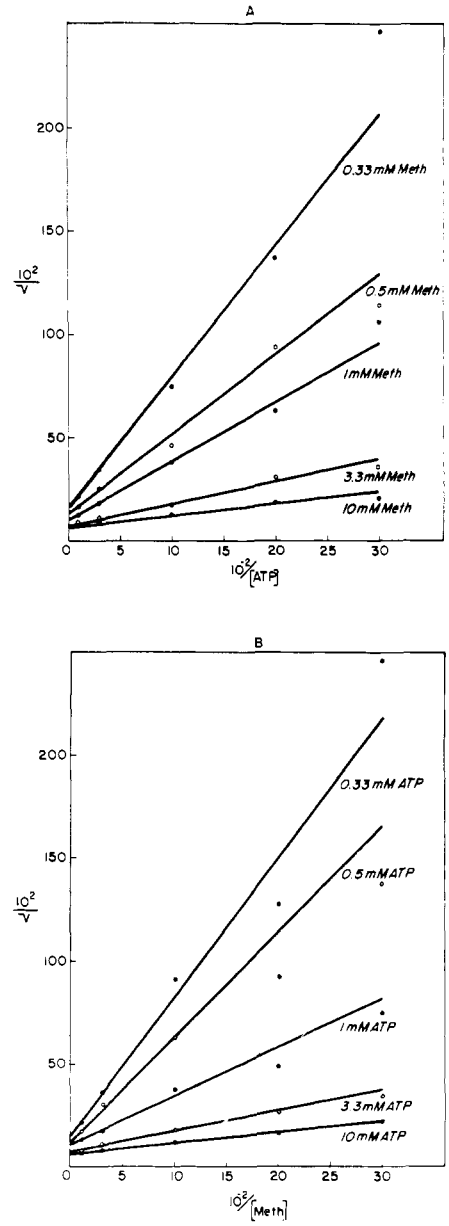

FIGURE 4: Kinetic experiments. (A) Plot of the reciprocal of the velocity of adenosylmethionine synthesis (millimicromoles per minute) *vs.* the reciprocal of the ATP concentration (molar) at several methionine concentrations. Incubation mixtures contain 0.15 M Tris (pH 7.6), 0.3 M $MgCl_2$, 0.3 M KCl, 0.008 M GSH, and ATP and methionine as indicated. Lines computed by the least-square fit to a hyperbola program of Cleland. (B) Plot of the reciprocal of the velocity of adenosylmethionine synthesis (millimicromoles per minute) *vs.* the reciprocal methionine concentration (molar) at several concentrations of ATP. Incubation conditions are the same as those given above.

[1] R. C. Greene, unpublished results.

[2] Abbreviations used are as follows: K_{app}, the apparent value of a Michaelis constant at the concentration of fixed substrate used in the experiment; $K_{I\,sl}$, inhibition constant affecting the slope of the reciprocal plot; $K_{I\,int}$, inhibition constant affecting the intercept of the reciprocal plot.

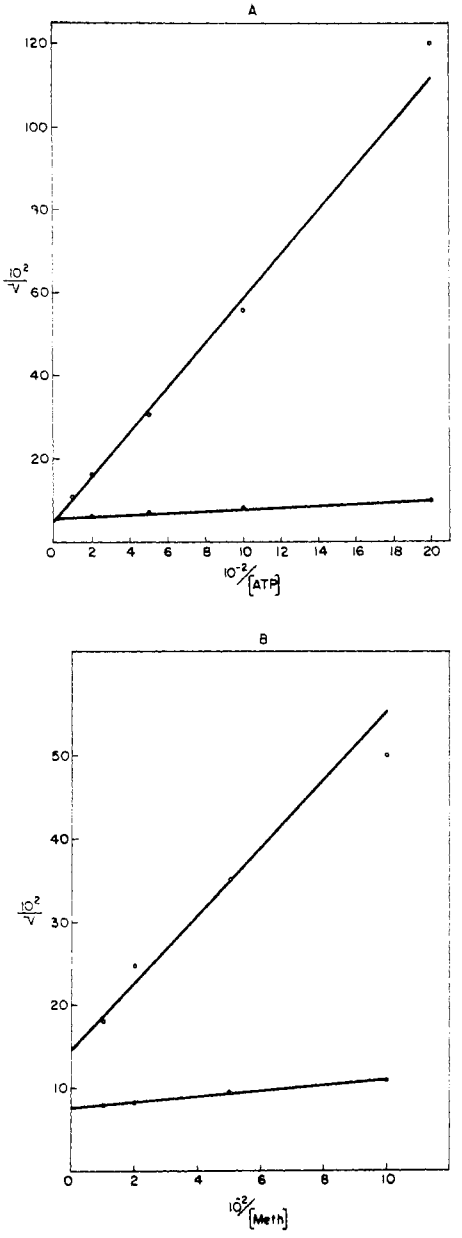

FIGURE 5: Inhibition of adenosylmethionine synthesis by tripolyphosphate. (A) With ATP as the variable substrate. Velocity is expressed in millimicromoles per minute and ATP concentration in moles per liter. Incubation mixtures contained 0.09 M Tris–0.09 M histidine (pH 9), 0.1 M KCl–0.005 M mercaptoethanol, 0.005 M excess $MgCl_2$, 0.01 M methionine, and ATP as indicated. The upper curve is from mixtures which also contained 0.0003 M PPP_i. Kinetic parameters are: V_{max} = 18.0 ± 0.3 mμmoles/min, $K_{ATP\ app}$ = (4.0 ± 0.3) × 10^{-4} M, and $K_{I\ sl}$ = (1.4 ± 0.1) × 10^{-5} M. (B) With methionine as the variable substrate. Velocity is expressed in millimicromoles per minute and methionine concentration in moles per liter. Incubation mixtures contained 0.09 M Tris–0.09 M histidine (pH 9), 0.1 M KCl, 0.005 M mercaptoethanol, 0.005 M excess $MgCl_2$, 0.005 M ATP, and methionine as indicated. The upper curve is from mixtures which also contained 0.0003 M PPP_i. Kinetic parameters are: V_{max} = 13.2 ± 0.2 mμmoles/min; $K_{Me\ app}$ = (4.4 ± 0.4) × 10^{-4} M, $K_{I\ sl}$ = (2.7 ± 0.4) × 10^{-5} M, $K_{I\ int}$ = (3.3 ± 0.4) × 10^{-4} M.

measured under three sets of experimental conditions are given in Table I. The Michaelis constants for ATP and methionine are considerably lower than those reported by Mudd (1963) under all conditions tested. The dissociation constant of the enzyme ATP complex $K_{i\ ATP}$ is in each case considerably larger than the Michaelis constant.

TABLE I: Kinetic Constants for Adenosylmethionine Synthetase.

A. 0.15 M Tris (pH 7.6), 0.3 M $MgCl_2$, 0.3 M KCl, and 0.008 M GSH
V_{max} = 17.0 ± 0.3 mμmoles/min
K_{ATP} = (6.2 ± 0.6) × 10^{-4} M
K_{Me} = (4.5 ± 0.6) × 10^{-4} M
$K_{i\ ATP}$ = (8.9 ± 1.5) ± 10^{-3} M

B. 0.09 M Tris–0.09 M histidine (pH 9), 0.03 M $MgCl_2$, 0.05 M KCl, and 0.005 M mercaptoethanol
V_{max} = 16.9 ± 0.5 mμmoles/min
K_{ATP} = (4.8 ± 0.5) × 10^{-4} M
K_{Me} = (1.4 ± 0.2) × 10^{-3} M
$K_{i\ ATP}$ = (1.3 ± 0.3) × 10^{-3} M

C. 0.09 M Tris–0.09 M histidine (pH 9), 0.005 M $MgCl_2$ in excess of ATP, 0.1 M KCl, and 0.005 M mercaptoethanol
V_{max} = 16.7 ± 0.3 mμmoles/min
K_{ATP} = (2.8 ± 0.2) × 10^{-4} M
K_{Me} = (3.1 ± 0.3) × 10^{-4} M
$K_{i\ ATP}$ = (1.0 ± 0.2) × 10^{-3} M

Figure 5 shows the effect of tripolyphosphate on the rate of adenosylmethionine synthesis when either ATP or methionine is the variable substrate. The kinetic constants computed from these data and their standard errors are given in the legend of Figure 5. When the concentration of methionine is held constant and that of ATP is varied, tripolyphosphate shows competitive inhibition (Figure 5A). In the inverse situation where ATP is held constant and methionine is varied the inhibition is noncompetitive (Figure 5B). As shown in Figure 6, pyrophosphate exhibits the same pattern of inhibition as tripolyphosphate.

Inhibition of the enzyme by adenosylmethionine is shown in Figure 7. Since rather high concentrations of adenosylmethionine must be added to the incubation mixtures to obtain significant inhibition, reaction rates were determined by measuring the incorporation of radioactive ATP into adenosylmethionine. As can be seen from these plots, adenosylmethionine is an uncompetitive inhibitor when either ATP or methionine is the variable substrate. The values of the inhibition constants measured in either type of experiment are approximately the same.

The inhibition of the enzyme by low concentrations of orthophosphate (0.01 M) is too small to be accurately measured. In the reaction media used here, it is not possible to incorporate much greater amounts of phosphate without formation of insoluble complexes with magnesium. Since valid kinetic measurements cannot be made after precipitation of some of the components of the medium, it has not been possible to determine the nature of the phosphate inhibition.

Kinetics of Tripolyphosphate Hydrolysis. Figure 8 shows a double-reciprocal plot of the velocity of tripolyphosphate hydrolysis *vs.* the concentration of tripolyphosphate in the absence of adenosylmethionine, and for comparison one in the

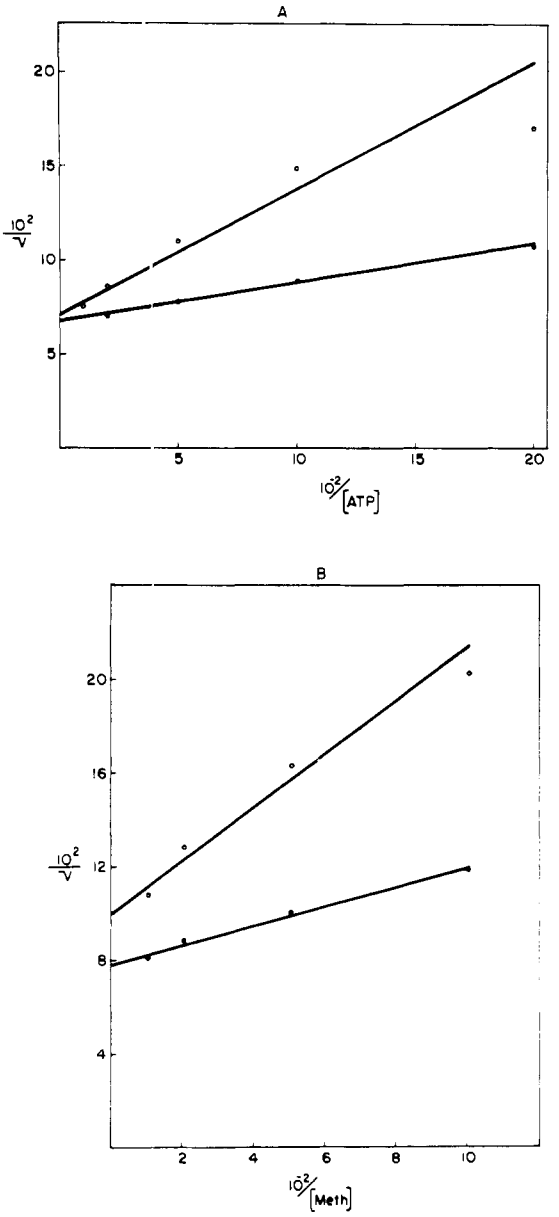

FIGURE 6: Inhibition of adenosylmethionine synthesis by pyrophosphate. (A) With ATP as the variable substrate. Velocity is expressed in millimicromoles per minute and ATP concentration in moles per liter. Incubation mixtures contained 0.09 M Tris–0.09 M histidine (pH 9), 0.1 M KCl, 0.005 M mercaptoethanol, 0.005 M excess $MgCl_2$, 0.01 M methionine, and variable ATP. The upper line is from mixtures which also contained 0.002 M PP_i. Kinetic parameters are $V_{max} = 14.6 \pm 0.4$ mμmoles/min, $K_{ATP\,app} = (2.8 \pm 0.4) \times 10^{-4}$ M, and $K_{I\,sl} = (7.1 \pm 1.2) \times 10^{-4}$ M. (B) With methionine as the variable substrate. Velocities are expressed in millimicromoles per minute and methionine concentrations in moles per liter. Incubation mixtures contained 0.09 M Tris–0.09 M histidine (pH 9), 0.1 M KCl, 0.005 M mercaptoethanol, 0.005 M excess $MgCl_2$, and 0.002 M ATP. The upper curve is from mixtures which also contained 0.002 M PP_i. Kinetic parameters are $V_{max} = 12.8 \pm 0.3$ mμmoles/min, $K_{Me\,app} = (5.4 \pm 0.6) \times 10^{-4}$ M; $K_{I\,sl} = (1.2 \pm 0.3) \times 10^{-3}$ M, and $K_{I\,int} = (7.3 \pm 1.3) \times 10^{-3}$ M.

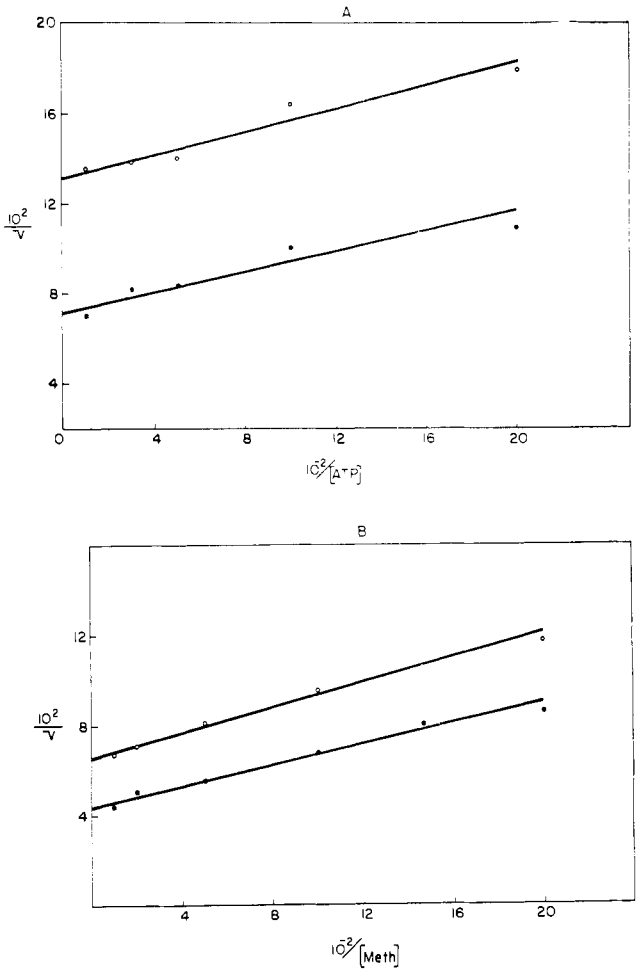

FIGURE 7: Inhibition of adenosylmethionine synthesis by adenosylmethionine. (A) With ATP as the variable substrate. Velocities are in millimicromoles per minute and ATP concentration in moles per liter. Incubation mixtures contained 0.09 M Tris–0.09 M histidine (pH 9), 0.1 M KCl, 0.005 M mercaptoethanol, 0.005 M excess $MgCl_2$, 0.01 M methionine, and variable ATP. The upper curve is from mixtures which also contained 0.003 M adenosylmethionine. Kinetic parameters are: $V_{max} = 14.1 \pm 0.3$ mμmoles/min, $K_{ATP\,app} = (3.3 \pm 0.4) \times 10^{-4}$ M, and $K_{I\,int} = (3.5 \pm 0.2) \times 10^{-3}$ M. (B) With methionine as the variable substrate. Velocities are in millimicromoles per minute and methionine concentrations in moles per liter. Incubation mixtures contained 0.09 M Tris–0.09 M histidine (pH 9), 0.1 M KCl, 0.005 M mercaptoethanol, 0.005 M excess $MgCl_2$, 0.009 M ATP, and variable methionine. The upper curve is from mixtures which also contained 0.003 M adenosylmethionine. Kinetic parameters are: $V_{max} = 23.2 \pm 0.6$ mμmoles/min; $K_{Me\,app} = (5.6 \pm 0.5) \times 10^{-4}$ M, and $K_{I\,int} = (5.4 \pm 0.4) \times 10^{-3}$ M.

presence of 0.0001 M adenosylmethionine, under the standard incubation conditions with the addition of 0.01 M NaF. The Michaelis constants and the maximal velocities of the reactions computed from these data are given in the legend of Figure 8. The kinetic constants computed from the reaction mixtures containing adenosylmethionine are in good agreement with those obtained in the absence of NaF. As has been reported by Mudd (1962), addition of adenosylmethionine to the incubation mixture causes a marked increase in the rate of tripolyphosphate hydrolysis. If the reciprocal of the rate of P_i release is plotted against the reciprocal of the tripolyphosphate concentration at several concentrations of adenosylmethionine, a series of convergent lines are obtained in which the intercepts and slopes decrease as adenosylmethionine is increased until inhibitory levels are reached which causes an increase in the intercepts. The intercepts and slopes of such plots and the standard error of these values at seven concentrations of adenosylmethionine are given in Table II. In each kinetic

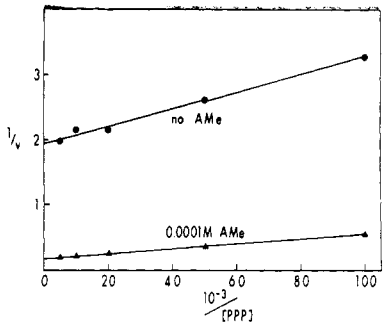

FIGURE 8: Double-reciprocal plot of tripolyphosphatase *vs.* PPP_i in the presence of 0.0001 M adenosylmethionine and without adenosylmethionine. Standard reaction conditions (pH 9.0) were used with the addition of 0.01 M NaF. Incubation mixtures with adenosylmethionine contained 0.05 unit of enzyme while those without contained 0.5 unit of enzyme but the velocities were corrected to 0.1 unit before plotting. The kinetic parameters of each line are: 0.0001 M adenosylmethionine, V (0.1 unit) = 5.11 ± 0.09 mμmoles/min, K_m = (1.8 ± 0.1) × 10^{-5} M; no adenosylmethionine, V (0.1 unit) = 0.515 ± 0.008 mμmole/min, K_m = (0.70 ± 0.006) × 10^{-5} M.

experiment, duplicate sets of tubes with five concentrations of tripolyphosphate were incubated together with appropriate blanks and standards. The number of experiments done at each concentration of adenosylmethionine is indicated in the table. Values of the slopes and intercepts extrapolated to infinite adenosylmethionine concentration were obtained from the intercepts of the lines shown in Figures 9 and 10. In Figure 9 the intercepts from Table II are plotted against the reciprocal of the adenosylmethionine concentration. The line was computed by means of a least-squares fit to the three points corresponding to 2 × 10^{-5}, 5 × 10^{-5}, and 10^{-4} M *S*-adenosylmethionine. The other points were not used because those obtained with higher concentrations of adenosylmethionine show inhibition and the point at the lowest adenosylmethionine concentration is expected to fall below the line due to the tripolyphosphatase activity of the enzyme in the absence of adenosylmethionine (see discussion). Inclusion of the point corresponding to the lowest concentration of adenosylmethionine in the computation does not greatly affect the slope or intercept of the line. The slopes from Table II are plotted *vs.* the reciprocal of the adenosylmethionine concentration in Figure

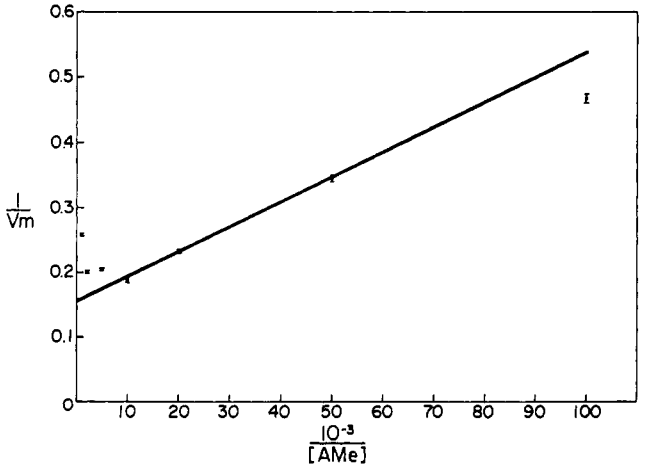

FIGURE 9: Plot of the intercepts from Table II *vs.* the reciprocal of the adenosylmethionine concentration. Lines above and below each point represent the standard error of that point.

TABLE II: Intercepts and Slopes of Reciprocal Plots of Tripolyphosphatase Activity at Different Concentrations of Adenosylmethionine.[a]

Adenosylmethionine Concn (× 10^5 M)	No. of Expt	Intercept ($1/V_m$)	Slope $(K/V)_{app}$ × 10^6
1	7	0.468 ± 0.007	8.33 ± 0.88
2	4	0.344 ± 0.005	5.60 ± 0.65
5	5	0.231 ± 0.002	5.03 ± 0.33
10	6	0.189 ± 0.005	5.12 ± 0.67
20	3	0.203 ± 0.002	3.95 ± 0.25
50	2	0.200 ± 0.002	4.16 ± 0.24
100	3	0.256 ± 0.003	4.24 ± 0.38

[a] Reaction vessels containing 0.1 unit of enzyme and variable MgPPP and adenosylmethionine were incubated under standard conditions. The kinetic parameters of the adenosylmethionine-stimulated tripolyphosphatase activity are: V_1 (0.1 unit of adenosylmethionine synthetase), 6.52 ± 0.15 mμmoles/min; $K_{adenosylmethionine}$, (2.50 ± 0.14) × 10^{-5} M; and K_{APP} (intercept, Figure 10/intercept, Figure 9), 2.6 × 10^{-5} M.

10, the line shown was obtained by a least-squares fit to all seven points. Though the precision of these points is much less than those of the intercepts and the degree of scatter is greater, they do not show the obvious increase at the higher adenosylmethionine concentrations that is seen in Figure 9.

The values of the extrapolated kinetic parameters obtained from these lines are also given in Table II. The reciprocal of the intercept of Figure 9 gives the maximal velocity of the adenosylmethionine-stimulated tripolyphosphate hydrolysis. This rate is approximately twelve times greater than that of the tripolyphosphatase reaction in the absence of adenosylmethionine which is similar to the result obtained by Mudd (1962). The Michaelis constant for adenosylmethionine was computed from the ratio of the slope to the intercept of Figure 9 and the Michaelis constant for tripolyphosphate was obtained by dividing the intercept of Figure 10 by the intercept of Figure 9.

From the reciprocal plot of the tripolyphosphatase activity in the presence and absence of 0.002 M pyrophosphate shown in Figure 11, it can be seen that pyrophosphate inhibition is competitive with tripolyphosphate.

Discussion

Adenosylmethionine synthetase of yeast has appeared to be a somewhat unusual enzyme in view of the high concentrations of potassium and magnesium which were reported to be necessary for maximum activity. The results presented here show that such behavior is only found in the lower pH range in the presence of high concentrations of ATP and potassium. At lower concentrations of ATP or potassium or at higher pH, high levels magnesium are inhibitory. In spite of the inhibitory action of excess magnesium, maximum adenosylmethionine

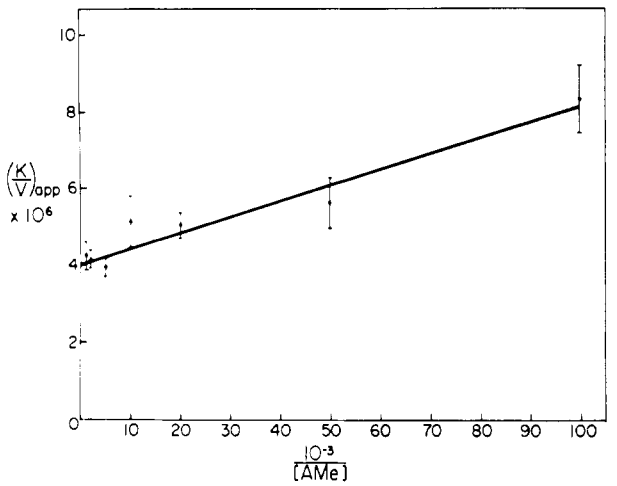

FIGURE 10: Plot of the slopes from Table II *vs.* the reciprocal of the adenosylmethionine concentration. Lines above and below each point represent the standard error of that point.

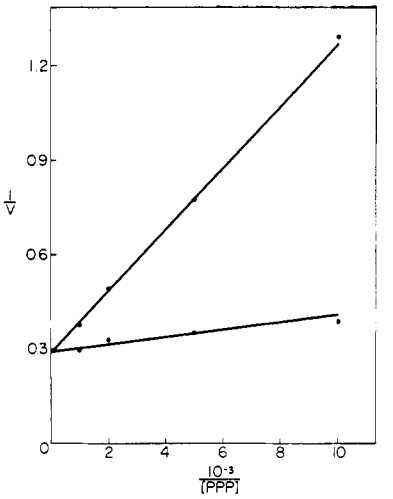

FIGURE 11: Inhibition of tripolyphosphatase by pyrophosphate. Standard incubation media at pH 9.0 containing 0.001 M PPP_i, 0.0001 M adenosylmethionine, 0.01 M NaF, and 0.1 unit of enzyme. The reaction mixtures plotted in the upper line also contained 0.002 M $MgPP^{2-}$. K_I for pyrophosphate is $(2.3 \pm 0.5) \times 10^{-4}$ M.

synthetase and tripolyphosphatase activities require a magnesium concentration greater than that of ATP or PPP_i. This observation suggests that magnesium plays a dual role, first to form magnesium complexes of ATP or PPP_i which are probably the true substrates, and second to interact directly with the enzyme. The observation of the synergistic behavior of magnesium and manganese tends to support this interpretation since it can be argued that each of the two cations is better at fulfilling one of the functions. At lower magnesium concentrations the adenosylmethionine synthetase activity shows a rather broad pH optimum with a peak at pH 9. In constrast the pH *vs.* activity curve of the tripolyphosphatase reaction is flat between pH 6 and 8.5 with no sign of a peak. The maximum velocity of the tripolyphosphatase reaction is about two-times greater than that of the adenosylmethionine synthetase reaction (which includes tripolyphosphate hydrolysis as one of the steps). As has been pointed out by Mudd and Mann (1963) this difference in rates indicates that the adenosylmethionine synthetase reaction must have two rate-limiting steps, one being the tripolyphosphate hydrolysis and the other unidentified (possibly the transfer of the adenosyl moiety from ATP to the sulfur of methionine). When selenomethionine is used as a substrate for adenosylmethionine synthetase, not only is the rate of the reaction increased about twofold (Mudd and Cantoni, 1957) but the pH *vs.* activity curve resembles that of the tripolyphosphatase rather than that of the adenosylmethionine synthetase with methionine as a substrate. The effect of selenoadenosylmethionine on the rate of the tripolyphosphatase reaction has not been evaluated because selenoadenosylmethionine is less stable than *S*-adenosylmethionine and an adequate procedure for isolation of the pure material has not been found. However, if it is assumed that both the sulfur- and selenium-containing adenosylmethionines have the same stimulating effect on the tripolyphosphatase reaction, it is reasonable to conclude that the rate of synthesis of selenoadenosylmethionine is limited by the tripolyphosphate hydrolysis. If this condition is true, then it is possible that the rate of the other limiting reaction (formation of adenosylmethionine and PPP?) may be increased to a much greater extent when selenomethionine is a reactant than is indicated by the twofold increase in rate of the over-all reaction.

The kinetic behavior of the adenosylmethionine synthetase and tripolyphosphatase activities, including the stimulation and inhibition of tripolyphosphate hydrolysis by adenosylmethionine, can be rationalized by the model given in Figure 12. The total rate equation for this model has been derived by the method of King and Altman (1956) but is is too complex to be generally useful (the denominator has 57 terms). In the individual experiments where the concentrations of some products are zero the appropriate simiplified rate equations are obtained by ignoring those reactions which are not operative. Without added tripolyphosphate the kinetics of adenosylmethionine synthesis can be described by the outer ring of reactions shown in Figure 12. This model with two substrates and three products is the inverse of one with three substiates and two products for which Cleland (1963a) has derived the rate equation. The initial velocity of the reaction in the absence of any products is described by the equation given below for an enzyme with two substrates derived by Alberty (1953) and modified to use the terminology of Cleland (1963a). Although

$$v = \frac{V_1}{1 + \frac{K_{ATP}}{[ATP]} + \frac{K_{Me}}{[Me]} + \frac{K_{i\,ATP}K_{Me}}{[ATP][Me]}}$$

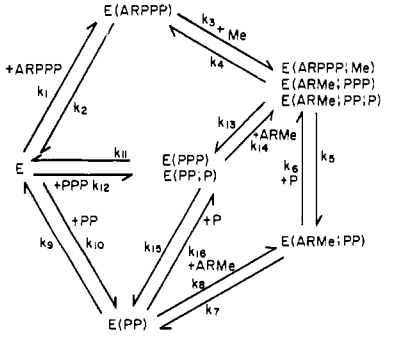

FIGURE 12: Proposed mechanism of yeast adenosylmethionine synthetase. Me is methionine, ARPPP is ATP, and ARMe is adenosylmethionine.

this equation predicts the convergent lines of the kinetic plot of Figure 4 the same behavior would be observed regardless of the order of addition of substrates. The pattern of product inhibition must be determined before any conclusions may be drawn about the sequence of substrate inhibition or product release.

The equations describing the inhibition by the various products are obtained from the total rate equation by dropping all terms which contain the concentrations of products other than the one being studied.

The equations for pyrophosphate and tripolyphosphate inhibition are similar and have the following form.

$$v = \frac{V_1}{1 + \frac{K_{Me}}{[Me]} + \left(\frac{K_{ATP}}{[ATP]} + \frac{K_{i\,ATP}K_{Me}}{[ATP][Me]}\right)\left(1 + \frac{I}{K_I}\right)}$$

For pyrophosphate inhibition, $[I]/K_I = [PP]/K_{i\,PPP}$; for tripolyphosphate inhibition, $[I]/K_I = [PPP]/K_{i\,PPP}$. The inhibition constant for tripolyphosphate obtained from this derivation is identical with the Michaelis constant for tripolyphosphate of the tripolyphosphatase reaction in the absence of adenosylmethionine and the inhibition constant for pyrophosphate is the dissociation constant of the enzyme–pyrophosphate complex. The above equation predicts that pyrophosphate and tripolyphosphate will inhibit competitively when ATP is the variable substrate and noncompetitively when methionine is the variable substrate. It also states that in experiments with variable methionine the apparent noncompetitive inhibition constants are not independent but are functions of the other kinetic constants and the ATP concentration. The competitive inhibition constants measured in experiments where ATP is the variable substrate are not affected by changes in the concentration of methionine, while changing the concentration of ATP in experiments with variable methionine causes the expected changes in the apparent noncompetitive inhibition constants. Cleland (1963a) has pointed out that product inhibition will be competitive when the variable substrate and the inhibiting product compete for the same form of the enzyme. In an ordered sequential mechanism this relationship would be seen between the first substrate to bind and the last product to dissociate since both would compete for the free enzyme.

The equation describing the inhibition by adenosylmethionine is as follows

$$v = \frac{V_1}{1 + \frac{K_{ATP}}{[ATP]} + \frac{K_{Me}}{[Me]} + \frac{K_{i\,ATP}K_{Me}}{[ATP][Me]} + \frac{[\text{adenosylmethionine}]}{K_{I\,\text{adenosylmethionine}}}}$$

Since the concentration of adenoslymethionine appears only in a term which does not contain the concentration of either substrate, the equation predicts that only the intercept will be changed by addition of adenosylmethionine to the incubation mixture which is consistent with the experimental results. Cleland (1963a,b) has pointed out that uncompetitive inhibition is seen when the variable substrate and the inhibiting product do not bind to the same form of the enzyme and when there is no reversible pathway connecting the two forms (dissociation of a product at very low concentration is sufficiently irreversible to cause this behavior). For an enzyme reaction with three products, uncompetitive inhibition is shown by the second product to dissociate. This observation further indicates that, in the catalytic reaction, adenosylmethionine does not bind to the free catalytic site since it shows the same type of inhibition for both substrates and regardless of their order of addition, at least one of them must bind to the free enzyme.

The rate equation for the tripolyphosphatase activity in the presence of adenosylmethionine derived by neglecting the reactions involving adenosine triphosphate and methionine and assuming initial conditions is given in eq 1. The term in the numerator containing the reciprocal of the adenosylmethionine concentration is required to account for the tripolyphosphatase activity in the absence of adenosylmethionine. Judging from the experimental results this term becomes negligible at adenosylmethionine concentrations greater than 2×10^{-5} M. When the concentration of adenosylmethionine is zero the rate equation reduces to the simple single substrate Michaelis–Menten equation. An unusual feature of the proposed mechanism for the tripolyphosphatase activity is the obligatory association and dissociation of adenosylmethionine during each catalytic cycle. According to this model adenosylmethionine behaves as a substrate and a product and the denominator of the rate equation therefore contains both $K_{\text{adenosylmethionine}}/[\text{adenosylmethionine}]$ and $[\text{adenosylmethionine}]/K_{I\,\text{adenosylmethionine}}$ terms. Because of these terms the rate equation predicts that adenosylmethionine will have both stimulatory and inhibitory effects as the experimental results have shown. The rate equation further predicts that with increasing adenosylmethionine the intercepts of the double-reciprocal plots will decrease and then increase while the slopes will continue to decrease. Although the measurement of the slopes of these plots is not sufficiently precise to draw any firm conclusions, the highest concentration of adenosylmethionine causes a marked increase in the intercept without a comparable effect on the slope. The value of the kinetic constant $K_{i\,PPP}$ can be computed in three ways from the rate equations given above, the Michaelis constant for tripolyphosphate hydrolysis in the absence of adenosylmethionine, the inhibition constant for the competitive inhibition of adenosylmethionine synthesis by tripolyphosphate, and the ratio of slopes of Figures 10 and 9. Values obtained by these procedures are in reasonably good agreement (7, 14, and 11 μM).

The action of a compound which has both stimulatory and inhibitory effects on an enzyme, such as those shown by adenosylmethionine on the tripolyphosphatase activity is most frequently explained by a model in which the enzyme can bind two molecules of the effector without obligatory dissociation of the effector molecules during catalysis. The binding of the

$$v = \frac{V_1\left(1 + \frac{k_{15}(k_5 + k_{13})}{k_5 k_{14}}\frac{1}{[\text{adenosylmethionine}]}\right)}{1 + \frac{K_{PPP}}{[PPP]} + \frac{K_{\text{adenosylmethionine}}}{[\text{adenosylmethionine}]} + \frac{K_{i\,PPP}K_{\text{adenosylmethionine}}}{[PPP][\text{adenosylmethionine}]} + \frac{[\text{adenosylmethionine}]}{K_{I\,\text{adenosylmethionine}}}} \quad (1)$$

first molecule presumably stimulates activity while the second inhibits it. This model cannot be ruled out, but in its simplest form it predicts that high concentrations of inhibitor will cause proportionate increases in the slopes and intercepts of the reciprocal plots. Further it has been reported that the K_I for the competitive inhibition of the tripolyphosphatase activity by ATP is not influenced by addition of adenosylmethionine (Mudd, 1963). This behavior is predicted by the model in Figure 12 in which ATP and tripolyphosphate are presumed to compete for the free enzyme prior to the binding of adenosylmethionine. On the other hand, if adenosylmethionine does not necessarily dissociate during the catalytic reaction and if it is assumed that the adenosyl moieties of adenosylmethionine and ATP each bind to the same site on the enzyme, the presence of bound adenosylmethionine would be expected to impair the binding of ATP and this difference should be reflected in an altered K_I.

Mudd and Mann (1963) have proposed a mechanism in which the association of substrates and release of products is random. In support of this proposal they have reported a lag during the early phases of adenosylmethionine synthesis which can be slightly diminished by the addition of 5×10^{-5} M adenosylmethionine. They believe this lag to be due to dissociation of adenosylmethionine prior to tripolyphosphate hydrolysis when the concentration of free adenosylmethionine is very low. In the work reported here, no special attempt has been made to study the early transient phases of the reaction but since no significant deviation of the rate of the synthetic reaction from linearity was observed any lag must be small. Mudd and Mann (1963) have also shown that dissociation of tripolyphosphate from the enzyme before it is hydrolyzed is about 2% of that which is produced under conditions where this dissociation may be expected to be maximal, *i.e.*, very low ATP (10^{-6} M), relatively high pyrophosphate (10^{-3} M), and very low adenosylmethionine. Addition of adenosylmethionine (4.8×10^{-4} M) to the incubation mixture reduces the amount of tripolyphosphate which is liberated. This amount of dissociation is too small to have a significant effect on the kinetic results. Under conditions where the enzyme can be shown to bind pyrophosphate no binding of ATP could be detected (Mudd, 1962). If the values of the dissociation constants for ATP, $K_{i\,ATP}$, reported here are correct, binding would not have been detected by the methods used. That the free enzyme can bind ATP is shown by the observation that ATP competitively inhibits tripolyphosphate hydrolysis in the absence of adenosylmethionine (Mudd and Mann, 1963). As has been mentioned above if dissociation of adenosylmethionine occurred after pyrophosphate release or before release of orthophosphate, product inhibition by adenosylmethionine would not be uncompetitive. It is difficult to rule out the possibility that part of the reaction may proceed with the binding of methionine before ATP is bound. However, the kinetic data do not suggest this sequence and there is no evidence to show that the free enzyme can bind methionine.

The model with a single binding site for each substrate or product was selected because it accounts for the kinetic properties of the enzyme in a simple and straightforward manner. While it is felt that this model is the best description of the major reaction sequence, there is no intent to suggest that alternate mechanisms cannot participate in the reaction to a minor extent.

Acknowledgment

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