Purification and Properties of β-Lysine Mutase, a Pyridoxal Phosphate and B_{12} Coenzyme Dependent Enzyme†

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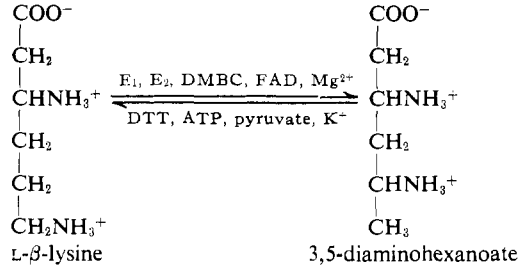
ABSTRACT: The cobamide coenzyme dependent β-lysine mutase has been purified to homogeneity by fractionation with ammonium sulfate and chromatography on DEAE-cellulose, Sephadex G-150, and DEAE-Sephadex A-50. The native enzyme is a tetramer (mol wt 170,000) composed of two subunits of approximately 32,000 and two of approximately 52,000 daltons. The enzyme exhibits an absolute dependency on pyridoxal phosphate for activity. During isolation there is degradation of enzyme-bound cobamide coenzyme to hydroxy(adenyl)cobamide which is a strong inhibitor and remains tightly bound to the protein. Incubation with cobalamin coenzyme, Mg^{2+}, a mercaptan, and pyridoxal phosphate displaces the hydroxycobamide and markedly activates the mutase. A monovalent cation (*e.g.*, K^+) is required for catalytic activity of the mutase. Low concentrations of hydroxylamine and other carbonyl reagents inhibit the mutase, presumably by reacting with pyridoxal phosphate. During catalysis under conditions where the mutase product, 3,5-diaminohexanoate, is not continuously removed there is extensive cleavage of cobalamin coenzyme to free 5′-deoxyadenosine and a cobalamin with concomitant inactivation of the enzyme. This inactivation is prevented by the addition of a sulfhydryl protein (E_2) and ATP. Inactivation of the mutase during catalysis is greatly accelerated by oxygen.

Partially purified preparations of the cobamide enzyme, L-β-lysine mutase, which catalyzes the conversion of 3,6-diaminohexanoate (L-β-lysine) to 3,5-diaminohexanoate, were found to require an unusually large number of cofactors for maximal catalytic activity (Stadtman and Renz, 1968; Stadtman and Grant, 1971). As shown in Scheme I, in addition

SCHEME I



to the cobamide protein (E_1), a sulfhydryl protein (E_2), cobalamin coenzyme (DMBC),[1] a thiol (dithiothreitol, DTT), ATP, pyruvate, FAD, a divalent metal ion (Mg^{2+} or Mn^{2+}), and a monovalent cation such as K^+ are required.

A more detailed study of this enzyme was facilitated by the development of a spectrophotometric assay procedure (Baker *et al.*, 1972b) that measures only the initial activity, and avoids many of the problems inherent in the longer assay previously employed which requires continued turnover of the enzyme and accumulation of micromole amounts of product. In the spectrophotometric assay, the product of the mutase reaction is oxidized to 3-keto-5-aminohexanoate in the presence of an excess of 3,5-diaminohexanoate dehydrogenase and either NAD or NADP, and the rate of formation of the reduced pyridine nucleotide is followed. In this report a method for isolation of β-lysine mutase in a homogeneous form is described, the absolute dependence of the purified mutase on yet another cofactor, pyridoxal-5′-P, is demonstrated and some information concerning the roles of the other cofactors is presented.

Experimental Procedure

Materials

Clostridium sticklandii cells, cultured, and harvested as described by Stadtman (1966) and stored at −80°, were employed as enzyme source. Samples of 5′-deoxyadenosine and [dA-U-^{14}C]DMBC (specific activity 8.2 Ci/mol) were gifts from Dr. B. Babior. Commercial preparations used were L-β-lysine from Cyclo Chemical Corp., 1,4-dithiothreitol from Calbiochem, DMBC from Pierrel, Sp.A., Milano, Italy, and NAD(H), ATP, and other nucleotides from P-L Biochemicals, Inc. Aquasol, [8-^{14}C]ATP, [U-^{14}C]ATP, and [γ-^{32}P]ATP were purchased from New England Nuclear Corp.

Methods

Standard Spectrophotometric Assay for β-Lysine Mutase Activity. ACTIVATION OF THE ENZYME. The activation mixture contained, per milliliter, 50 mM triethanolamine-HCl buffer (pH 7.8), 2 mM dithiothreitol, 5 mM $MgSO_4$, 40 μM pyridoxal-5′-P, 3.8 μM DMBC, 0.4 mM NADP (or as indicated in some experiments, 0.4 mM NAD), an excess (about 0.1 unit) of 3,5-diaminohexanoate dehydrogenase that had been purified through the hydroxylapatite step (J. J. Baker and C. van der Drift, manuscript in preparation), and 0.5–250 μg of protein (depending on purity). The mixture, 0.9 ml, in a 1.0-ml cuvet, open to the air, was incubated for 90 min at 37° in the dark.

DETERMINATION OF ACTIVITY. After activation of the enzyme the reaction was started by the simultaneous addition of

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[1] Abbreviations used are: DMBC, 5′-deoxyadenosylcobalamin or α-(5,6-dimethylbenzimidazolyl)cobamide coenzyme; 3,5-DAH, 3,5-diaminohexanoate; $N(EtOH)_3$, triethanolamine.

L-β-lysine and KCl to a final concentration of 1 and 80 mM, respectively. The reaction rate at 37° was measured by following NADPH formation with a Gilford Model 2000 multiple-sample absorbance recorder. The rate was a linear function of enzyme concentration up to at least 0.06 unit/assay. One unit of β-lysine mutase is defined as that amount which allows the formation of 1 μmol of NADPH/min in the coupled assay system.

Long-Term Assay for Mutase Activity. Occasionally β-lysine mutase activity was determined by the procedure of Stadtman and Renz (1968) with the exception that the reaction mixture was supplemented with 40 μM pyridoxal-5′-P and unless otherwise indicated FAD was omitted. The amount of 3,5-DAH formed from β-lysine in 90 min under anaerobic conditions was estimated by reaction with acid ninhydrin.

Preparation of β-Lysine Mutase Labeled with [dA-U-^{14}C]-DMBC. The mutase (0.96 mg; specific activity 19.5) was labeled by incubation in a standard activation mixture (11 ml) in which 0.79 μM ^{14}C-labeled DMBC (130,000 cpm; specific activity 8.2 Ci/mol) was substituted for unlabeled DMBC. After activation for 80 min at 37°, glycerol was added to 10% (v/v), and the solution was chilled to 0° and applied to a DEAE-cellulose column (0.6 × 4 cm) equilibrated with 20 mM NaEDTA buffer (pH 6.8) in 10% glycerol. A 1-ml wash with the same buffer removed free [^{14}C]DMBC from the column, and the absorbed ^{14}C-labeled mutase was then eluted with 1 ml of buffer containing 1 M NaCl. The NaCl eluate (0.6 ml) contained 18,900 cpm and this labeled enzyme was used in the experiments described in Figure 10.

Coenzyme Synthetase Assay. A radiochemical assay based on procedures described by other investigators (Brady *et al.*, 1962; Peterkofsky and Weissbach, 1963; Vitols *et al.*, 1966) was employed for estimation of B_{12} coenzyme synthetase activity of E_2 preparations.

Reaction mixtures (250 μl in 7 × 50 mm test tubes) contained 80 mM 2-methylimidazole·HCl (pH 8.2), 8 mM K_2HPO_4, 40 mM KCl, 4 mM MgCl, 2 mM [8-^{14}C]ATP (1 μCi), 88 μM hydroxy-B_{12}, 40 μM FAD, 8 mM 1,4-dithiothreitol, 4 mM KBH_4, and enzyme (usually 50 μg to 1 mg of protein, depending on purity).

The borohydride solution, prepared in deoxygenated water, was added last under a stream of argon to samples that already had been flushed with argon and the tubes were immediately closed with tight-fitting rubber stoppers. Incubation was at 36° in the dark for 90 min. Reactions were terminated by addition of 40 μl of 1 M $HClO_4$, the samples were chilled in ice and, after a few minutes, were adjusted to pH 6–7 with 2 N KOH. Following centrifugation, 20-μl aliquots of the clear supernatant solutions were applied to cellulose thin-layer plastic backed sheets (Eastman 6064). Each sample was cochromatographed with adenosine and DMBC which had already been applied to the spot to serve as markers. The chromatograms were developed first in a solvent consisting of 1-butanol–glacial acetic acid–water (80:20:50). DMBC, α-(adenyl)hydroxycobamide, adenosine, 5′-deoxyadenosine, adenine, and hypoxanthine all migrate at approximately the same rate in this solvent (R_F 0.65–0.69) but separate satisfactorily from ATP, ADP, AMP, and inosine (R_F 0.12, 0.2, 0.3, and 0.5, respectively). To separate the labeled coenzyme product from any contaminating radioactive adenosine, 5′-deoxyadenosine, adenine, or hypoxanthine formed from the labeled ATP in side reactions catalyzed by crude enzyme preparations, the chromatograms, after brief drying, were run in the reverse direction in a second solvent consisting of *tert*-butyl alcohol–methyl ethyl ketone–glacial acetic acid–water (40:30:15:15). In this system DMBC does not migrate whereas adenosine, 5′-deoxyadenosine, adenine, and hypoxanthine are all moved away from the DMBC spot. Although α-(adenyl)hydroxycobamide does not separate completely from the coenzyme it is not radioactive and therefore does not interfere when the coenzyme spots are cut from the chromatograms and examined for ^{14}C. All of the above manipulations are carried out in the dark or in dim light until after the final chromatogram is developed; then the various compounds are located under uv light. Development in the second solvent is rapid and requires only 1–2 hr. If desired, the chromatogram can be dried briefly and run a second time in solvent 2 to achieve even wider separation of the DMBC from contaminating labeled bases and nucleosides.

The E_2 preparations used in this study were obtained as described by Stadtman and Renz (1968).

Protein was determined according to Lowry *et al.* (1951) using bovine serum albumin as standard.

Radioactivity measurements were made with a scintillation spectrometer (Beckman Model LS-250) using Bray's solution (1960) or aquasol as scintillant.

Polyacrylamide gel electrophoresis was done at pH 8.5 with a continuous buffer of 25 mM asparagine. The buffer was prepared by adjusting 100 mM asparagine to pH 8.5 with Tris base. Sodium dodecyl sulfate gel electrophoresis was performed by the procedure of Weber and Osborn (1969) with the modifications (Baker, 1970; Baker *et al.*, 1972a) that 5% gels were used and *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid adjusted to pH 7.1 with Tris base was substituted for phosphate buffer. With a final buffer concentration of 25 mM, the electrophoresis takes about 1 hr at 2 mA/tube. Linear results are observed at least for monomers with molecular weights between 15,000 and 68,000 daltons.

Results

Purification of the Enzyme. CELL-FREE EXTRACT. Frozen *C. sticklandii* cells (103 g) were thawed in 250 ml of a pH 7.2 buffer containing 100 mM potassium phosphate, 20 mM Na-EDTA, and 10% glycerol. The suspended cells were sonicated with cooling in 40-ml batches for 1.5 min, cooled 1.5 min, and sonicated another 1.5 min with a Branson Sonifier (Model LS-75). The broken cell suspension was centrifuged for 45 min at 18,000*g* and the supernatant was used for the next step. All subsequent purification steps were performed at 4° and unless otherwise specified, the standard buffer was 20 mM NaEDTA–10% glycerol (pH 6.7).

AMMONIUM SULFATE PRECIPITATION. Solid ammonium sulfate (32.1 g/100 ml) was added slowly to bring the supernatant to 53% saturation. After 30 min the precipitate was removed by centrifugation at 30,000*g* for 45 min and additional ammonium sulfate (17.6 g/100 ml) was added to bring the supernatant to 80% saturation. After 30 min the precipitate was collected by centrifugation and dissolved in a minimal amount of standard buffer. This fraction, which contained 76% of the total β-lysine mutase activity and over 70% of the 3,5-diaminohexanoate dehydrogenase activity (J. J. Baker and C. van der Drift, manuscript in preparation), was used for the next step.

DEAE-CELLULOSE CHROMATOGRAPHY. The 53–80% ammonium sulfate fraction was desalted by passage through a Sephadex G-25 column (4 × 43 cm) equilibrated with standard buffer. The desalted protein solution was applied to a DEAE-cellulose column (4.2 × 32 cm) which had been equilibrated with standard buffer.

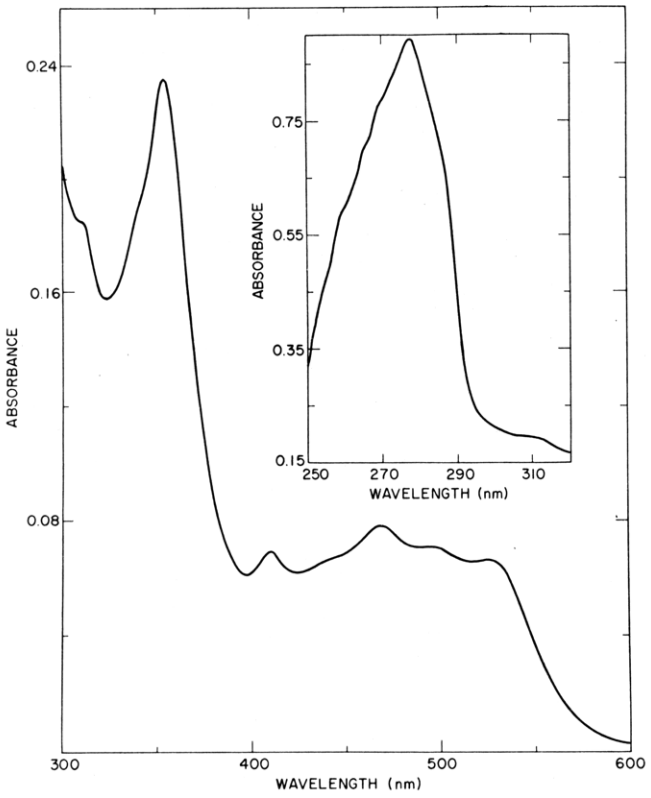

FIGURE 1: Visible spectrum and ultraviolet spectrum (inset) of L-β-lysine mutase. Protein (1.1 mg/ml) was dissolved in a pH 6.8 buffer containing 20 mM NaEDTA and 0.4 M NaCl. Spectra were recorded at 22° using 1-cm cuvets with a Cary 17 spectrophotometer.

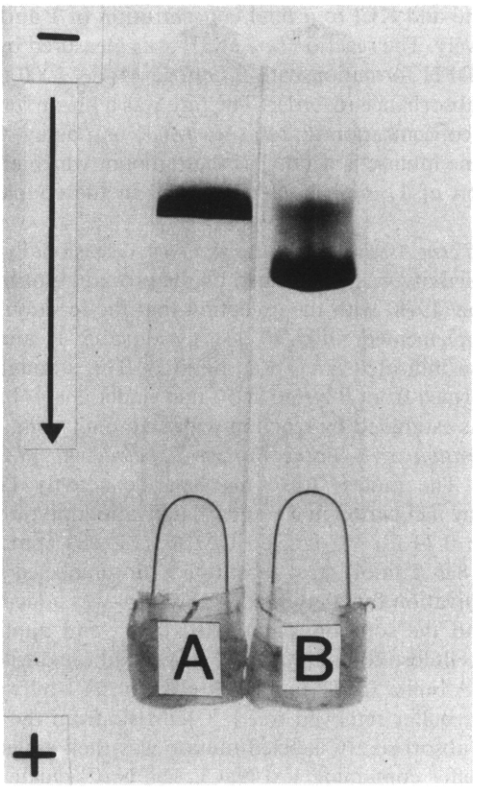

FIGURE 2: Polyacrylamide gel electrophoresis of L-β-lysine mutase. The electrophoresis was done as described under Methods with 7.5% gels and 25 mM Tris-asparagine buffer (pH 8.5). The protein was stained with Amido Black.

A 1-l. linear gradient of NaCl (0–0.25 M) in standard buffer was used to elute 3,5-diaminohexanoate dehydrogenase, which was saved for a separate study (J. J. Baker and C. van der Drift, manuscript in preparation). A second 1-l. linear gradient of NaCl (0.3–0.5 M) in standard buffer was used to elute the β-lysine mutase. Fractions of 6 ml were collected. The mutase activity eluted between 0.35 and 0.40 M NaCl. Fractions containing more than 2 units/ml were pooled and concentrated by vacuum dialysis.

GEL FILTRATION ON SEPHADEX G-150. The concentrated protein solution from the preceding step was applied to a Sephadex G-150 column (4 × 100 cm), which had been equilibrated with 20 mM NaEDTA buffer (pH 6.7). The protein was eluted with the same buffer and 3-ml fractions were collected. Fractions containing over 3 units/ml were pooled.

DEAE-SEPHADEX CHROMATOGRAPHY. The mutase fraction obtained in step 4 was applied to a DEAE-Sephadex A-50 (mesh 40–120 μ) column (3 × 25 cm), which had been equilibrated with a pH 6.8 buffer composed of 20 mM NaEDTA and 0.2 M NaCl. Protein was eluted with a 200-ml linear gradient of NaCl (0.3–0.6 M) in buffer and 2-ml fractions were collected. A single protein peak was eluted. All of these protein fractions exhibited an identical pattern when analyzed by disc gel electrophoresis. The peak tubes were pooled and this material was used for further studies.

TABLE I: Purification of L-β-Lysine Mutase.

	Vol (ml)	Total Protein (mg)	Total Act. (Units)	Sp Act. (Units/mg)
1. Cell extract	307	14,200	3220	0.23
2. 53–80% ammonium sulfate fraction	69	4,760	2430	0.51
3. DEAE-cellulose	96	240	1412	5.9
4. Sephadex G-150	84	70	1320	18.9
5. DEAE-Sephadex A-50	12	66	1290	19.5

A summary of the entire purification procedure is shown in Table I. The final chromatographic step employing DEAE-Sephadex, which serves principally as an indication of extent of purity of the enzyme, can be omitted in most procedures as it gives very little additional purification.

The enzyme is a bright orange colored protein when isolated by the above procedure; its absorption spectrum is shown in Figure 1. If instead enzyme was purified through steps 1–4 in a pH 6.8 buffer containing 20 mM potassium phosphate and 3 mM NaEDTA, *i.e.*, in the absence of glycerol and at a lower EDTA concentration, the mutase was red rather than orange in color. This latter type of preparation is referred to as red mutase.

Both the orange and the red mutase preparations must be activated as described in Methods in order to obtain full activity. Depending upon the preparation, the residual activity prior to activation varies from 1 to 30%. This appears to be due both to the presence of varying amounts of bound hydroxycobalamin which is inhibitory and to loss of pyridoxal-5′-P, an essential cofactor.

Homogeneity. The homogeneity of the isolated mutase was investigated by electrophoresis on polyacrylamide gels. Figure

2A shows that a single, darkly stained band is obtained when 150 μg of mutase (specific activity 19.5) were subjected to electrophoresis in a 7.5% gel. With some mutase preparations a minor band resolved from the major band. This is illustrated in Figure 2B which shows the relative proportions of the two bands which separated from 120 μg of one of these protein preparations. Both bands were visible before staining; the major band was bright orange in color, and the minor band faint orange in color. When these bands were cut from an unstained gel and the protein extracted from the sections and assayed, β-lysine mutase activity was detected in both, but not between the bands. The mutase extracted from the major band was rerun on a 7.5% gel, and again the minor band appeared. It is apparent that both bands are β-lysine mutase, but their exact relationship to each other is not yet clear.

Stability. The purified mutase can be stored at −22° for at least 3 months without loss of activity. After 6 months, with repeated freezing and thawing, two-thirds of the original activity remained. Such partially inactivated preparations exhibit as many as eight minor protein bands in addition to the major mutase band when analyzed by disc gel electrophoresis.

Molecular Weight. A molecular weight value of 170,000 was determined for the mutase by gel filtration on a Sephadex G-150 column calibrated with catalase, alkaline phosphatase, bovine serum albumin, cytochrome *c*, and aldolase (Andrews, 1965). Electrophoresis in gels in the presence of sodium dodecyl sulfate (see Methods) shows the mutase to be composed of two nonidentical subunits having molecular weights of approximately 32,000 and 52,000. On the basis of this subunit composition, the native enzyme therefore must be a tetramer composed of two of each type of subunit.

K_m for β-Lysine. The K_m value for β-lysine was determined after activation of the enzyme under standard conditions, by adding variable amounts of β-lysine with the KCl. A hyperbolic response to increasing concentrations of β-lysine was observed and a K_m of 0.3 mM was extrapolated from a Lineweaver–Burk plot.

Effects of Prior Incubation Conditions on Extent of Activation of the Mutase. The set of conditions that were adopted for routine activation of the isolated β-lysine mutase are described under Methods. The extent of activation is dependent on the time and temperature of incubation as well as nature of the buffer, pH, and presence of cofactors in the activation mixture.

Effects of Buffers and pH. Data from some experiments wherein buffer, pH, temperature, and incubation time were varied are shown in Figure 3. Of the buffers shown in Figure 3A, $N(EtOH)_3 \cdot HCl$ is the most effective. The optimum pH in this buffer (Figure 3B) is 7.8 and the optimum temperature (Figure 3C) is 37°.

Other buffers and conditions were tested; compared to the activation achieved in $N(EtOH)_3 \cdot HCl$ at pH 7.8, the maximal activity observed in potassium phosphate pH 7.4 was 25%, in potassium arsenate pH 8.3 30% or pH 8.7 63%, and in diethanolamine·HCl, either at pH 8.4 or 8.8, 2%. The latter buffer is a potent inhibitor.

DMBC and Mercaptan Requirements. The effects of varying concentrations of DMBC and dithiothreitol on extent of activation of the enzyme are shown in Figure 4A,B, respectively. If either of these cofactors was omitted from the prior incubation mixture and instead added at the same time as the substrate, no appreciable increase in activity of the enzyme was observed.

Divalent Cation Effects. Activation of β-lysine mutase also is dependent on a divalent cation. As shown in Figure 5, Mg^{2+} (2–5 mM) is somewhat more effective than Mn^{2+}. Several other divalent metal ions also serve as cofactor for the activation process but they were of limited use in the spectrophotometric assay because a precipitate formed when they were added in concentrations exceeding 1 mM. When compared to Mg^{2+} at the same concentration (1 mM or less), Ca^{2+} was found to be 59% as effective, Zn^{2+} was 69%, and Cu^{2+} was 32%.

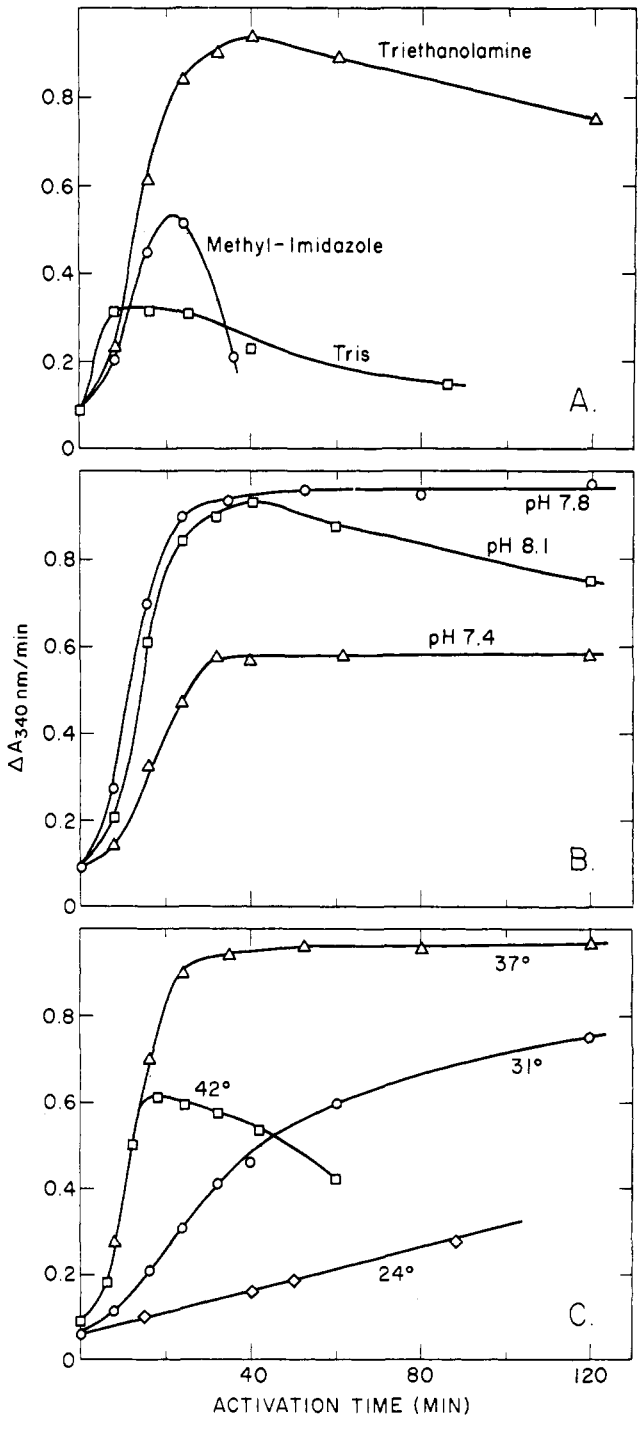

FIGURE 3: Effect of buffer (A), pH (B), or temperature (C) on the extent of activation as a function of incubation time. For part A, activity was determined at pH 8.1 in 100 mM of the indicated buffer. Each determination was done at the indicated time with 20 μg of β-lysine mutase (specific activity 15). Except for the indicated differences and the omission of pyridoxal-5′-P, other conditions were as given under Methods.

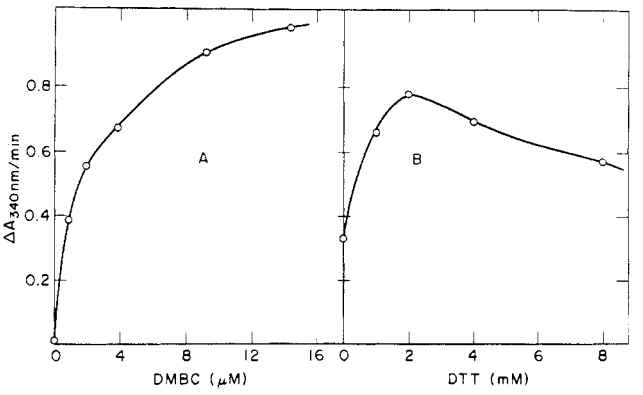

FIGURE 4: The dependence of the activation on DMBC (A) or dithiothreitol (B). Ten micrograms of β-lysine mutase (specific activity 15) was used for each determination. The buffer was 100 mM 2-methylimidazole hydrochloride (pH 8.1) and the activation time was 30 min. Other conditions were as given under Methods except pyridoxal-5′-P was not present.

Requirement for Pyridoxal Phosphate. The highly purified mutase used in the present studies depends on yet another cofactor (pyridoxal-5′-P) that had not been previously recognized as a requirement. The effect of pyridoxal-5′-P on the activation of both the normal orange colored mutase and the red mutase is shown in Figure 6. Whereas the red form of the mutase is markedly stimulated (9-fold) by the addition of pyridoxal-5′-P, the orange form shows only a 1.7-fold stimulation. The K_m for pyridoxal-5′-P (0.22 μM) was determined using the more pyridoxal-5′-P-dependent red mutase preparation. The data for the reciprocal plot shown in the inset of Figure 6 were obtained under standard conditions except that the pyridoxal-5′-P concentration in the prior incubation mixture was varied from 0.13 to 4 μM.

Addition of pyridoxal-5′-P affects both the extent of activation and the time required to reach full activation (Figure 7). It is seen that although the activity of the orange mutase reaches a maximum within 45 min in the absence of pyridoxal-5′-P, the activation achieved ultimately (after about 90 min) in the presence of a saturating level of pyridoxal-5′-P is almost twice as great.

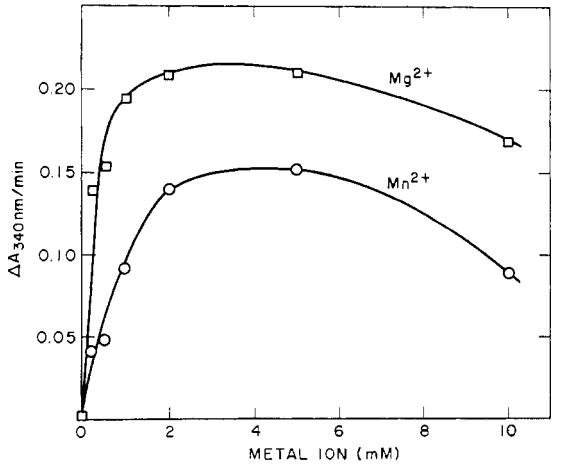

FIGURE 5: Effect of magnesium or manganese ion on activation of the mutase. Experiments were performed under standard assay conditions with 4 μg of β-lysine mutase (specific activity 15) and the indicated amount of divalent cation.

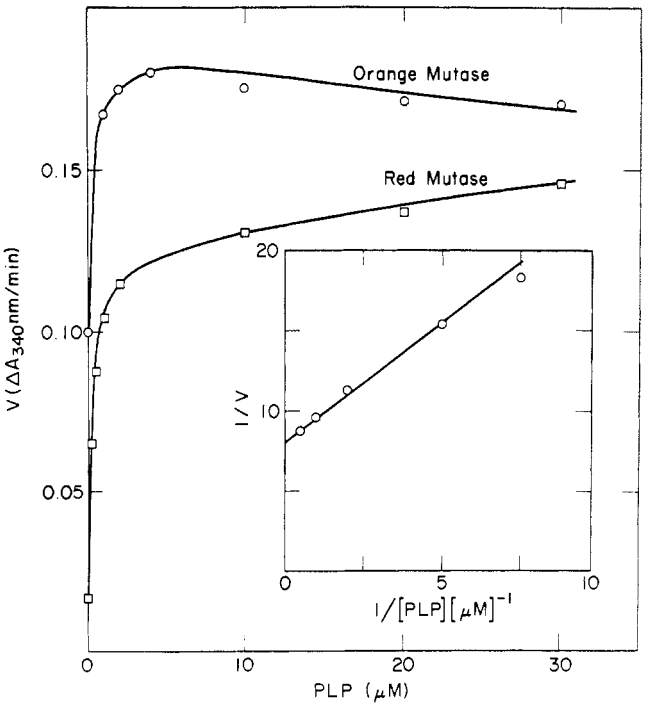

FIGURE 6: Effect of pyridoxal-5′-P concentration on the activation of L-β-lysine mutase. Activation was performed under standard conditions in the presence of the indicated amount of pyridoxal-5′-P. For each determination 2 μg of the normal orange mutase (specific activity 15) or 12.4 μg of the red mutase (specific activity 1.9) was used.

The requirement for pyridoxal-5′-P is specific. Pyridoxal, pyridoxine, pyridoxamine, and pyridoxamine-5′-P have no effect on the activity of the mutase.

Enzymic activity can be made completely dependent on added pyridoxal-5′-P by treatment of the mutase, after activation, with hydroxylamine. Incubation with 10 μM NH_2OH for 15 min at 37° results in complete inhibition of enzyme activity. When this treatment is followed by a 20-min incubation with 50 μM pyridoxal-5′-P, 80% of the activity is restored. Although hydroxylamine is the most effective inhibitor, other carbonyl reagents also inhibit the mutase. Table II shows the concentration of NH_2OH, phenylhydrazine, hydrazine, isonicotinic acid hydrazide, and $NaBH_4$ necessary to give half-maximal inhibition.

Addition of the components of the coupled assay system, NADP, and 3,5-diaminohexanoate dehydrogenase, to the standard prior incubation mixture had no effect on the activation of the enzyme. Similarly, activation of the mutase was not influenced by inclusion of ATP, pyruvate, FAD, and E_2 (a partially purified preparation of the sulfhydryl protein component required for activity in the long-term assay).

Factors Affecting the Catalytic Activity of the Mutase during the Spectrophotometric Assay. Once the β-lysine mutase was converted to its activated form by incubation with DMBC, a thiol, pyridoxal-5′-P, and a divalent metal ion under the conditions described above, there was still little or no activity observed upon the addition of substrate unless a monovalent cation such as K^+ was also present. As shown in Table III, the most effective monovalent cations are K^+, Li^+, and Rb^+; NH_4^+, Na^+, and Cs^+ also stimulate appreciably. Similar effects have been observed in the long-term assay using 2-methylimidazole·HCl buffer rather than the $N(EtOH)_3$·HCl

TABLE II: Effect of Carbonyl Reagents on β-Lysine Mutase Activity.[a]

Compound	Concn of Reagent Needed for Half-maximal Inhibn (μM)
Hydroxylamine	0.5
Phenylhydrazine	1.5
Hydrazine	5.3
Isonicotinic acid hydrazide	100
$NaBH_4$	350

[a] Activation of β-lysine mutase (2 μg/sample; specific activity 15) was carried out under standard conditions except that pyridoxal-5′-P was omitted. Inhibitors were added after activation for 90 min and enzyme activity was assayed spectrophotometrically 10 min after addition of inhibitor.

buffer used here except that Li^+ and Na^+ were relatively ineffective (Bray and Stadtman, 1968).

β-Lysine mutase activity as a function of potassium ion concentration is shown in Figure 8 where it is seen that the optimal concentration is 70–100 mM. The stimulatory effect of KCl (100 mM) is about 20% less if the cation is included in the standard activation mixture instead of being added along with substrate to start the reaction. Although low concentrations (5–10 mM) of NH_4Cl give a 1.5-fold higher initial reaction rate than does 100 mM KCl, the activity of the enzyme falls off more rapidly than it does with K^+ with the result that the total conversion of β-lysine to 3,5-DAH may be as much as 80% less. It thus appears that ammonium ion acts not only as an activator but also as an inhibitor of β-lysine mutase activity.

The activity of β-lysine mutase, as determined spectrophotometrically, is not stimulated by the addition of pyruvate, ATP, FAD, or E_2 or combinations thereof. This is in contrast to the stimulatory effects of these substances on the activity of many enzyme preparations in the long-term β-lysine mutase assay (Stadtman and Renz, 1968). The components of the coupled assay, 3,5-diaminohexanoate dehydrogenase (0.1 unit) and NADP (0.4 mM) are saturating and the initial reaction rate is unaffected by their addition in excess of these amounts.

Inactivation of the Enzyme during Catalysis of the Mutase Reaction. In view of the fact that β-lysine mutase activity as determined by initial rate measurements in the spectrophotometric assay is not further stimulated by the addition of pyruvate, ATP, FAD, or E_2 or combinations thereof, whereas all of these components are usually required for maximum activity in the long-term assay, experiments were designed to test the effects of these substances on continued activity of the enzyme under the conditions of the spectrophotometric assay. The enzyme, activated by a prior incubation with the reagents described in the legend of Figure 9, was then allowed to react with the substrate under the following three conditions: (1) wherein the product accumulated in the absence of E_2 and ATP; (2) wherein the product accumulated in the presence of E_2 and ATP; and (3) wherein the product was removed in the early part of the reaction by the action of 3,5-diaminohexanoate dehydrogenase and NAD. As shown in curve 1 of

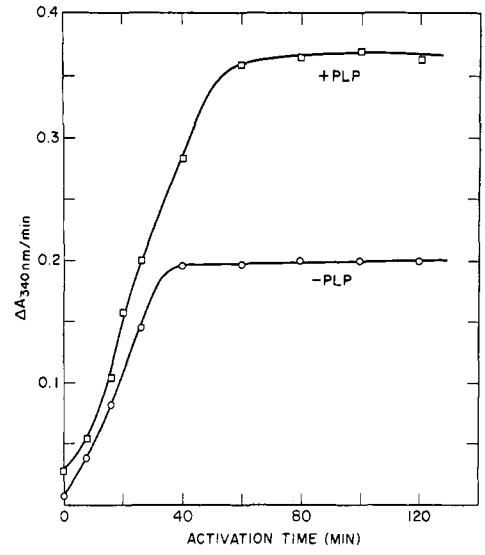

FIGURE 7: Comparison of the extent of activation of β-lysine mutase as a function of time with or without pyridoxal-5′-P. Activation was carried out under standard conditions with (□) and without (○) pyridoxal-5′-P present. Four micrograms of β-lysine mutase (specific activity 15) was used for each determination.

TABLE III: Effects of Monovalent Cations on L-β-Lysine Mutase Activity.[a]

Addns	$\Delta A_{340\,nm}$/min
None	0.03
LiCl	0.282
NaCl	0.155
KCl	0.310
RbCl	0.280
CsCl	0.139
NH_4Cl	0.222

[a] Each sample contained 4 μg of β-lysine mutase (specific activity 15) that had been activated as described under Methods. The reaction was started by the addition of β-lysine (1 mM) and the desired cation (100 mM).

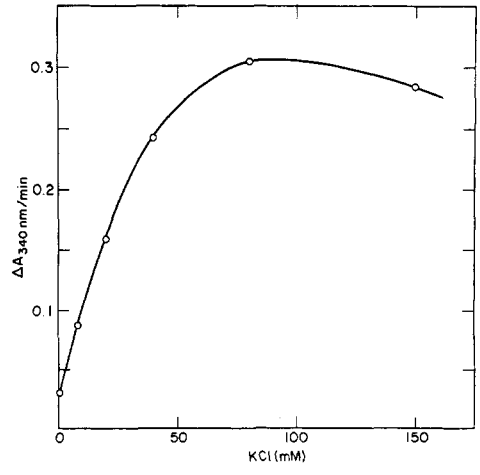

FIGURE 8: Effect of KCl concentration on the mutase activity. Activity was determined as described under Methods. Each reaction mixture contained 2 μg of β-lysine mutase (specific activity 15) and the indicated KCl concentration.

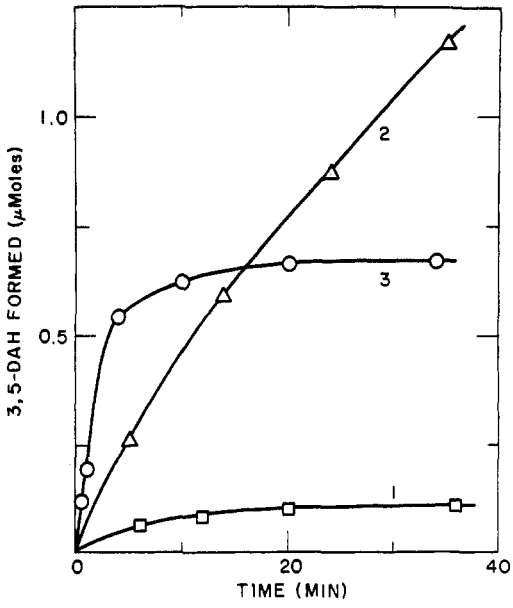

FIGURE 9: Effect of E_2, ATP, and NAD on the continued conversion of β-lysine to 3,5-DAH in the presence of 3,5-DAH-dehydrogenase. For each of the three experiments, 120 μg of β-lysine mutase (specific activity 14) was activated in an open 1-ml cuvet for 30 min at 37° in the dark in 1 ml of a pH 8.1 solution containing 100 mM 2-methylimidazole hydrochloride, 3.8 μM DMBC, 2 mM dithiothreitol, 5 mM MgCl, and 0.2 unit of 3,5-DAH-dehydrogenase. For expt 1 (□), 20 mM β-lysine was added to start the reaction and, at the indicated times, 50-μl aliquots were removed for determination of DAH by the spectrophotometric assay method of Baker *et al.* (1972). Experiment 2 (△) was done as expt 1, except 4 mM ATP and 0.65 mg of E_2 were added with the β-lysine. For expt 3 (○), 0.4 mM NAD was added with the β-lysine. In this sample, which contained both NAD and DAH-dehydrogenase, the amino acid product did not accumulate until NAD became limiting.

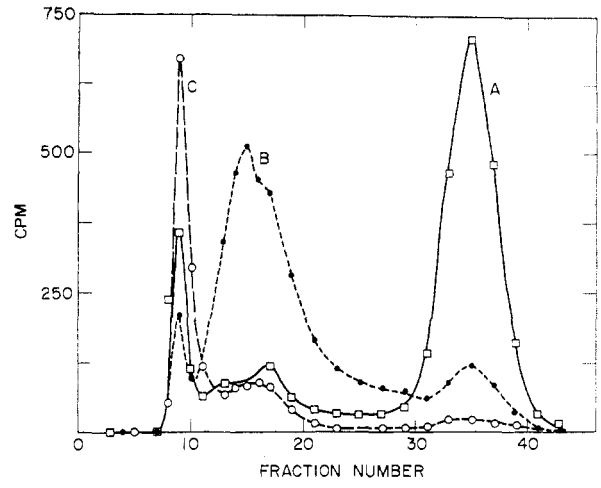

FIGURE 10: Separation of ^{14}C-labeled β-lysine mutase, DMBC, and a deoxyadenosyl fragment by molecular sieve chromatography on Sephadex G-15. The experimental details for curve A (□), curve B (●), and curve C (○) are given in the text.

Figure 9 very little β-lysine was converted to 3,5-DAH by the unsupplemented, activated enzyme and this occurred only in the first few minutes of the reaction. In contrast a rapid rate of reaction was observed in the coupled enzyme system (curve 3) as long as NAD remained for oxidation of the product, but when the NAD became limiting and 3,5-DAH accumulated, the reaction ceased. Under conditions similar to those used for the long-term assay, *e.g.*, when ATP and E_2 were added together with the substrate (curve 2), product formation continued for the duration of the experiment although the rate was not as rapid initially as in the coupled enzyme system. Hence it appears that in order to maintain activity of β-lysine mutase for continued catalysis of the reaction, either the product, 3,5-DAH, must be removed continuously by oxidation to 3-keto-5-aminohexanoate or E_2 protein and/or ATP must also be present in the reaction mixture. In separate experiments it was determined that NADH generated in the coupled assay did not stimulate the mutase reaction. With the enzyme and the conditions used for the experiments of Figure 9, there was no effect of FAD and pyruvate.

Liberation of 5′-Deoxyadenosine from Enzyme-Bound DMBC. One plausible explanation of the rapid cessation of the mutase reaction observed in Figure 9 (curves 1 and 3) would be destruction of enzyme-bound DMBC with concomitant conversion of the mutase to an inactive form. To investigate this possibility the stability of enzyme-bound DMBC, labeled with ^{14}C in the deoxyadenosyl moiety, was tested in various types of reaction mixtures. The labeled enzyme was prepared as described under Methods. Aliquots were incubated as described below, and then examined for retention or loss of coenzyme by chromatography on Sephadex G-15. Three aliquots of the labeled enzyme were incubated in 1-ml reaction mixtures at 37° in the dark for 17 min. Sample A contained 0.33 mg of mutase (6600 cpm) in 2 mM dithiothreitol, 5 mM $MgCl_2$, 1 mM L-β-lysine, 5 mM $(NH_4)_2SO_4$,[2] and 70 mM $N(EtOH)_3 \cdot HCl$ buffer (pH 7.8). Sample B was the same as A except that 0.27 mg of mutase (5400 cpm) was used and L-β-lysine and $(NH_4)_2SO_4$ were omitted. Sample C contained 0.12 mg of mutase (2400 cpm) in 15 mM EDTA–7.5% glycerol buffer (pH 6.8). After incubation each solution was applied to a Sephadex G-15 column (1.2 × 22 cm) and the ^{14}C-containing compounds were eluted with water except for sample C which was eluted with 20 mM EDTA–10% glycerol. The elution profiles (Figure 10) show that in the presence of β-lysine and NH_4^+ (curve A) most of the ^{14}C appeared in the fractions where low molecular weight substances such as adenosine occur. In the absence of β-lysine and NH_4^+ (curve B) most of the ^{14}C was eluted from the column earlier in the region where free authentic DMBC appears. When the enzyme was kept in EDTA–glycerol rather than in water throughout the experiment (curve C), the ^{14}C remained with the protein and was eluted in the exclusion volume of the column.

To establish that the low molecular weight labeled compound cleaved from the enzyme-bound DMBC in the presence of substrate was, as suspected, 5′-deoxyadenosine, fractions 30–40 containing the ^{14}C from sample A (Figure 10) were concentrated and examined chromatographically as described by Babior (1970b). Figure 11 shows that almost all of the ^{14}C cochromatographed with authentic 5′-deoxyadenosine. Thus under conditions where the mutase catalyzes conversion of β-lysine to 3,5-DAH, there is extensive cleavage of DMBC to yield 5′-deoxyadenosine which is liberated from the protein.

Role of E_2 and ATP. Since a rapid inactivation of β-lysine mutase with concomitant loss of the deoxyadenosyl group occurs during the catalytic reaction, it seems likely that the stimulatory effects of ATP and E_2 on mutase activity (curve 2, Figure 9 and Stadtman and Renz, 1968) might be due at least in part to regeneration of DMBC by B_{12} coenzyme synthetase

[2] Ammonium ion was used as the monovalent cation in this experiment.

TABLE IV: Effect of Heat Treatment on the Ability of Partially Purified E_2 to Activate the Mutase or to Act as Coenzyme Synthetase.[a]

Addn	DAH Formed (mM)	cpm Bound Covalently Mutase
None	1.0	200
E_2	1.38	6200
E_2 heated	0.8	6500

[a] The heat treatment was 10 min at 60°. All incubations were done under argon. The reaction solutions used to determine the ability of E_2 to activate the mutase contained the standard activation mixture, 51 μg of E_1 (specific activity 19.5), 2 mM ATP, and where indicated, 55 μg of E_2. After the normal activation time, 20 μmol of β-lysine (20 μl) and 80 μmol of KCl (20 μl) were added, and the solution was incubated for 1.5 hr. The enzymic reaction was stopped by chilling to 0°, and the amount of 3,5-DAH was measured spectrophotometrically as described in Figure 9. The reaction solutions used to determine the coenzyme synthetase activity contained 48 μM [8-^{14}C]ATP (2.5 μCi), 260 μg of E_1, 8 mM dithiothreitol, 5 mM $MgSO_4$, 40 μM pyridoxal-5′-P, 35 mM $N(EtOH)_3$ (pH 7.8), and where indicated, 110 μg of E_2. The final volume was 1 ml. After incubation at 37° for 1.5 hr, the cpm covalently bound to the mutase were determined as follows. The reaction solution was chilled to 0° and glycerol was added to 10% (v/v). The solution was passed over a DEAE-cellulose column (0.6 × 4 cm) equilibrated with a pH 6.8 buffer composed of 20 mM EDTA–10% glycerol. The column was washed with three column volumes of a pH 6.8 buffer composed of 20 mM EDTA, 10% glycerol, and 0.125 M NaCl. This wash removed E_2 from the column, but not the mutase. The mutase was removed by eluting the column with 1 M NaCl dissolved in the pH 6.8 buffer. The red-orange mutase (0.5 ml or less) was separated from residual low molecular weight ^{14}C compounds by chromatography on a Sephadex G-25 column (1.2 × 25 cm). The buffer used for the elution was 20 mM NaEDTA–10% glycerol (pH 6.8). The cpm eluting in the void volume with the protein were taken as the amount of [^{14}C]DMBC bound to the mutase. The ^{14}C emerging with the protein was identified as [^{14}C]DMBC by demonstrating (as described for expt 10) that the counts are released as 5′-deoxyadenosine in the presence of β-lysine and as DMBC in the absence of β-lysine. E_2 interferes with the Sephadex G-25 separation from [^{14}C]ATP and must be removed by the DEAE procedure.

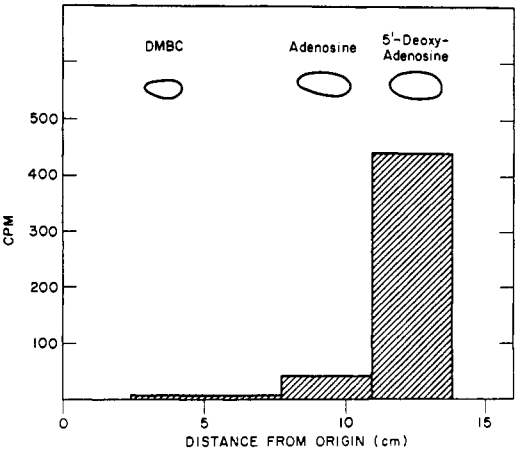

FIGURE 11: Chromatographic comparison of the unknown labeled compound derived from the deoxyadenosyl moiety of DMBC with authentic DMBC, adenosine, and 5′-deoxyadenosine. Descending chromatography on Whatman No. 1 paper in 1-butanol–acetic acid–water (4:1:5, v/v, upper layer).

in the E_2 preparations. *C. sticklandii* is an especially rich source of B_{12} coenzyme synthetase and most E_2 preparations exhibit this activity. However, it was noted in earlier experiments that the stimulatory effect of E_2 on β-lysine mutase activity was destroyed by brief heating at 55–60° whereas the B_{12} coenzyme synthetase from *Clostridium tetanomorphum* is not inactivated when heated at 60° for 10 min (Vitols *et al.*, 1966). Therefore the possibility that the E_2 and B_{12} coenzyme synthetase activities of the E_2 preparations derived from *C. sticklandii* might be differentiated on the basis of their sensitivity to heat was tested.

In the experiments shown in Table IV the effect of heat treatment on the ability of an E_2 preparation to (1) activate the mutase or (2) synthesize enzyme-bound [^{14}C]DMBC from [^{14}C]ATP was compared. The untreated E_2 preparation stimulated 3,5-diaminohexanoate formation and also catalyzed the synthesis of an appreciable amount of mutase-bound ^{14}C-labeled DMBC. The heated E_2 preparation formed the same amount of ^{14}C-labeled DMBC on the mutase protein but failed to stimulate β-lysine conversion to 3,5-diaminohexanoate. In a similar experiment in which [γ-^{32}P]ATP was substituted for [^{14}C]ATP, the mutase was not labeled with ^{32}P.

The stability of B_{12} coenzyme synthetase to the heat treatment was also established in assays wherein the amount of [^{14}C]DMBC formed from free hydroxycobalamin and [^{14}C]-ATP was determined following its isolation by thin-layer chromatography (see Methods). Parallel samples were prepared in which the hydroxycobalamin was reduced either with dithiolthreitol plus FAD or with dithiothreitol, FAD, and 4 mM KBH_4. The extent of the reaction was measured both in the presence and in the absence of borohydride in view of the report that a separate labile reductase protein is required to reduce hydroxycobalamin to B_{12s} when only thiols and FAD are supplied as a reducing system (Vitols *et al.*, 1966). As shown in Table V, B_{12} coenzyme synthetase activity was unaffected by heat treatment of the E_2 preparation and neither the heated nor the untreated enzyme was stimulated by the inclusion of borohydride. However, in these experiments also the heat treatment destroyed completely the activating effect of the E_2 preparation on β-lysine mutase activity.

Discussion

Purification of β-lysine mutase has been followed using two different assay procedures. Originally (Stadtman and Renz, 1968) the amount of 3,5-DAH formed from β-lysine during a 90-min incubation period was measured by the acid ninhydrin method of Chinard. The spectrophotometric method reported in this paper, which measures only the initial rate of the mutase-catalyzed reaction, is more rapid and much more sensitive. The specific activity of the mutase as determined in the spectrophotometric assay is significantly higher, particularly after a preliminary incubation of the enzyme with

TABLE V: Effect of Heat Treatment on Ability of E_2 Preparation to Stimulate β-Lysine Mutase and to Synthesize B_{12} Coenzyme.[e]

E_2 Added	3,5-DAH[a] Formed in β-Lysine Mutase Assay		DMBC[b] Formed in Coenzyme Synthetase Assay	
	$-KBH_4$ (μmol)	$+KBH_4$ (μmol)	$-KBH_4$ (cpm)	$+KBH_4$ (cpm)
None	0.51		0	0
Untreated E_2				
46 μg	0.95		735	795
92 μg	1.82	1.16	1380	*c*
Heated E_2[d]				
46 μg	0.48		870	855
92 μg	0.47	0.41	1330	1440

[a] Total 3,5-diaminohexanoate formed in long term assay. FAD (40 μM) was included in each assay mixture. [b] Total [^{14}C]DMBC formed from [8-^{14}C]ATP; determined directly on uniform area spots cut from thin layer sheets using a Geiger-Müller end window counter (cpm not corrected for self-absorption). [c] Sample lost. [d] Heated 10 min at 58–60° in 25 mM K_2HPO_4; protein concentration, 1.53 mg/ml. [e] All samples were incubated 100 min at 36° in the dark under argon. Where indicated, KBH_4 (4 mM) was added.

DMBC, pyridoxal-5′-P, Mg^{2+}, and a mercaptan. Moreover, under the conditions of the spectrophotometric assay, there is no dependency on several factors, namely E_2, ATP, FAD, and pyruvate, which stimulate mutase activity when the product is allowed to accumulate in the long-term incubations.

Pure preparations of β-lysine mutase are bright orange in color when isolated in the presence of glycerol, but when glycerol is omitted from the buffer solutions, the resulting mutase preparations are red in color. The spectrum exhibited by the latter in the long uv and visible range is typical of hydroxycobalamin but the orange mutase shows an additional prominent absorption peak in the visible region at 470 nm (Figure 1). The absorbancy at 470 nm is at too long a wavelength to be assigned to pyridoxal-5′-P which is also bound to the enzyme. Although the red mutase preparations exhibit greater dependency on pyridoxal-5′-P and presumably contain less of this factor, any contribution of pyridoxal-5′-P to the spectrum of either preparation is completely masked by the cobamide. A similar absorbancy peak at 470 nm is exhibited by certain isolated preparations of ethanolamine deaminase (Kaplan and Stadtman, 1968) and methionine synthetase (Weissbach and Taylor, 1967). For both of these enzymes it was suggested that interaction of bound cobamide with a sulfhydryl group on the protein might account for the peak at 470 nm. If this is the case with β-lysine mutase, only a small percentage of the bound cobamide interacts because the 353-nm absorbancy peak which is typical of a hydroxycobamide clearly dominates the spectrum (Figure 1).

In spite of precautions taken to protect the mutase from exposure to light or to acidic pH during the isolation procedure, preparations always contain considerable amounts of bound hydroxycobamide which must be exchanged for DMBC to achieve full activity. Not only is this activation process affected by the time and temperature of incubation, the nature of the buffer and pH (Figure 3), but also it is totally dependent on DMBC, dithiothreitol, Mg^{2+}, and pyridoxal-5′-P. Although the precise roles of dithiothreitol and Mg^{2+} are not known, it is possible that by reducing the bound hydroxycobamide they facilitate its displacement from the enzyme. Because of the low K_m for pyridoxal-5′-P (0.2 μM), complete dependence upon pyridoxal-5′-P can be demonstrated only after treatment of the mutase with a carbonyl group inhibitor such as hydroxylamine. The partially dependent enzyme preparation shown in Figure 7 required the addition of pyridoxal-5′-P to the activation mixture in order to observe stimulation (1.7-fold) of mutase activity. If instead the pyridoxal-5′-P was added after the activation step, either before or with the β-lysine to start the reaction, it was completely ineffective. It is possible that binding of pyridoxal-5′-P to the mutase also facilitates displacement of the inhibitory hydroxycobamide with concomitant activation of the enzyme.

The pyridoxal-5′-P requirement is specific; all other related compounds tested were inactive and they did not act as competitive inhibitors. The extreme sensitivity of the mutase to hydroxylamine, hydrazine, phenylhydrazine, isonicotinic acid hydrazide, and $NaBH_4$ indicates that Schiff base formation with the aldehyde group of pyridoxal-5′-P is essential to catalysis of the mutase reaction.

Once the mutase has been activated, additional dithiothreitol, Mg^{2+}, and DMBC do not appear to be directly required for catalytic activity. Removal of the excess of any of the three by dilution of concentrated, activated enzyme into a standard activation solution that is missing the cofactor being tested, has no effect on the initial reaction rate. However, the rate decays exponentially to zero within 1 min; the latter phenomenon is typical of oxygen inactivation which can be prevented by doing the assay under anaerobic conditions. The mutase is sensitive to oxygen only during catalysis.

Experiments of the type shown in Figure 9 (to be reported in more detail elsewhere) indicate that it is primarily the product, 3,5-diaminohexanoate, which inactivates the mutase. When the product itself is added or when the reaction is allowed to proceed under conditions where the product accumulates, the enzyme is inactivated even under anaerobic conditions and this is accompanied by the cleavage of the carbon–cobalt bond of enzyme-bound DMBC. In the experiments of Figure 10 it was shown that DMBC labeled in the deoxyadenosyl moiety was cleaved to [^{14}C]5′-deoxyadenosine and this readily dissociated from the enzyme. This is of particular interest since evidence is accumulating with other cobamide coenzyme dependent enzymes (Babior, 1970a,b; Yamada *et al.*, 1971; Brodie *et al.*, 1972; Finley *et al.*, 1972) that the hydrogen-carrying function of the coenzyme requires breaking and remaking of the carbon–cobalt bond with the transient formation of 5′-deoxyadenosine and B_{12r}. The other enzymes investigated so far, in contrast to β-lysine mutase, maintain the deoxyadenosine moiety tightly bound to the enzyme and it is displaced only when the protein is denatured. Although there is no clearcut evidence as yet on this point, the role of the E_2 protein and possibly also ATP, which are both required in the long-term assay of mutase activity or for continued turnover in the spectrophotometric assay, may be to maintain the enzyme in a form which prevents dissociation of 5′-deoxyadenosine during the catalytic event.

The monovalent cation requirement, which is particularly pronounced in the spectrophotometric assay of β-lysine mutase activity, may be related to maintenance of an active conformational form of the enzyme. Although a monovalent cation has been reported (Toraya *et al.*, 1971) to be required

for the binding of DMBC to diol dehydratase, a direct effect on the binding of DMBC to β-lysine mutase seems less likely since potassium addition is most effective after the enzyme is activated rather than in the process during which DMBC is bound.

Unlike diol dehydratase (Toraya *et al.*, 1971), the relative effectiveness of the various monovalent cations in stimulating β-lysine mutase activity correlates poorly with their ionic radii. Although the relative activities of K^+, Rb^+, Na^+, and Cs^+ do correlate well there is appreciable deviation with Li^+ and NH_4^+. Li gives a higher value than expected. At low concentrations, ammonium ion also is more effective than would be predicted on the basis of its size. At high concentrations ammonium ion is inhibitory.

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Inhibition of Pancreatic Ribonuclease A by Arabinonucleotides†

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ABSTRACT: Changes in the carbohydrate, the base, or the phosphate component of nucleosidic or nucleotidic ligands to pancreatic ribonuclease A have been evaluated. The association constants with ribonuclease A of various compounds which can be obtained by combinations of uracil, cytosine, ribose, arabinose, and the phosphate group were determined. The hydrolysis of Cyd-2′:3′-P was studied spectrophotometrically at 25°, pH 7.0, at an ionic strength of 0.2. Kinetic rate constants were determined at three substrate concentrations and four inhibitor concentrations. In general, higher K values were obtained for the arabino derivatives than for the ribo derivatives, the highest being that of ara-Cyt-3′-P. These findings which can be interpreted primarily by geometric considerations add new emphasis to the importance of the 2′-hydroxyl group in the mechanism of RNase binding.

Knowledge of the tertiary crystalline structure of pancreatic ribonuclease (Kartha *et al.*, 1967; Wyckoff *et al.*, 1967) has undoubtedly contributed to the continued interest in the mechanism of action of this enzyme. Inhibition studies have proven very useful to the understanding of how ligands bind to the active center. The possible inhibition of RNase action during the isolation of various RNAs also presents an important practical aspect of such studies. It was found that small inorganic anions, particularly phosphate and nitrate (Nelson *et al.*, 1962; Anderson *et al.*, 1968), exhibit a moderate binding to RNase by virtue of Coulombic interactions with the active site. More effective inhibitors are the polyanions such as polyphosphates (Anderson *et al.*, 1968) and polyglucose sulfate (Mora, 1962). Not unexpectedly, the best inhibitors of small molecular weight are the mononucleotidic end products of RNA hydrolysis. The association constants of these compounds with RNase have been determined by several authors (Anderson *et al.*, 1968; Barnard and Ramel, 1962; Harries *et al.*, 1962; Hummel and Dryer, 1962).

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