Purification and Characterization of Avian Liver Mevalonate-5-pyrophosphate Decarboxylase†

Marysol Alvear,‡ Ana María Jabalquinto,§ Jaime Eyzaguirre, and Emilio Cardemil*

ABSTRACT: Mevalonate-5-pyrophosphate decarboxylase [ATP:5-diphosphomevalonate carboxy-lyase (dehydrating), EC 4.1.1.33] has been purified 5800 times from chicken liver and obtained in a stable and highly purified form. The protein is a dimer of molecular weight 85400 ± 1941, and its subunits were not resolved by gel electrophoresis in denaturing conditions. The purified enzyme does not require the presence of SH-containing reagents for either activity or stability. The enzyme shows a high specificity for adenosine 5′-triphosphate (ATP) and requires for activity a divalent metal cation, Mg^{2+} being most effective. The optimum pH for the enzyme ranges from 4.0 to 6.5. Inhibitory effects for the enzyme activity were detected by citrate, phthalate, and phosphate. The isoelectric point, as determined by column chromatofocusing, is 4.8. The kinetics are hyperbolic for both substrates, showing a sequential mechanism; true K_m values of 0.0141 mM and 0.504 mM have been obtained for mevalonate-5-pyrophosphate and ATP, respectively.

One of the first steps in the biosynthesis of cholesterol from mevalonate is catalyzed by mevalonate-5-pyrophosphate decarboxylase [ATP:5-diphosphomevalonate carboxy-lyase (dehydrating), EC 4.1.1.33]. The decarboxylase catalyzes a bimolecular reaction between MVAPP[1] and ATP to form isopentenyl pyrophosphate, inorganic phosphate, ADP, and CO_2. There is no agreement on the mechanism of this reaction (Beytía & Porter, 1976), and the studies directed to this problem have been hampered by the lack of a stable and highly purified preparation. Physiological studies, however, have been performed with the enzyme; it has been shown that its activity decreases, as does hepatic cholesterogenesis, when rats are fasted, made diabetic, or fed cholesterol and increases when rats are fed cholestyramine (Jabalquinto & Cardemil, 1981, 1982).

The enzyme has been partially purified from yeast (Bloch et al., 1959), latex of *Hevea brasiliensis* (Skilleter & Kekwick, 1971), pig liver (Hellig & Popják, 1961; Popják, 1969), and recently rat liver (Shama Bhat & Ramasarma, 1980). This last preparation is homogeneous but extremely unstable. We report here a procedure for the isolation of the enzyme from chicken liver in a stable and highly purified state, as well as some of its properties. This preparation has a specific activity of 6.3 μmol min^{-1} (mg of protein)$^{-1}$; this value is approximately 100-fold greater than the specific activity reported for the homogeneous rat liver decarboxylase (Shama Bhat & Ramasarma, 1980). A brief description of some of these results has been given earlier (Alvear et al., 1980).

Experimental Procedures

Materials. Nucleotides, lactic dehydrogenase, pyruvate kinase, protamine sulfate grade X, Blue Dextran, cytochrome *c*, enzyme grade $(NH_4)_2SO_4$, marker proteins for molecular weight determination by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, Tris base, and Mes were from Sigma Chemical Co. Acrylamide, bis(acrylamide), Temed, glycine, ammonium persulfate, bromophenol blue, Coomassie blue, and hydroxylapatite were supplied by Bio-Rad Laboratories. Sephadex G-200, PBE-94, and Polybuffer 74 were from Pharmacia Fine Chemicals. DEAE-cellulose (DE-22) and phosphocellulose were from Whatman. Blue Dextran–Sepharose was a gift of Dr. M. A. Valenzuela (Universidad de Chile). The synthesis of MVAPP and the source of all other materials have been described earlier (Jabalquinto & Cardemil, 1980).

Spectrophotometric Assay. The reaction mixture (1 mL final volume) contained 0.1 M Tris-HCl buffer, pH 7.0, 0.1 M KCl, 5 mM ATP, 5 mM $MgCl_2$, 0.5 mM PEP, 0.23 mM NADH, 6.5 units of pyruvate kinase, 11.8 units of lactic dehydrogenase, MVAPP decarboxylase, and 0.071 mM MVAPP, added to start the reaction. The spectrophotometric assays were performed at 30 °C in a Perkin-Elmer 550 spectrophotometer. This method cannot be used at pH values below 5 (Lowry et al., 1961) or in crude preparations due to the presence of NADH oxidases and phosphatase activities.

Radioactive Assay. The reaction mixture (0.6 mL final volume) contained 0.1 M Tris-HCl buffer, pH 7.0, 5 mM ATP, 5 mM $MgCl_2$, 0.41 mM [3-^{14}C]MVAPP (2.45×10^5 cpm/μmol), and MVAPP decarboxylase. The reaction mixture was incubated at 30 °C for 10 min in rubber-stoppered 15-mL centrifuge tubes. The reaction was stopped by the addition of 5.5 units of bovine intestine alkaline phosphatase in 0.5 mL of 1 M Tris-HCl buffer, pH 8.4, and further incubated for 2 h at 30 °C. The incubation mixture was then extracted twice with 1.0 mL of petroleum ether (bp 40–60 °C). The radioactivity was measured as previously described (Jabalquinto & Cardemil, 1980). One unit of enzyme activity was defined as the amount of enzyme required to decarboxylate 1 μmol of MVAPP/min under the assay conditions.

Enzyme Purification. All steps were performed at 4 °C. Unless otherwise indicated, the buffer used in the purification was potassium phosphate buffer (pH 7.0)–10 mM 2-mercaptoethanol–0.1 mM EDTA.

† From the Facultad de Medicina, Universidad de Chile, Santiago, Chile (A.M.J., M.A., and E.C.), the Departamento de Química, Universidad de Santiago de Chile, Casilla 5659, Correo 2, Santiago, Chile (A.M.J. and E.C.), and the Laboratorio de Bioquímica, Universidad Católica de Chile, Casilla 114-D, Santiago, Chile (J.E.). *Received May 7, 1982.*

* Address correspondence to this author at the Departamento de Química, Universidad de Santiago de Chile, Casilla 5659, Correo 2, Santiago, Chile.

‡ Present address: Laboratorio de Bioquímica, Universidad Católica de Chile, Casilla 114-D, Santiago, Chile.

§ Present address: Departamento de Química, Universidad de Santiago de Chile, Casilla 5659, Correo 2, Santiago, Chile.

[1] Abbreviations: MVAPP, mevalonate 5-pyrophosphate; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane; Mes, 2-(*N*-morpholino)ethanesulfonic acid; Temed, tetramethylethylenediamine; DEAE, diethylaminoethyl; PEP, phosphoenolpyruvate.

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(*a*) *Crude Extract.* Chicken livers were obtained immediately after slaughter, brought to the laboratory on ice, and frozen. Frozen livers (1 kg) were cut into small pieces and homogenized in a Waring Blendor in 3 L of 0.1 M buffer–1 mM EDTA. After filtration through cheesecloth and glass wool, a 30 mg/mL solution of protamine sulfate was added to the homogenate to a final concentration of 1.5 mg/mL and was stirred for 2 min. The slurry was centrifuged for 15 min at 23500*g*, and the clear supernatant was decanted and filtered through cheesecloth and glass wool. The pH of the supernatant was adjusted to 5.3 with glacial acetic acid and immediately centrifuged at 23500*g* for 5 min. The supernatant was filtered through cheesecloth and glass wool and its pH was readjusted to 7.0 with 10 M KOH.

(*b*) *Ammonium Sulfate Fraction.* Solid $(NH_4)_2SO_4$ was added to the supernatant to 35% saturation, the pH being maintained constant at 7.0 with 10 M KOH. After being stirred for 30 min, the preparation was centrifuged at 23500*g* for 15 min. The supernatant was brought to 60% saturation with solid $(NH_4)_2SO_4$, stirred for 30 min, and centrifuged as before. The precipitate was resuspended in 400 mL of 1 mM buffer and dialyzed for 18 h against 8 L of the same buffer.

(*c*) *DEAE-cellulose Column Chromatography.* The dialyzate was diluted with 1 mM buffer to a conductivity of 1.5 mS and applied to a DEAE-cellulose column (6.5 × 27 cm) equilibrated with 10 mM buffer. The column was washed with 4 L of 50 mM buffer, and the enzyme was eluted with a linear gradient of 2 L each of 60 and 140 mM buffer; 20-mL fractions were collected. The active fractions were pooled (EDTA was added to a final concentration of 1 mM), and the enzyme was precipitated by addition of $(NH_4)_2SO_4$ to 80% saturation. After centrifugation for 15 min at 23500*g*, the precipitate was resuspended in 50 mL of 10 mM buffer containing 0.2 M KCl.

(*d*) *Sephadex G-200 Gel Filtration.* The DEAE-cellulose fraction was applied to a Sephadex G-200 column (5 × 92 cm) equilibrated with 10 mM buffer containing 0.2 M KCl and then washed with the same buffer. Fractions of 20 mL were collected at an upward flow rate of 50 mL/h. The active fractions were combined (EDTA was added to a final concentration of 1 mM), and the enzyme was precipitated by addition of $(NH_4)_2SO_4$ to 75% saturation. After centrifugation at 23500*g* for 15 min, the precipitate was resuspended in 5 mM buffer and dialyzed for 18 h against 2 L of the same buffer. Glycerol was added to the dialyzate to a final concentration of 20%, the pH of this solution was adjusted to 6.0 with concentrated HCl, and the solution was then centrifuged for 10 min at 23500*g*. The supernatant was diluted with 1 mM buffer (pH 6.0)–20% glycerol to a conductivity of 0.7 mS.

(*e*) *Phosphocellulose Column Chromatography.* The enzyme was applied to a phosphocellulose column (3.7 × 21 cm) equilibrated with 10 mM buffer (pH 6.0)–20% glycerol. The column was washed with 350 mL of the same buffer, and the enzyme was eluted with a linear gradient of 750 mL each of 20 and 250 mM buffer (pH 6.0)–20% glycerol. Fractions of 18 mL were collected at a flow rate of 60 mL/h. The pH of the pooled active fractions was adjusted to 7.0 with 10 M KOH, and the fractions were concentrated by ultrafiltration. The concentrated enzyme was diluted with 1 mM buffer and again concentrated by ultrafiltration. Glycerol was added to a final concentration of 20%, and the conductivity of the enzyme solution was adjusted with buffer to 15% above the conductivity of the hydroxylapatite column (see below).

(*f*) *Hydroxylapatite Column Chromatography.* The volume of the hydroxylapatite column was calculated according to the following ratio: milliliters of hydroxylapatite/milligrams of protein = 0.4. The enzyme was applied to a hydroxylapatite column equilibrated with 13 mM buffer–20% glycerol. The column was washed with the same buffer, and 1.2-mL fractions were collected. The enzyme is obtained in this washing.

(*g*) *Blue Dextran–Sepharose Column Chromatography.* The enzyme was applied to a column (2.5 × 10 cm) of Blue Dextran–Sepharose prepared according to Ryan & Vestling (1974), equilibrated with 10 mM Tris-HCl buffer (pH 7.0)–20% glycerol–0.1 mM EDTA, without 2-mercaptoethanol. The column was washed with the same buffer until the absorbance at 280 mm was 0. The enzyme was eluted with 75 mM KCl in the same buffer as above, and 5-mL fractions were collected. The active fractions were pooled and concentrated by ultrafiltration. The enzyme was stored frozen at −20 °C.

Protein Determination. For crude extracts the biuret method was used. Otherwise, the micromethod of Bensadoun & Weinstein (1976) was employed.

Polyacrylamide Gel Electrophoresis. Electrophoresis and staining of native proteins were performed as described by Gabriel (1971). Separating gels contained 7.5% acrylamide; the stacking gel was omitted. MVAPP decarboxylase activity in the gels was measured by the radioactive method. Sodium dodecyl sulfate electrophoresis was performed according to Weber & Osborn (1969) in 10% acrylamide gels.

Isoelectric Point Determination. The isoelectric point was determinated by chromatofocusing (Sluyterman & Elgersma, 1978). A column (1 × 4 cm) of PBE-94 was equilibrated with 25 mM imidazole buffer (pH 6.4)–0.1 mM EDTA. The enzyme, previously dialyzed against the same buffer, was applied to the column at a flow rate of 50 mL/h. The enzyme was eluted with 100 mL of Polybuffer 74, pH 4.0. Fractions of 3 mL were collected, and immediately after the pH of these fractions was measured, 0.5 mL of 1 M potassium phosphate buffer, pH 7.0, was added. The activity of the enzyme was measured by the spectrophotometric assay.

Molecular Weight Determination. Three methods were used. The first method was gel filtration in a Sephadex G-200 column, according to Andrews (1965). A column (2.6 × 83 cm) was equilibrated with 25 mM potassium phosphate buffer (pH 7.0)–0.2 M KCl–0.1 mM EDTA. Separate samples of cytochrome *c* (M_r 12400), bovine serum albumin (M_r 67000), yeast alcohol dehydrogenase (M_r 141000), rabbit muscle pyruvate kinase (M_r 237000), or MVAPP decarboxylase were applied to the column. Cytochrome *c* and bovine serum albumin were determined spectrophotometrically at 412 and 280 nm, respectively. Alcohol dehydrogenase and pyruvate kinase activities were determined spectrophotometrically according to Vallee & Hoch (1955) and to Cardemil & Eyzaguirre (1979), respectively. MVAPP decarboxylase activity was determined by the spectrophotometric assay. The second method was sucrose gradient centrifugation. Gradients were prepared from 5 to 20% in potassium phosphate buffer (pH 7.0)–0.1 M KCl–0.1 mM EDTA. Centrifugation was performed at 135000*g* in a Beckman Model L 5 40 centrifuge. The same standards as those in the gel filtration method were used. The third method was sodium dodecyl sulfate–polyacrylamide gel electrophoresis according to Weber & Osborn (1969); cross-linked hemoglobin (M_r 16000–64000) was used as a standard.

Results

Enzyme Purification. Purification of chicken liver MVAPP decarboxylase was carried out as described in Table I. The enzyme activity could not be detected with the spectrophotometric assay before the DEAE-cellulose step because of the

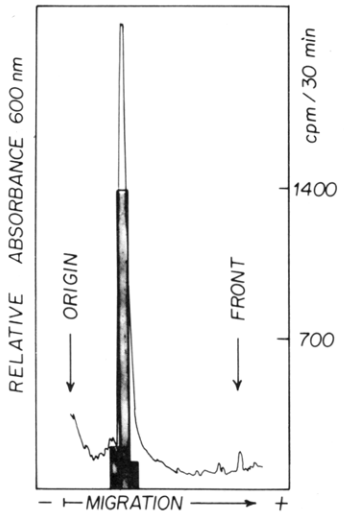

FIGURE 1: Polyacrylamide gel electrophoresis of MVAPP decarboxylase and localization of enzyme activity in the gel. 10 μg of protein of the Blue Dextran–Sepharose chromatography step was applied to two gels. One was stained for protein, and scanning was performed at 600 mm in a Varian-Techtron 635 spectrophotometer. A duplicate unstained gel was sliced into 0.33-cm sections. Each slice was cut twice and assayed radiochemically for enzyme activity as described under Experimental Procedures except that 0.024 mM [2-^{14}C]MVAPP (2.93 10^6 cpm/μmol) was used and the assay was run for 30 min.

presence of contaminating enzymes. Several preparations, however, indicate that when the radioactive method of assay in these early steps is used, the recovery of enzyme units and the purification of the enzyme before the DEAE-cellulose column are 51% and 50-fold, respectively. When these values are taken into account, the final yield of the purification procedure is 4.8% and the total purification factor is 5800. The enzyme could not be eluted from the Blue Dextran–Sepharose column by 1 mM ATP, suggesting that the binding of the enzyme may not be specific. Several commercially available ATP–agaroses were found to be ineffective as purification steps, since the enzyme did not bind to any of them.

Purity of the Enzyme. The purity was established by polyacrylamide gel electrophoresis. Figure 1 shows that a homogeneous preparation is obtained after Blue Dextran–Sepharose chromatography. The figure also shows that the protein band corresponds to the position of the enzyme activity in the gel. On sodium dodecyl sulfate–polyacrylamide gels one major band and one trace band were observed. The latter may be due to a trace of a contaminating protein.

Table I: Purification of Mevalonate-5-pyrophosphate Decarboxylase from Chicken Liver

fraction	protein (mg)	units	sp act. (units/mg)	purification (x-fold)	yield (%)
DEAE-cellulose	1141.8	64.6	0.055		100
Sephadex G-200	246.1	40.4	0.164	3.0	62.3
phosphocellulose	8.3	14.0	1.68	30.5	21.7
hydroxylapatite	1.8	7.6	4.14	75.3	11.7
Blue Dextran–Sepharose	1.0	6.2	6.26	114.0	9.6

Stability of the Enzyme. The purified enzyme does not require the presence of sulfhydryl reagents in order to protect its activity. Preparations at the DEAE-cellulose step are stable for several months when stored at −20 °C in 10 mM potassium phosphate buffer (pH 7.0)–50% glycerol. The highly purified enzyme is stable for 1 month when kept at −20 °C in Tris-HCl buffer (pH 7.0)–20% glycerol–75 mM KCl.

Molecular Weight and Isoelectric Point. The molecular weight of the native enzyme was determined by three methods. Gel filtration in Sephadex G-200 gave a value of 87 600. With sucrose density gradient centrifugation a value of 81 500 was obtained. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis gave a value of 43 500, showing that the enzyme is composed of two subunits. These three methods give an average M_r of 85 400 ± 1941 (SEM). The isoelectric point of MVAPP decarboxylase is 4.8 as determined by chromatofocusing. The recovery of activity in this experiment was 8%, indicating that the enzyme is unstable at this pH value.

Initial Velocity Studies. MVAPP decarboxylase shows hyperbolic kinetics with both MVAPP and ATP. Hanes plots at variable concentrations of ATP and at different, fixed concentrations of MVAPP gave a family of intersecting lines. The interception occurs to the left of the vertical axis and below the horizontal axis as shown in Figure 2. Similar results were obtained when MVAPP was varied at fixed levels of ATP, indicating a sequential mechanism for the enzyme. True K_m values of 1.41×10^{-5} M for MVAPP and 5.04×10^{-4} M for ATP were obtained from secondary plots according to Cornish-Bowden (1979).

Effects of pH and Inhibitors. Figure 3 shows that the pH–activity profile had a maximum between pH 4.0 and pH 6.5, with pronounced decreases in activity below or above these values. The same figure (broken line) shows that citric acid inhibits the enzyme below pH 4.8, and that the pH value for

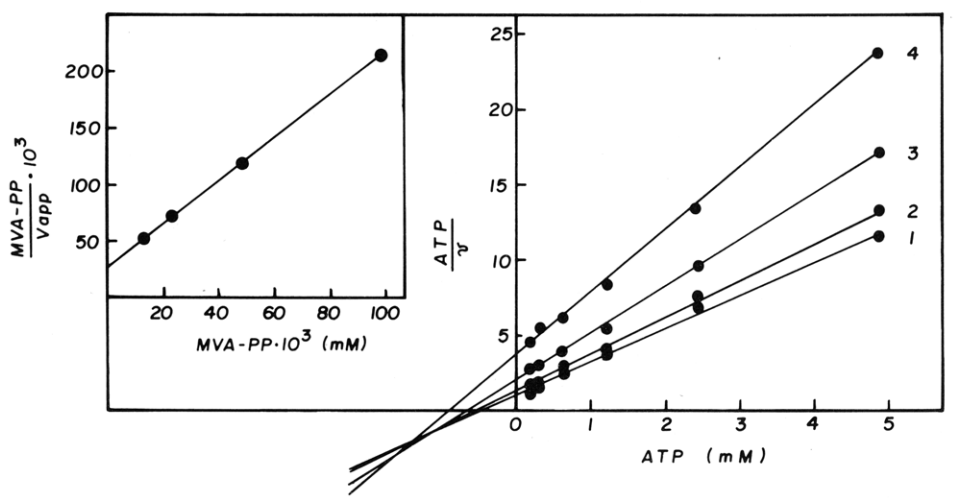

FIGURE 2: Hanes plot of variable concentrations of ATP at different fixed concentrations of MVAPP. Fixed substrate concentrations were (1) 98.2, (2) 48.3, (3) 23.5, and (4) 12.9 μM. 9 milliunits of enzyme (sp act. 2.67 units/mg) was used. (Inset) Secondary plot of the data.

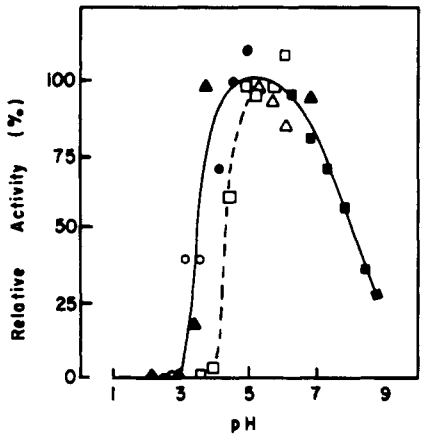

FIGURE 3: Effect of pH and inhibitory effect of citrate on activity of MVAPP decarboxylase. The following buffers (100 mM concentration) were used: (▲) glycine–HCl, (○) phosphoric acid–KOH, (●) formic acid–KOH, (□) citric acid–sodium citrate, (△) Mes–HCl, and (■) Tris-HCl. The reaction mixture contained 13 milliunits of enzyme (sp act. 4.37 units/mg) and the rest of the components of the radioactive assay, as described under Experimental Procedures.

Table II: Effect of Several Divalent Cations on Activity of Chicken Liver Mevalonate-5-pyrophosphate Decarboxylase[a]

metal ion	rel act. (%)	metal ion	rel act. (%)
$MgCl_2$	100	$CaCl_2$	29.0
$MnCl_2$	96.7	$BaCl_2$	16.2
$CoCl_2$	89.5	---	10.4
$ZnCl_2$	85.1	--- + EDTA (2 mM)	1.6
$CdCl_2$	56.9	--- + EDTA (16 mM)	0.2

[a] The activity of MVAPP decarboxylase was measured by the radioactive method as described under Experimental Procedures, with 13 milliunits of MVAPP decarboxylase (sp act. 4.37 units/mg). The concentration of the corresponding metal ion was 5 mM.

50% inhibition is around 4.4. Phosphate was also found to be an inhibitor at pH values around 7. The activity determined with 0.1 M potassium phosphate, pH 6.4, in the assay was 50% of that found in Tris-HCl at the same pH, while no activity at all was detected in 0.1 M potassium phthalate from pH 2.65 to pH 4.0 (data not shown).

Substrate and Cofactor Requirements. All the preparations of MVAPP decarboxylase so far reported require a bivalent metal cation for their activity, the best being Mg^{2+} or Mn^{2+} (Bloch et al., 1959; Skilleter & Kekwick, 1971; Shama Bhat & Ramasarma, 1980). Table II shows that at an equimolar concentration of ATP and metal ion of 5 mM, Mg^{2+} is the best activator for the enzyme, followed by Mn^{2+}. When the Mg^{2+} concentration is varied (using 5 mM ATP), it is found that the activity reaches a maximum at 1 mM Mg^{2+} and keeps constant up to at least 6 mM Mg^{2+} (data not shown).

Studies performed with the purified chicken liver enzyme show that at 5 mM, GTP has 8.7% and CTP or UTP 5% of the activity found in the presence of a similar concentration of ATP. It has been reported that when rat liver mVAPP decarboxylase is assayed in the presence of GTP, its activity is 29% of that obtained in the presence of ATP and that the enzyme is inactive with either UTP or CTP (Shama Bhat & Ramasarma, 1980). None of these nucleotides can replace ATP for the partially purified decarboxylase from *H. brasiliensis* (Skilleter & Kekwick, 1971).

Discussion

The purification of chicken liver MVAPP decarboxylase reported in this paper represents the first preparation of a stable and highly purified form of this enzyme. MVAPP decarboxylase was previously purified to near homogeneity from rat liver (Shama Bhat & Ramasarma, 1980). This preparation has a very low specific activity (0.056 unit/mg of protein) as compared to that found by us for the chicken enzyme (6.3 units/mg of protein). This large discrepancy may be explained, at least in part, because of the high instability of the rat enzyme.

Chicken liver decarboxylase shows a molecular weight of 85 400 ± 1941 and is constituted by two undistinguishable subunits. Its molecular weight and subunit structure are very different from those informed for the rat liver enzyme, which is composed of four subunits of molecular weight 35 000 each (Shama Bhat & Ramasarma, 1980).

Purified chicken liver MVAPP decarboxylase does not require thiol compounds for either activity or stability. This agrees with the report of Skilleter & Kekwick (1971) for the partially purified decarboxylase from *H. brasiliensis* but differs from both the partially purified pig liver enzyme (Popják, 1969) and the homogeneous rat liver decarboxylase (Shama Bhat & Ramasarma, 1980). Similar differences in thiol compound requirements have also been detected for mevalonate kinase and phosphomevalonate kinase. In the last case, the purified enzymes from pig liver require 2-mercaptoethanol for both activity and stability (Beytía et all., 1970; Bazães et al., 1980), while this is not so when these enzyme activities are measured in neonatal chick liver homogenate (García-Martínez et al., 1978).

The chicken liver enzyme has an absolute requirement for bivalent metal cations, as has also been found for all other MVAPP decarboxylases so far studied (Bloch et al., 1959; Popják, 1969, Skilleter & Kekwick, 1971; Shama Bhat & Ramasarma, 1980) and also for many other nucleotide-dependent decarboxylases (Utter & Kolenbrander, 1972). With 5 mM ATP, the optimum activity of the chicken decarboxylase is reached at a Mg^{2+}/ATP ratio of 0.2. Previously, Skilleter & Kekwick (1971) had found for the *Hevea* enzyme that the molar ratio of Mg^{2+}/ATP giving maximal activity was 0.5. This point deserves further investigation since it is a common observation that for most enzymes where the substrate is MgATP, maximal activation is obtained at a Mg^{2+}/ATP ratio above 1 (Beytía et al., 1970; Bazães et al., 1980; Palella et al., 1980).

The enzyme shows hyperbolic kinetics for both substrates, and the data support a sequential mechanism, but product inhibition studies are needed to specify the type of sequential mechanism involved. The isoelectric point obtained (4.8) is in agreement with the fact that the enzyme is retained during the purification procedure by the DEAE-cellulose column run at pH 7.0. There have been no isoelectric point determinations carried out with other MVAPP decarboxylases.

The observations reported here about the inhibitory effects of compounds generally employed as buffers strengthen the need of using several buffer systems when doing a pH–activity profile. The pH-dependent inhibitory effect of citric acid is about 50% at pH 4.4, which is close to the value for one of its three pKs (pK_1 = 3.09, pK_2 = 4.75, and pK_3 = 5.41) (Mahler & Cordes, 1966). This suggests that the molecular species responsible for this effect is that with one unprotonated primary carboxyl group. Inhibitory effects of phosphate are rather common on enzymes catalyzing reactions in which inorganic phosphate or phosphate-containing substrates or products participate [i.e., Barnard & Popják (1981)].

The optimum pH has been found to be in the range 4.0–6.5, which is somewhat different from that reported for the rat liver

enzyme (Shama Bhat & Ramasarma, 1980). Nonetheless, since the pH curve for this last enzyme was determined with only citrate–phosphate buffers for the pH range 3–7, the possibility remains that citric acid may also be inhibitory for this enzyme below pH 5, as we have shown for the chicken liver enzyme. This possibility is currently been explored in our laboratory, and preliminary experiments indicate that this is the case when rat liver MVAPP decarboxylase is assayed in crude extracts.[2] The stable and highly purified preparation of avian liver MVAPP decarboxylase reported in this communication will be of great value for future investigations of its reaction mechanism, initiated by the pioneering studies of Bloch et al. (1959) and Cornforth et al. (1966).

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