Streptococcus faecalis Aspartate Transcarbamylase. I. Purification and General Properties*

Lansing M. Prescott† and Mary Ellen Jones‡

ABSTRACT: Bethell and Jones ((1969), *Arch. Biochem. Biophys. 134*, 352) studied the size and kinetic characteristics of aspartate transcarbamylase of different bacteria and found only three molecular size groups, designated A, B, and C. Since each size group appeared also to possess a distinctive set of kinetic properties, it seemed that an aspartate transcarbamylase of each group should be characterized. *Escherichia coli* aspartate transcarbamylase (Gerhart and Pardee (1962), *J. Biol. Chem. 237*, 891) belonging to group B is well characterized, but enzymes from groups A and C have not been purified. We wish to report here the extensive (300-fold) purification of the aspartate transcarbamylase of *Streptococcus faecalis* (group C) which may be representative of a number of aspartate transcarbamylases having a molecular weight between 120,000 and 140,000. This group of low molecular weight aspartate transcarbamylases are completely insensitive to nucleotide regulation (Bethell and Jones (1969), *Arch. Biochem. Biophys. 134*, 352). The pH optimum for enzymatic activity varies with temperature. At 25°, the optimum is broad and extends from pH 8.7 to 10.3. At 37°, the optimum lies at pH 9.8–9.9. The aspartate and carbamyl phosphate saturation curves are hyperbolic at pH 8.5. The Michaelis constants at 25° are 1.8×10^{-4} M for carbamyl phosphate and 1.9×10^{-2} M for aspartate. There is inhibition of enzyme activity by high levels of aspartate, but not by carbamyl phosphate. The enzyme is fairly stable at high temperatures. Half the activity is lost in 30 min at 66.7° when the enzyme preparation is at a concentration of 0.05 mg/ml or above. There is a sharp pH optimum for temperature stability which lies at pH 6.6–6.7. Mercuric chloride, mersalyl acid, and *p*-mercuribenzoate all inhibit the enzyme activity, but in quite different ways. Mersalyl inhibits completely at concentrations above 1×10^{-4} M, mercuric chloride never completely inhibits activity, and *p*-mercuribenzoate inhibits enzyme activity 80% at 3×10^{-5} M but at a higher concentration, 3×10^{-4} M, only 40% inhibition is observed. Low concentrations of substrates, products, and inhibitors will protect the enzyme against mersalyl inhibition, and one substrate, carbamyl-P, activates the enzyme in the presence of mersalyl.

The aspartate transcarbamylase (carbamoyl phosphate: L-aspartate carbamoyltransferase, EC 2.1.3.2) of many, but not all, bacterial species is regulated by nucleotides (Gerhart and Pardee, 1962; Neumann and Jones, 1964; Bethell and Jones, 1969). Bethell and Jones (1969) have grouped these enzymes into three classes (A, B, and C) based upon their kinetic properties and behavior upon gel filtration columns. Class A enzymes such as the enzyme from *Pseudomonas fluorescens* are very large molecules with a Stokes radius of about 8.5 mμ and a molecular weight between 400,000 and 500,000.[1] The *P. fluorescens* aspartate transcarbamylase is inhibited by UTP, CTP, and ATP. This inhibition is not selectively abolished by heating and other mild denaturing procedures. The substrate saturation curves in the absence of nucleotides are hyperbolic. The carbamyl phosphate curve becomes sigmoidal in the presence of nucleotide inhibitors.

The aspartate transcarbamylase from *E. coli* is the most thoroughly studied class B enzyme. The class B enzymes have Stokes radii of about 6.5 mμ and molecular weights in the order of 300,000. The *E. coli* enzyme is inhibited by CTP and activated by ATP (Gerhart and Pardee, 1962). Its substrate saturation curves are sigmoidal in the absence of nucleotides (Gerhart and Pardee, 1962; Bethell *et al.*, 1968). The presence of ATP abolishes the sigmoidicity of both substrate curves, while the presence of CTP does not. The effects of CTP and ATP are selectively abolished by heating and other treatments (Gerhart and Pardee, 1962). It is clear that the enzymes in classes A and B are quite different from one another even though both groups are affected by nucleotides.

A third class of bacterial aspartate transcarbamylases, *i.e.*, class C, differ from the above two groups in that these enzymes are much smaller than the enzymes of classes A and B and are not affected by nucleotides (Neumann and Jones, 1964; Bethell and Jones, 1969). They have Stokes radii of about 4.0 mμ and molecular weights of approximately 100,000 to 140,000. In order to learn more about the nature of the differences between the enzymes of these

* From the Graduate Department of Biochemistry, Brandeis University, Waltham, Massachusetts 02154, and the Department of Biochemistry, School of Medicine, University of North Carolina, Chapel Hill, North Carolina 27514. Publication number 731 from the Graduate Department of Biochemistry, Brandeis University. *Received October 27, 1969.* The investigation was supported by National Institutes of Health Grant HD-02148 and National Science Foundation Grant GB-5354. L. M. P. has also been supported as a National Science Foundation Graduate Fellow. This work was submitted in partial fulfillment of the requirements for the Ph.D. degree at Brandeis University. A preliminary account of some of these results has already been published (Prescott and Jones, 1969a).

† Present address: Department of Biology, Augustana College, Sioux Falls, S. D. 57102.

‡ Present address: Department of Biochemistry, School of Medicine, University of North Carolina, Chapel Hill, N. C. 27514; to whom to address correspondence.

[1] Unpublished results of Linda B. Adair and M. E. Jones.

three groups, the aspartate transcarbamylase from *Streptococcus faecalis* has been selected for more detailed study as a representative of the class C enzymes. This enzyme has been purified to a limited extent in the past (Jones, 1962). In the present paper, a more extensive purification procedure and the stability of the purified enzyme to heat, pH, and mercurials are presented.

Experimental Section

Reagents and Chemicals. Streptomycin sulfate was purchased from Nutritional Biochemicals Corp. DEAE-cellulose and hydroxylapatite, when the commercial products were used, were obtained from the Bio-Rad Laboratories. The DEAE-cellulose was washed prior to use by the method of Peterson and Sober (1962). The reagents employed in acrylamide gel electrophoresis (acrylamide, *N,N'*-methylenebisacrylamide, *N,N,N',N'*-tetramethylethylenediamine, and sodium dodecyl sulfate) were all purchased from the Eastman Organic Chemicals Co. The coomassie brilliant blue R250 dye came from Colab Laboratories.

Color assay reagents came from several sources. Elon (monomethyl *p*-aminophenol sulfate) was purchased from the Eastman Kodak Co. The phenol reagents used in protein determinations and Brij 35 came from the Fisher Scientific Co. Eastman Chemicals Co. supplied 2,3-butanedione 2-monoxime and *p*-diphenylaminesulfuric acid, disodium salt. Antipyrine was obtained from the Matheson Coleman and Bell Co. The protein used as a standard in the protein assay was crystalline bovine serum albumin, A grade, from the California Corp. for Biochemical Research (Calbiochem). Calbiochem also supplied L-aspartic acid and carbamyl phosphate. Carbamyl-D,L-aspartic acid came from the Nutritional Biochemicals Corp.

Purine and pyrimidine nucleotides came from either Sigma Chemical Co. or P-L Biochemicals, Inc. The proteins used as standards in acrylamide gel electrophoresis were obtained from two sources. Porcine thyroglobulin (Type II) came from Sigma Chemical Co. Bovine albumin, crystalline bovine pancreatic trypsin, and ovalbumin were purchased from Calbiochem.

The saturated ammonium sulfate used for purifying the enzyme was prepared by saturating a solution of 0.05 M KH_2PO_4 and 1×10^{-4} M EDTA at 4° with recrystallized ammonium sulfate. The pH of the cold, saturated solution was then adjusted to pH 6.6 ammonium hydroxide. The pH was determined at room temperature on 1 to 10 dilutions with distilled water of the saturated solution. Just before use, the saturated ammonium sulfate solution was made 1×10^{-3} M in mercaptoethanol.

Determination of Enzyme Activity. The activity of the enzyme was followed by measurement of either orthophosphate or carbamyl aspartate (CAA)[2] production. Inorganic orthophosphate was determined by a modification of the Fiske–Subbarow method (Jones and Spector, 1960; Prescott, 1969). CAA was measured to a limited extent by the use of the method of Gerhart and Pardee (1962), but it was usually determined by a new modification of the Fearon reaction developed during the course of these studies (Prescott and Jones, 1969b). Unless otherwise noted, this new procedure was used.

The protein concentration was determined using Oyama and Eagle's modification of the Lowry method (Oyama and Eagle, 1956). Crystalline bovine serum albumin was used as the standard. When the amount of protein being eluted from a column was to be determined, the absorbance at 280 mμ was followed using either a Zeiss PMQII spectrophotometer with the quartz cells of 1-cm path length or a LKB 8300A Uvicord II photometer with a 6520A chopper bar recorder.

Enzyme assays were run in duplicate or triplicate. Incubation periods were generally 5 or 10 min in length. A preincubation period of 1 min was used for temperature equilibrium before the incubations were started with the addition of either CAP or enzyme. The reaction was stopped with the addition of twice the incubation volume of either 1 N perchloric acid or 0.1 M sodium acetate buffer (pH 4.1) (where P_i was to be measured (Weitzman and Wilson, 1966)). Aliquots were then removed for product measurement. Minus aspartate or minus enzyme blanks were used to correct for background color. The composition of the incubation mixtures is given in the data.

When determining kinetic constants by the use of Lineweaver–Burk and 1/velocity *vs.* substrate concentration plots, straight lines were fitted to the data by the method of least squares using a General Electric computer time-sharing system equipped with a GE-265 computer.

The unit of enzyme activity is defined as that amount of enzyme which catalyzes the formation of 1 μmole of carbamyl aspartate or phosphate per min under the appropriate assay conditions. The specific activity of the enzyme is the number of units of activity per milligram of protein.

Hydroxylapatite and DEAE-cellulose Chromatography. In addition to using commercially produced hydroxylapatite, large quantities were prepared using a modification of the methods of Siegelman *et al.* (1965) and Sizer and Jenkins (1962) as previously described (Prescott, 1969). This method yielded hydroxylapatite which gave the same results as the commercial material, but with a slower flow rate due to a smaller particle size.

Columns containing hydroxylapatite, either the commercial product or that made in this laboratory, were prepared by suspending the hydroxylapatite in 1×10^{-3} M potassium phosphate buffer (pH 6.6) and this suspension was used to pour the column. Chromatography tubes with fritted-glass supports were used. The inside diameters of these tubes were usually 7.6 cm. The fritted-glass support was covered with a circle of Whatman No. 1 filter paper, the column outlet was clamped so that no buffer could run out, and the tube was filled with a few centimeters of 1 mM phosphate buffer. Some of the hydroxylapatite suspension was added carefully and the buffer was allowed to begin running from the column slowly. More gel suspension was continually added as the hydroxylapatite settled until the desired bed height was reached. Usually the bed height was about 7.5 cm. If the gel bed was made too high, the flow rate became impractically slow. After the gel had settled, a Whatman No. 1 filter paper disk was very carefully placed on top of it in order to prevent the surface from being disturbed. The void volume of the column was taken to be about 75%

[2] The abbreviations used are: CAP, carbamyl phosphate; CAA, carbamyl aspartate; *p*MB, *p*-mercuribenzoate.

of the bed volume in accordance with the information provided by Bio-Rad Laboratories for their hydroxylapatite.

The DEAE-cellulose chromatography was carried out in a chromatography tube with an insider diameter of 1.9 cm and a fritted-glass support was used. The glass support was covered with a filter paper disk before pouring the column. A slurry of washed DEAE-cellulose in 0.01 M potassium phosphate buffer (pH 6.6) was used to pour the column. The column was poured in the same way as the hydroxylapatite column and capped with filter paper. The bed height was about 12 cm. The column was washed with 0.01 M potassium phosphate buffer (pH 6.6) containing 1×10^{-4} M EDTA before use. The void column was taken as approximately 40% of the bed volume. The concentration of the enzyme eluted from the large DEAE-cellulose column was accomplished by a batchwise absorption and elution of the enzyme from a small DEAE-cellulose column (stage 6, Table I). The small DEAE-cellulose column was poured at 4° using a glass wool–sand support. These columns usually were 0.9 cm in diameter, and the DEAE beds were from 3.0 to 6.0 cm in height depending upon the amount of protein to be applied. The column was washed thoroughly with 5×10^{-3} M potassium phosphate buffer (pH 6.6) containing 1×10^{-4} M EDTA. The dialyzed DEAE-cellulose column eluate was then applied at a slow rate, and 1.0-ml fractions were collected. The A_{280} of these fractions were monitored carefully. If the absorbency began to rise, the column was stopped and more washed DEAE-cellulose was added to the top before continuing protein addition. After protein application was complete, the protein was eluted in a small volume by washing the column with 0.01 M potassium phosphate buffer (pH 6.6) containing 1×10^{-4} M EDTA and 0.5 M KCl. One-milliliter fractions were collected during the elution, and the A_{280} of each fraction was read on a Zeiss spectrophotometer. The protein normally came out in three or four fractions. These peak fractions were combined and dialyzed overnight at 4° against 3 l. of 0.05 M potassium phosphate buffer (pH 6.6) containing 1×10^{-4} M EDTA and 1×10^{-3} M mercaptoethanol.

Electrophoresis of Aspartate Transcarbamylase. Polyacrylamide gel electrophoresis of the native enzyme was carried out using an Analytical polyacrylamide vertical gel electrophoresis apparatus, no. 3-1750, and a regulated power supply, no. 3-1014A (Buchler Instruments, Inc.). The electrophoresis was performed as described by Davis (1964). All electrophoresis runs were made at room temperature with cooling by tap water. The proteins were fixed in trichloroacetic acid and stained with coomassie blue dye according to the method of Chrambach *et al.* (1967). In order to locate enzymatic activity, an acrylamide gel was sliced into approximately 1.6-mm thick disks by the use of a gel sectioning device (Chrambach, 1966). Proteins were eluted from each section by soaking it overnight at 5° in 0.5 ml of 0.05 M potassium phosphate buffer (pH 6.6) containing 10^{-4} M EDTA and 10^{-3} M mercaptoethanol. The solutions were then assayed for enzymatic activity.

The enzyme was also reduced and denatured in mercaptoethanol–sodium dodecyl sulfate solution. The denatured preparation was then subjected to electrophoresis on polyacrylamide gels in the presence of sodium dodecyl sulfate as described by Shapiro *et al.* (1967).

Growth and Disruption of S. faecalis. *S. faecalis* (American Type Culture Collection no. 8043) was grown by the procedure already employed in this laboratory (Jones, 1962). This procedure was simply scaled up for use with the 500-l. fermenter at the New England Enzyme Center. Lyophilized bacteria from the American Type Culture Collection was used. Five-hundred liters of medium yielded about 2000–2300 g of bacterial paste. This paste was stored frozen at −20° until use.

Portions of the bacterial paste were suspended in 0.02 M potassium phosphate buffer (pH 6.6) containing 10^{-4} M EDTA. The cells were then disrupted using glass beads and a large colloid mill at the New England Enzyme Center. The homogenate was clarified by passing it twice through Sharples centrifuges. Even better results were obtained by the use of a Braun Model MSK mechanical cell homogenizer (Bronwill Scientific) with 0.17–0.18-mm glass beads according to the method of Bleiweis *et al.* (1964). The centrifuged homogenate was stored frozen at −20° until use. It has been used for 3 years with no loss of activity.

Results

Purification Procedure. The first step in the purification of *S. faecalis* ATCase (Table I) was the removal of nucleic acids from the homogenate. A portion of the frozen homogenate which ranged from 100 to 1000 ml in volume was thawed and dialyzed overnight at 4° against 5 l. of 0.02 M potassium phosphate buffer (pH 6.6) which was changed once. A 5% (w/v) streptomycin sulfate solution was added very slowly to the dialyzed homogenate which was being stirred vigorously by a magnetic mixer. The temperature was maintained at 4° by an ice–water bath. A volume of streptomycin sulfate solution equivalent to one-fifth of the homogenate volume was added dropwise. If the streptomycin sulfate was added too quickly, the ATCase precipitated out with the nucleic acids. The large amount of precipitate which formed was centrifuged down in a Sorvall RC2-B centrifuge at 0° either for 20 min at 13,200*g* with the GSA head or for 15 min at 34,800*g* with the SS-34 head.

The ATCase can be enriched 3-fold, concentrated, and separated from the carbamyl phosphokinase and ornithine transcarbamylase of the crude extract by acid precipitation at pH 4.8 using the conditions described (Jones, 1962). These studies showed that in addition to the details given by Jones (1962) that the 0.5 N acetic acid, used to bring the pH to 4.8, must be added as quickly as thorough mixing permits. The time usually required was 10–20 min. Approximately 30 ml of 0.5 N acetic acid was required to adjust the pH of 1 l. of streptomycin sulfate supernatant. As soon as the acid addition was complete, the precipitate was centrifuged down in the same way as before. The pH precipitate was then resuspended in 0.10 M potassium phosphate buffer (pH 6.6) using a tissue homogenizer with a Teflon pestle. Normally a volume of buffer about 0.05 the volume of streptomycin sulfate solution was used. The resuspended pH 4.8 precipitate was then dialyzed overnight at 4° against two 3-l. changes of 0.05 M potassium buffer (pH 6.6). The pH 4.8 precipitate did not immediately dissolve completely in the 0.10 M buffer. However, the precipitate did completely go into solution during dialysis. The dialyzed pH 4.8 precipitate solution was usually stored frozen at −20° until it could be further purified. The enzyme was very stable in this condition.

TABLE I: Purification of Aspartate Transcarbamylase from *S. faecalis*.

Purification Stage	Vol (ml)	Protein (mg/ml)	Total Protein (mg)	Total[a] Act. (units)	Sp. Act. (units/mg)	Recov (%)
1. Homogenate	376	14.0	5264	1337	0.25	100
2. Streptomycin sulfate supernatant	416	9.5	3955	1510	0.38	113
3. pH 4.8 precipitate	33.0	31.8	1049	1356	1.3	101
4. Hydroxylapatite column eluate (0.050 M P_i wash)	297	0.73	224	1590	7.1	119
5. DEAE-cellulose column eluate	174	0.21	36.5			
6. DEAE-cellulose column concentrate	4.40	6.4	28.8	760	26.5	57
7. Ammonium sulfate fraction (43–54% cut)	1.20	8.0	10.6	803	76.1	60

[a] A unit is defined as that amount of enzyme which catalyzes the formation of 1 μmole of carbamyl aspartate/min under the standard assay conditions (25° incubation temperature, 5×10^{-2} M aspartate, 1×10^{-2} M CAP, and 0.10 M Tris-Cl buffer, pH 8.5).

The enzyme was further purified by use of hydroxylapatite column chromatography at 4°. The solution of the pH 4.8 precipitate (stage 3, Table I) was thawed and dialyzed overnight against one or two changes of 1×10^{-3} M potassium phosphate buffer (pH 6.6). A volume of buffer approximately 100 times the volume of pH 4.8 precipitate solution was used for dialysis. Enough solution was dialyzed to give an amount of total protein equivalent to about 4.0 mg of protein/ml bed volume. The dialyzed enzyme solution was then carefully applied to the top of the bed and allowed to slowly run in. After the protein had entered the gel, the top of the bed was washed with a small volume (about one-tenth of the bed volume) of the 10^{-3} M phosphate buffer. The column was then washed with 0.050 M potassium phosphate buffer (pH 6.6) until all protein which could be eluted with this buffer had come off. This usually took a volume of buffer approximately equal to twice the bed volume. During the elution of enzyme, 10-ml fractions were collected. The flow rate during elution was about 1.5 ml/min. The enzyme activity usually began coming off slightly after the void volume. Activity was followed by assaying selected fractions using the CAA assay. The fractions containing the enzyme were pooled and dialyzed overnight against two 3-l. changes of 0.01 M potassium phosphate buffer (pH 6.6) containing 1×10^{-4} M EDTA and 1×10^{-3} M 2-mercaptoethanol.

The great majority of the protein was retained by the hydroxylapatite under these conditions and was washed off with 0.40 M potassium phosphate buffer (pH 6.6). The column was then washed with 1×10^{-3} M potassium phosphate buffer (pH 6.6) and the gel was saved for future reuse.

The ATCase eluted from hydroxylapatite was further purified by DEAE-cellulose column chromatography at 4°. The dialyzed eluate from the hydroxylapatite column containing from 120 to 220 mg of protein was applied to the DEAE column at a flow rate of about 2 ml/min. After the protein solution has been applied, the top of the column was washed with a small aliquot of the 0.01 M potassium phosphate buffer. The protein was then eluted with a linear potassium chloride gradient running from 0.00 to 0.50 M KCl. The flow rate during elution was kept at about 1 ml/min, and 5-ml fractions were collected. Enzyme activity was followed with the Pl colorimetric assay. The fractions with peak activity were pooled and dialyzed overnight at 4° against about 30 times their volume of 5×10^{-3} M potassium phosphate buffer (pH 6.6) containing 1×10^{-3} M mercaptoethanol and 1×10^{-4} M EDTA. A representative DEAE-cellulose gradient is presented in Figure 1. It was very important to run the DEAE-cellulose column fairly rapidly. The enzyme was not completely stable on the column, and activity loss increased with time. Normally the gradient was run with a period of 10–15 hr. The enzyme's stability also seemed to decrease with decreasing the protein loads on the column. The eluate sometimes lost activity upon freezing and thawing. An aliquot of the DEAE-cellulose eluate was therefore not saved for future assay.

The dialyzed eluate from the large DEAE-cellulose column was concentrated on a small DEAE-cellulose column as described in the Experimental Section. The protein concentration of this concentrate ranged from 4.3 to 6.4 mg per ml. The recovery of activity was essentially complete if this concentration step was properly executed. The DEAE-cellulose concentrate was usually stored frozen until the final purification step could be carried out.

The final step in the purification procedure was ammonium sulfate fractionation of the DEAE concentrate at 4°. The tube of DEAE-cellulose concentrate was thawed. A micro-magnetic stirring bar was placed in the bottom of the tube of solution. The enzyme solution was then carefully brought to 43% saturation with the saturated ammonium sulfate solution. This was done by very slow addition of the saturated solution over a period of 15–20 min with thorough mixing. The opaque suspension which formed was allowed to sit at 4° for 1 hr. The precipitate was then centrifuged down in the Sorvall at 4° for 5 min at 12,000*g*. The 43% supernatant was clear and had a faint yellow tinge.

The cold 43% supernatant was then brought to 54% saturation by the slow addition of saturated ammonium sulfate solution with thorough mixing. The solution was slightly opalescent by the end of the ammonium sulfate addition. The micro magnet was removed, and the test tube was tightly capped with Parafilm. It was allowed to sit at 4° overnight (approximately 17–18 hr). During this period

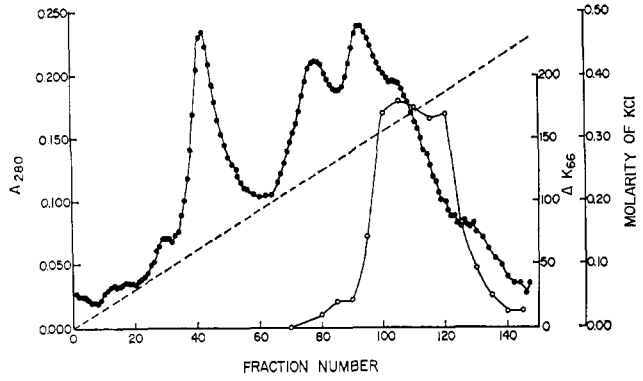


FIGURE 1: DEAE-cellulose fractionation of hydroxylapatite eluate protein. The eluate from a hydroxylapatite column was dialyzed overnight at 4° against 5.5 l. of 0.01 M potassium phosphate buffer (pH 6.6) containing 1×10^{-4} M EDTA and 1×10^{-3} M mercaptoethanol. The dialyzed eluate (297 ml, 217 mg of protein) was applied to a 1.9 × 12.3 cm DEAE-cellulose column with a flow rate of 2 ml/min. After application, the protein was eluted with a linear KCl gradient which extended from 0.00 to 0.50 M KCl. The cylindrical gradient bottle connected to the column contained 400 ml of 0.01 M potassium phosphate buffer (pH 6.6) plus 1×10^{-3} M mercaptoethanol and 1×10^{-4} M EDTA at the start of the gradient. It was stirred by a magnetic mixing bar. The second cylindrical gradient bottle was connected to the first and contained 400 ml of the same buffer plus 0.50 M KCl. The flow rate during elution was 1.3 ml/min. Five-milliliter fractions were collected. The protein elution pattern (●-●) was determined by reading the A_{280} of each fraction. The ATCase activity (O-O) was determined using the P_i assay and is expressed as ΔK_{66}. The approximate KCl concentration is denoted by the dashed line. Fractions 95–130 were combined and used for further purification. DEAE-cellulose columns were prepared using Cellex-D (exchange capacity, 0.70 mequiv/g; control number 4471). The separation properties of DEAE vary with the lot, as well as with brand. Therefore, the same lot of DEAE was used in all purifications.

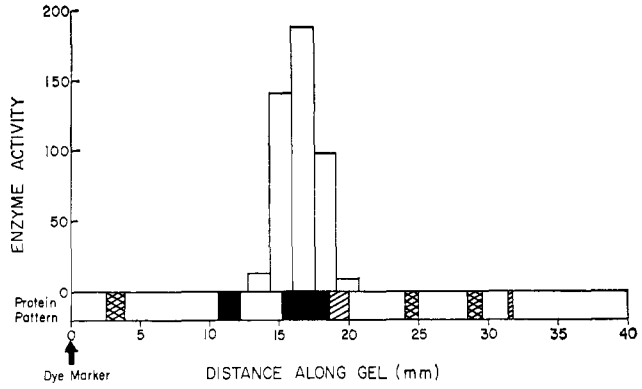


FIGURE 2: Acrylamide gel electrophoresis of the purest enzyme fraction. Acrylamide gel electrophoresis of the most purified aspartate transcarbamylase preparation was carried out by the procedure of Davis as described in the Experimental Section. Enzyme solution containing 154 μg of protein was applied to the top of each of two gels. Electrophoresis was performed with a constant current of 3 mA/gel for 1 hr and 40 min. One gel was stained for protein, and the other gel was sliced into approximately 1.6-mm thick disks for determination of enzyme activity. Activity was determined using the Gerhart–Pardee CAA assay. It is expressed as μmoles of CAA formed per 10 min per ml of eluate at 37°. The intensity of staining of protein bands is indicated by the following patterns: black bands, very intense staining; cross-hatched bands, much less intense staining; and bands with parallel lines, least stained. The recovery of activity from this gel was approximately 76%.

a white, flocculent precipitate formed and settled out. The next morning, the precipitate was resuspended and centrifuged down in the same way as before. The 43–54% precipitate was dissolved in 1 or 2 ml of 0.05 M potassium phosphate buffer (pH 6.6) containing 1×10^{-4} M EDTA and 1×10^{-3} M 2-mercaptoethanol. It was then dialyzed overnight at 4° against 3 l. of the same buffer. This dialyzed solution constituted the purest preparation available and was used for almost all the studies to be reported. The enzyme solution was separated into 0.5-ml aliquots and stored frozen at −20° until use. The enzyme appears to be stable indefinitely when frozen at −20°. The summary of a representative purification sequence is presented in Table I. This purification procedure has been successfully scaled up to handle several times as much starting material.

Electrophoresis of the Purest Fraction. The enzyme preparation which resulted from the purification procedure outlined in Table I was analyzed by acrylamide gel electrophoresis as outlined in the Experimental Section and in the legend of Figure 2. Two samples of enzymes were run at the same time. One gel was stained for protein, and the second was sliced into disks. The enzyme was eluted by soaking the gel disk in buffer, and the enzyme activity of each disk was then determined by the CAA assay. The results are shown in Figure 2.

The most highly purified preparation of the enzyme is clearly not homogeneous. The enzyme activity does migrate with one band of protein and this band is in the major protein component. The enzyme activity is distributed rather symmetrically over this major protein band suggesting that the entire major dark band is ATCase. However, it is not possible to be certain that the enzyme activity exactly parallels protein concentration or that the band definitely represents only one protein species. The reasons are: (1) that one can measure enzyme activity when little or no protein is visible, *i.e.*, the activity measurement is more sensitive than the protein stain, (2) that the activity distribution is not as precise as could be desired because the gel slices are fairly thick relative to the protein band widths, and (3) that there may be more than one protein in the band, if the component proteins were not resolved under these particular electrophoresis conditions. If we assume, with these reservations in mind, that the protein band does represent aspartate transcarbamylase alone, the enzyme would appear to be 40 to 50% pure.

Some idea of the structure or purity of the enzyme may be obtained by denaturing and reducing the enzyme, then electrophoresing it in the presence of sodium dodecyl sulfate (Shapiro *et al.*, 1967). This technique allows the determination of the molecular weights of polypeptide chains in pure enzyme preparations. With proteins which are only 50% pure the method cannot give reliable data on subunit structure particularly if the native protein is composed of two (or more) unlike subunits and if there is one or two additional major proteins in the preparation. However, if the molecular weight of the enzyme is known and if all the protein observed within the Shapiro system are of smaller molecular weight than the native enzyme, then it is reasonable to assume

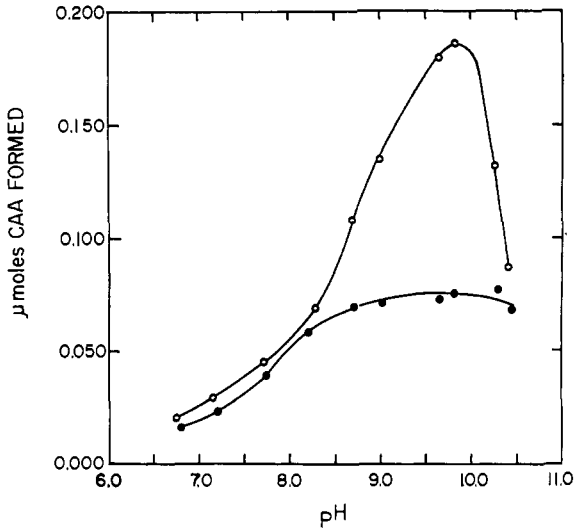


FIGURE 3: pH dependence of enzyme activity. The assay incubation mix contained 100 mM buffer at the proper pH, 50 mM aspartate, 20 mM CAP, and enzyme in a total volume of 1.00 ml. The buffers used were the following: 0.20 M imidazole-Cl, pH's 6.50, 7.00, and 7.50; 0.20 M Tris-Cl, pH's 7.50, 8.00, 8.50, and 9.00; and 0.20 M potassium carbonate buffer, pH's 9.00, 9.50, 10.00, 10.50, and 11.00. The following aspartate solutions were used at the pH's indicated in parentheses: 0.50 M aspartate, pH 7.00 (pH's 6.50, 7.00, 7.50); 0.50 M aspartate, pH 8.50 (pH's 8.00, 8.50, 9.00); 0.50 M aspartate, pH 9.70 (pH's 9.50, 10.00); and 0.50 M aspartate, pH 10.50 (pH's 10.50, 11.00). At 25° (●-●), 0.32 μg of protein (specific activity 76.1) per mix was used; 0.20 μg of protein was used at 37° (O-O). The incubation time at both temperatures was 10 min. The pH of half of each assay mix was measured after the incubation, and this pH was taken as the true pH. The other half of each mix was used in the CAA determination. The 25° pH curve values have been adjusted to those expected for 0.20 μg in order to facilitate comparison of the curves.

that the enzyme contains subunits. When the same preparation and same amount of *S. faecalis* ATCase as was used in Figure 2 was subjected to electrophoresis according to Shapiro *et al.* (1967) five protein bands were observed. From comparison with the standard proteins listed in the Experimental Section, the molecular weight of these bands were 120,000, 104,000, 73,000, 46,000, and 32,000. Since the data of Bethell and Jones (1969) yielded molecular weight for *S. faecalis* ATCase of 120,000 to 140,000, only one band, the first of the five bands listed above, had a similar molecular weight. This band, mol wt 120,000, represented less than 10% of the applied protein as did the band having a molecular weight of 104,000. The 32,000 band was also a minor component. The 73,000 band was the major component and appeared to contain two times as much protein as the 42,000 band. These results lead to two mutually exclusive alternatives. If the *S. faecalis* enzyme is about 50% pure, it must have subunits and it is possible that it is a dimer of the 73,000 molecular weight polypeptides for only this band contained 50% of the applied protein. The second alternative is that *S. faecalis* ATCase does not have subunits and contains a single polypeptide chain with a molecular weight of about 120,000. The second alternative would also mean that the protein is not 50% pure and would have to be purified at least 10-fold further than the purest preparation in Table I.

General Properties of the Enzyme Reaction. The enzyme

TABLE II: Substrate Requirements of the ATCase Reaction.[a]

Assay System	μmoles of CAA Formed
Complete	0.144
−Aspartate	0.027
−CAP	0.007
−Aspartate and CAP	0.005
−Enzyme	0.030

[a] The complete assay incubation mixture contained 100 mM Tris-Cl buffer (pH 8.5), 50 mM L-aspartate (pH 8.5), 10 mM CAP, and 0.21 μg of enzyme (Specific activity 76.1) in a total volume of 0.50 ml. The assay mix was incubated at 25° for 10 min. The amount of CAA formed was determined by the modified color determination procedure (Prescott and Jones, 1969).

from *S. faecalis* is similar to all other aspartate transcarbamylases studied in that it requires only L-aspartate and CAP for activity as shown in Table II. The apparent CAA present in the minus aspartate and minus enzyme control is most probably due to urea, a common trace contaminate of CAP. The same specific activity for an enzyme sample is obtained with either the orthophosphate or the CAA assays. Therefore equivalent amounts of the two reaction products are produced. The rate of CAA formation is constant for at least 20 min at both 25 and 37°. The amount of CAA produced in 10 min increases in direct proportion to the amount of enzyme present at both temperatures.

The dependence of enzyme activity upon pH at both 25 and 37° is illustrated in Figure 3. The pH curves at these two incubation temperatures are quite different in shape. The curve at 25° has a very broad optimum which extends from around pH 8.7 to about pH 10.3. At 37°, the pH curve has a sharp optimum at 9.8–9.9. The difference in shape of the two curves results in a pH optimum for stimulation of activity at 37°. In all studies to be reported, the enzyme assays have been run at pH 8.5. This pH has been used, even though it is not optimal at 37° but is essentially optimal at 25°, in order to avoid the serious problems which would arise from the increased rate of CAP hydrolysis at pH values above 8.8 (Allen and Jones, 1964). This is not a trivial reason for working below the pH optima. For example, at 37° and pH 9.86, the apparent 37° pH optimum, 30 and 50% of the added CAP is chemically decomposed in 5 and 10 min, respectively, so that whenever the CAP was not above saturation a significant change in concentration would occur during either of the usual incubation periods. In experiments dealing with variations of enzyme activity with pH, an attempt was made to minimize the effect of CAP hydrolysis by using a CAP concentration much above saturation and by employing as short an assay incubation period as possible.

The kinetic constants for the enzyme have been determined from substrate saturation studies by the use of Lineweaver–Burk and 1/velocity *vs.* substrate concentration plots as outlined in the Experimental Section. The aspartate saturation curve at 25°, together with the

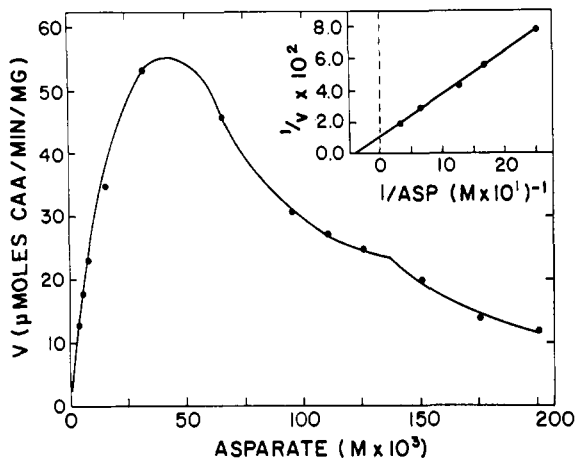


FIGURE 4: Aspartate saturation curve at 25°. The assay incubation mixtures contained 100 mM Tris-Cl buffer (pH 8.5), varying amounts of aspartate (pH 8.5), 5 mM CAP, and 0.28 μg of enzyme protein (specific activity 55.7). The incubation was for 10 min. CAA formation was measured. The insert depicts the double-reciprocal plot of the data at low aspartate concentrations.

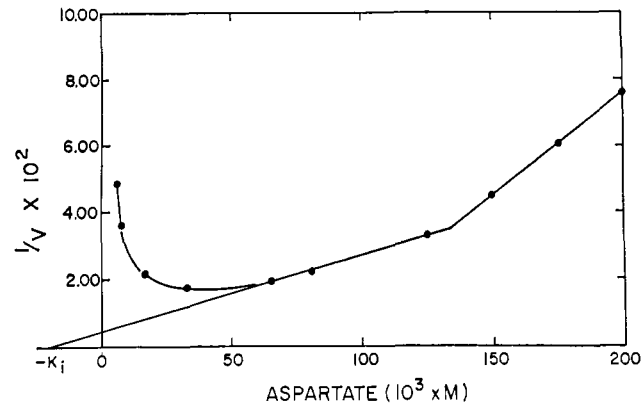


FIGURE 5: Single-reciprocal plot of aspartate saturation curve at 25°. The experiment depicted in this figure was run in the same way and with the same quantity of enzyme as the experiment described in Figure 4. The extrapolated line intercepts the abscissa at a point whose value is equivalent to $-K_i$.

double-reciprocal plot of the data at low apsartate concentrations, is given in Figure 4. The K_m (app) for aspartate is $1.9 \pm 0.5 \times 10^{-2}$ M, and the V_{max} (app) is 93 ± 14 μmoles of CAA/min per mg (average of seven experiments). The aspartate saturation curves do not appear sigmoidal at low substrate concentrations. The aspartate curve is different from that of CAP in that substrate inhibition is present. Furthermore, the substrate inhibition effect is biphasic in nature. The discontinuity in the aspartate curve at 25° has been consistently seen. It occurs at an aspartate concentration of about 130–140 mM.

It is possible to obtain a value for the inhibition constant, K_i, graphically if a fairly simple substrate inhibition mechanism is assumed (Dixon and Webb, 1964). This mechanism involves two separate binding sites. Binding of substrate to the inhibitory site alters the catalytic site so that it will not catalyze the formation of product. K_i is the dissociation constant for the inhibitory site. According to this mechanism a straight line is generated when the reciprocal of the velocity is plotted against the substrate concentration. The intercept of this line on the abscissa is equivalent to K_i. Even though this mechanism may not fully describe the actual situation, the K_i values derived from these plots can serve as a measure of the efficiency of aspartate inhibition under various conditions. A single-reciprocal plot from a representative aspartate saturation curve at 25° is shown in Figure 5. The biphasic nature of the aspartate inhibition is dramatically demonstrated here by the two straight-line segments intersecting at a point approximately equivalent to 133 mM aspartate. The x intercept of the second line located at higher aspartate levels is always on the positive side of the origin. The K_i (app) values have all been obtained from the line representing inhibition at lower aspartate concentrations. In general the values produced in this way are somewhat more variable and qualitative in nature than the K_m (app) and V_{max} (app) values which result from double-reciprocal plots.

When the kinetic analysis outlined above is employed, two different K_i values are obtained at 25°. Normally an enzyme preparation is divided into approximately 0.5-ml portions which are stored frozen. When this enzyme is thawed once and the K_i determined the value is 1.6×10^{-1} M. However, as the preparation is repeatedly frozen and rethawed this K_i value changes and becomes 1.3×10^{-2} M (Prescott, 1969). Presumably repeated freezing and thawing of the enzyme or some other factor involved in using it repeatedly for enzyme assays causes a selective change in the enzyme structure such that the apparent affinity of the aspartate inhibitory site for aspartate is markedly enhanced. No change is seen in the Michaelis constants or the V_{max} (app) in these preparations.

In Figure 6 a representative CAP saturation curve and the resulting double-reciprocal plots are presented. When the substrate saturation studies are conducted at 25°, the K_m (app) for CAP is $1.8 \pm 0.3 \times 10^{-4}$ M.[3] There is no inhibition of enzyme activity at high CAP levels, and the CAP saturation curves do not appear to be sigmoidal.

Effect of Nucleotides. Preliminary studies with fairly crude enzyme fractions indicated that the *S. faecalis* enzyme was not affected at all by nucleotides (Neumann and Jones, 1964). It was very important to confirm these preliminary observations. Both the pH 4.8 precipitate enzyme (specific

[3] The V_{max} (app) for these experiments is 48 μmoles of CAA/min per mg, a value lower than obtained from the aspartate saturation curves. This is expected because it is not possible to saturate the enzyme with aspartate because of the aspartate inhibition. An attempt has been made to study the variation in the saturation curve for one substrate when the concentration of the other substrate is changed at 25°. Due to the technical problems with the enzyme assay, the results obtained are only qualitative. The Lineweaver–Burk plots appear to intersect on or near the x axis when data from the saturation curves for one substrate are plotted. The slopes and y intercepts from these primary plots have been replotted in order to obtain dissociation and Michaelis constants for the substrates (Mahler and Cordes, 1966). The secondary plots are linear, but with an undesirable degree of scattering in some cases. From the experiments in which the aspartate saturation curves have been run at various CAP concentrations, the K_{asp} is 1.5×10^{-2} M and the K_{CAP} is 1.4×10^{-4} M. When the CAP curves were run at various aspartate concentrations, the K_{asp} is 2.4×10^{-2} M and the K_{CAP} is 1.5×10^{-4} M. The results, though only qualitative, are approximately the same as the Michaelis constants calculated from regular substrate saturation studies.

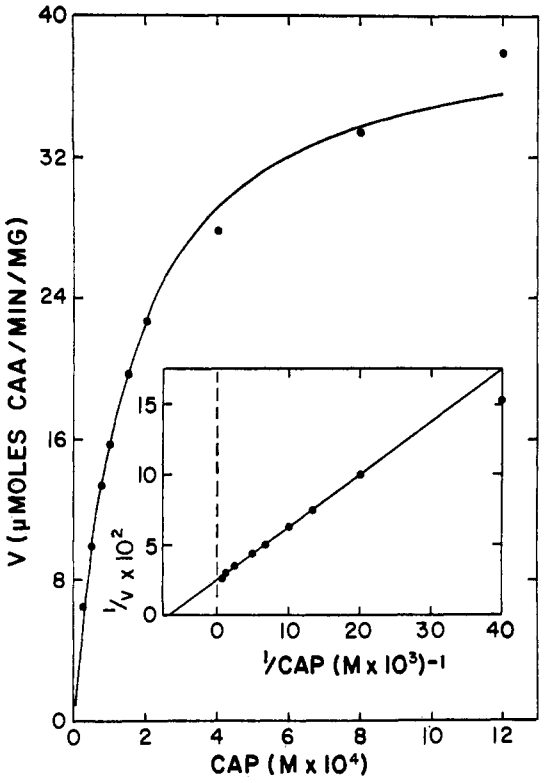


FIGURE 6: CAP saturation curve at 25°. The assay incubation mixture contained 100 mM Tris-Cl buffer (pH 8.5), 40 mM aspartate (pH 8.5), varying amounts of CAP, and 0.14 μg of enzyme protein (specific activity 55.7) in a total volume of 0.50 ml. The incubation period was 10 min with a 1-min preincubation period. The incubation was terminated with the addition of 1.50 ml of 0.5 N perchloric acid, and a 1.00-ml aliquot was assayed for CAA. The concentration of CAP stock solutions was determined by measurement of the phosphate concentration before and after hydrolysis of the CAP in 2 N KOH at room temperature for 10 min.

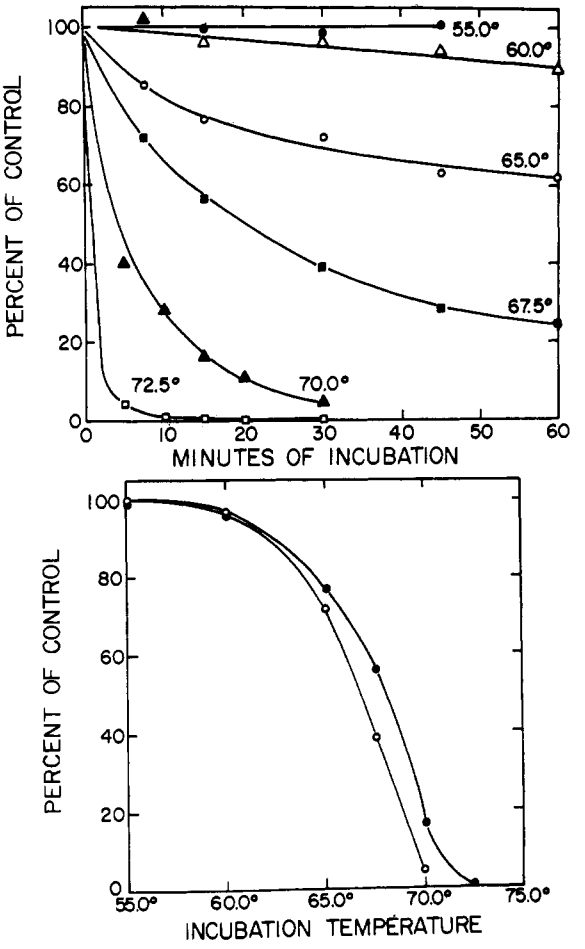


FIGURE 7: Temperature stability of the enzyme activity. Highly purified enzyme (specific activity 55.7) was incubated at a concentration of 0.05 mg/ml in 0.050 M potassium phosphate buffer (pH 6.6) for the intervals and at the temperatures given in the figure. The incubation at a particular temperature was started by the addition of the appropriate amount of concentrated enzyme solution to the preheated buffer. Aliquots of the incubation solution were removed at the specific intervals and chilled in ice water. They were kept on the ice until they could be assayed. There was no visible precipitate formed. The protein solutions were incubated in a Haake constant-temperature circulator bath, Model NBe. The control solution was prepared in the same way as the experimentals and kept on ice during the experimental incubations. The enzyme assay incubation mix contained 100 mM Tris-Cl buffer (pH 8.5), 40 mM aspartate (pH 8.5), 5 mM CAP, and enzyme solution containing 28 μg of protein. The assay incubation time was 10 min at 25°. The activity was determined by measurement of the CAA formed. The data for the lower graph were obtained from the values in the upper graph after 15 min (●-●) and 30 min (O-O) of incubation.

activity 0.98) and the highly purified enzyme (specific activity 76.1) were assayed in the presence of 1 and 5 mM concentrations of various nucleotides at 25°. A CAP concentration of 3×10^{-4} M, slightly over half-saturating concentration, was used in order to improve the chances of seeing any inhibition. No inhibition or activation was seen at either concentration with the following nucleotides: CTP, ATP, UTP, GTP, CMP, AMP, UMP, and GMP. This finding confirms the preliminary study and clearly distinguishes the *S. faecalis* aspartate transcarbamylase from the class A and class B forms (Bethell and Jones, 1969).

Stability of the Enzyme. The purified enzyme is quite stable to high temperatures at low protein concentrations as shown in Figure 7. Only 10% of the enzyme activity is lost after 1 hr at 60°. Above 60°, the enzyme stability rapidly declines over the span of a few degrees. The temperature at which half the activity is lost is 68.0° for a 15-min incubation period and 66.7° when a 30-min incubation period is used. A plot of the log of the per cent of control *vs.* time is not linear. Therefore the inactivation process is higher than zero order. The same pattern of temperature stability is seen when a crude enzyme fraction (specific activity 1.9) is incubated under the same conditions.

Because of the fact that the crude enzyme fractions show the same pattern of sensitivity, several temperature sensitivity studies with crude enzymes (specific activity 2.1) will be reported. The solutions were incubated at 67° for 50 min in 0.05 M potassium phosphate buffer (pH 6.6). The amount of activity remaining was 50, 49, and 33% when the protein concentrations were 0.10-, 0.05-, and 0.01-mg per ml solutions, respectively. The enzyme stability is independent of protein concentration when it is at or above 0.05 mg/ml.

Studies with crude enzyme preparation have also indicated that there is a very sharp pH optimum for temperature

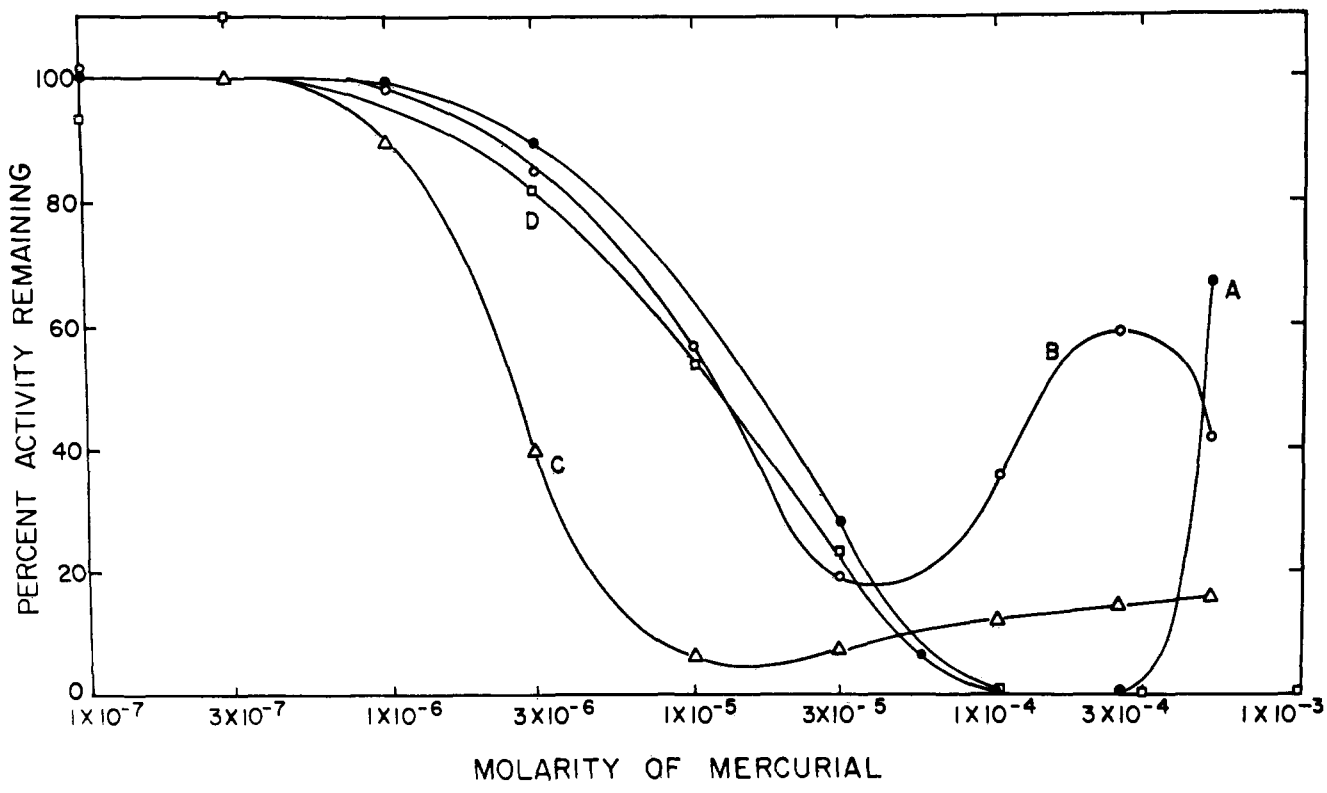


FIGURE 8: The effect of mercurials on aspartate transcarbamylase activity. All mercurial stock solutions were prepared in 0.01 M potassium phosphate buffer (pH 6.6). This same buffer was used to make all dilutions of the stock solutions, so that all incubations were carried out in this buffer. The enzyme sample, 5 or 10 μl, was added to 0.50 ml of each mercurial buffer solution to start the incubation. The mixture was incubated in a water bath at 25° for 30 min, and the incubation was terminated by immersion in an ice–water mixture. The experiment involving a crude enzyme fraction (specific activity 1.0) had a final protein concentration in the incubation mix of 0.78 mg/ml. When "pure" enzyme (specific activity 55.7) was used, the final protein concentration was 33.6 μg/ml. The enzyme activity was assayed immediately after the mercurial incubation. The crude enzyme mix was diluted 1:3 with cold distilled water for assay, and the "pure" enzyme mix, 1:10 with cold water. One-tenth-milliliter aliquots of these dilutions were incubated at 25° in assay incubation mixes containing 100 mM Tris-Cl (pH 8.5), 40 mM aspartate (pH 8.5), and 5 mM CAP. The formation of CAA was measured. The following experiments are presented: curve A (●-●), the effect of *p*MB on the crude enzyme; curve B (○-○), *p*MB effect on "pure" enzyme; curve C (△-△), $HgCl_2$ effect on "pure" enzyme; and curve D (□-□), mersalyl effect on "pure" enzyme.

stability. This optimum lies in the region of pH 6.6–6.7 when the enzyme is incubated with 0.05 M potassium phosphate buffers for 50 min at 67°. The enzymatic activity is only half as stable at either pH 6.0 or 7.1 as it is at pH 6.6.

Effect of Mercurials on Enzyme Activity. The effects of a series of mercurial compounds on aspartate transcarbamylase activity have been studied. The enzyme has been incubated at 25° for 30 min with various concentrations of each mercurial, and then the activity determined by assaying a dilution of this incubation mixture. The results are presented in Figure 8. The effect of *p*-mercuribenzoate (*p*MB) and mersalyl acid on the pure enzyme (curves B and D) are very similar up to a concentration of 3×10^{-5} M. When the mercurial concentration rises above this level, mersalyl completely inhibits the activity while *p*MB causes an increase in activity which peaks at about 3×10^{-4} M (curve B). Mercuribenzoate does completely inhibit the crude enzyme (curve A.) However, as the *p*MB concentration is further increased, the same activation is seen as with the purer preparation. Large amounts of extraneous protein may shift the *p*MB curve to higher values by binding the mercurial and thus lowering the concentration of the unbound mercurial capable of reacting with the enzyme.

The effect of mercuric chloride on the activity of the purer preparation differs from that seen with either organic mercurial (curve C). $HgCl_2$ is a much more effective inhibitor of activity at low concentrations than either of the other two compounds. The activity is inhibited 50% by 2.6×10^{-6} M $HgCl_2$. Thus mercuric chloride is about 4.5 times as effective as the organic mercurials. At higher concentrations, $HgCl_2$ is not completely inhibitory, for there is a slight rise in activity with increasing $HgCl_2$ concentration. This effect at higher $HgCl_2$ levels appears to be intermediate between the effects of mersalyl and *p*MB.

An attempt has been made to mimic the *p*MB effects by incubating the enzyme with a mixture of mercuric chloride and benzoic acid in equal amounts. The mercuric chloride curves in the presence and absence of benzoic acid are identical, and benzoic acid itself has no effect on the activity. Therefore, the benzoic acid will not interact with the enzyme to induce $HgCl_2$ binding and subsequent activation in the same way as *p*MB.

The inhibition by *p*MB and mersalyl is totally prevented by the addition of an equal amount of 2-mercaptoethanol to the mercurial solution before the addition of enzyme. About half the $HgCl_2$ inhibition is prevented. This lack

TABLE III: The Effect of Substrates, Products, and Inhibitors on Mersalyl Inhibition.[a]

Compound Added	% Act. Remaining	
	Expt I	Expt II
None	43	16
0.01 M P_i		42
0.05 M P_i	92	79
0.20 M P_i	103	
30 mM L-aspartate	95	90
100 mM L-aspartate	87	
30 mM L-aspartate + 0.20 M P_i	106	
0.5 mM CAP		63
5.0 mM CAP	178	124
2.5 mM D,L-CAA		65
2.5 mM D,L-CAA + 0.05 M P_i		94
0.20 M fluoride	93	

[a] The enzyme was incubated with 1×10^{-5} M mersalyl and assayed in the same way as described in Figure 7. Various compounds were present in the incubation mix where indicated. Highly purified enzyme (specific activity 55.7) was added to each mersalyl incubation mixture to give a final concentration of 33.6 μg of protein/ml. In expt I, the mersalyl solution contained 0.01 M potassium phosphate buffer (pH 6.6) so that the final phosphate concentration in each mersalyl incubation mix was 5 mM. In expt II, the mersalyl solution contained 1 mM buffer, and the final phosphate concentration in each mixture was 0.5 mM. The 100% control was prepared by diluting enzyme into 0.01 M (expt I) or 1 mM (expt II) potassium phosphate buffer (pH 6.6) and incubating in the same way as in the experiments with mersalyl present.

of total mercaptoethanol protection from $HgCl_2$ may be due to the inhibitory effectiveness of $HgCl_2$ and incomplete removal of the mercurial by mercaptoethanol. When the enzyme is incubated for 30 min at 25° with a maximally inhibitory concentration of mercurial and then incubated at 25° with an excess of mercaptoethanol for another 30 min, mersalyl and *p*MB inhibition is completely reversed; however mercuric chloride inhibition is only decreased from 92 to 84% inhibition. The final concentration of mercaptoethanol in the mix during the second incubation was twice that of the *p*MB concentration and seven times that of either $HgCl_2$ or mersalyl. Thus mercuric chloride is not only a more effective inhibitor, but its action is much less rapidly reversed.

The influence of substrates, products, and inhibitors on the inhibition of enzyme activity by mersalyl has been examined, and the results from two preliminary experiments are presented in Table III. These results indicate that low concentrations of substrates and products protect the enzyme against mersalyl attack. Fluoride, which is an effective inhibitor of enzymatic activity, also protects against mersalyl inhibition. The most marked effect is that of CAP. CAP binding, unlike aspartate or phosphate binding, seems to alter the action of mersalyl such that the enzyme is actually stimulated. The significance of these results will be discussed later.

Discussion

The purification procedure presented here results in about 300-fold purification with satisfactory overall yields. Each step is easily reproducible. In the course of the purification process a second minor aspartate transcarbamylase component has sometimes been observed. It is eluted from the DEAE-cellulose column by 0.15 M KCl. This minor component is very unstable, and the activity is lost upon sitting at 4° for 1 day. It constitutes less than 5% of the total enzymatic activity. The characteristics of this activity have not been examined due to its small quantity and great instability. It might be either another species of aspartate transcarbamylase or possibly some sort of association–dissociation product of the native enzyme.

It is clear from these studies that the *S. faecalis* aspartate transcarbamylase differs significantly from the class A and B enzymes. Differences are seen in such properties as pH optima, nucleotide effects, the shape of substrate saturation curves, mercurial sensitivity, temperature stability, and size. Since these differences have been discussed at some length previously (Bethell and Jones, 1969), they will not be dealt with in detail at this point.

The pattern of inhibition at low concentrations of mercurials followed by activation of the enzyme at higher concentrations is very unusual. There is precedent for it in the work of Owens on pancreatic amylase (Owens, 1953). Owens found that incubation of pancreatic amylase preparations in phosphate buffer (pH 7.0) with increasing concentrations of phenylmercuric acetate would result in increasing inhibition up to a mercurial concentration of about 5×10^{-8} M. With a further rise in the mercurial level, an increase in enzyme activity resulted. Above a concentration of 5×10^{-6} M, the activity again dropped with increasing mercurial concentration. This complex effect was not seen when pancreatic amylase was treated with mercuric chloride or when malt amylase was treated with either mercurials.

The complex mercurial inhibition curves seen with *S. faecalis* aspartate transcarbamylase and pancreatic amylase may be the result of the existence of more than one mercurial binding site which influences the activity of these enzymes. Mercuric chloride, mersalyl, and *p*MB all affect aspartate transcarbamylase activity differently at high concentrations. The *p*MB curve, in particular, appears to be due to the presence of two (or more) sites of different reactivity or accessibility toward *p*MB. At low concentrations, 10^{-5}–10^{-6} M, one would expect that the mercurials were probably binding to sulfhydryl groups. However, the effects which occur at higher mercurial levels may be the result of binding to other groups on the enzyme, possibly carboxylate or imidazole groups. Drenth has recently found during the course of crystallographic studies on papain structure that various mercurials bind to different groups on this enzyme (Drenth *et al.*, 1968). The mercury from $HgCl_2$ attaches to the active site sulfhydryl group. However, the mercurials, *p*-mercuribenzoate, *p*-mercuribenzenesulfonate, and *p*-mercurianiline, bind next to the two histidine residues present in papain.

The *S. faecalis* aspartate transcarbamylase is protected against inhibition by 1×10^{-5} M mersalyl acid by substrates, products, and inhibitors (Table III). The substrates, aspartate or CAP, protect the enzyme at concentrations similar to those required for saturation of the active site. Aspartate

protects at low concentrations, but may destabilize the enzyme at high levels which normally inhibit activity. CAP, in contrast with aspartate, P_i, and CAA, actually causes mersalyl to activate the enzyme. CAP seems to be inducing a different conformational change in the enzyme than aspartate or the reaction products do. If the protection of *S. faecalis* enzyme by substrates is due to binding at the active site, the experiments presented here may indicate that each substrate can bind to the enzyme in the absence of the second substrate since the presence of either substrate alone affords good protection at concentrations close to those required to achieve the maximum velocity of the reaction. With *E. coli* ATCase (or its catalytic subunit) either substrate can also bind to the enzyme alone (von Fellenberg, 1968; Collins and Stark, 1969); however the binding of the substrates during the enzymatic reaction may be ordered rather than random (Porter *et al.*, 1969).

Protection of the *S. faecalis* enzyme by reaction products also occurs at low concentrations. It has been shown in previous studies in this laboratory that carbamyl-D-aspartate does not bind to the enzyme and inhibit activity. Only carbamyl-L-aspartate will bind. Thus the effective concentration of CAA in expt II of Table III is actually only 1.25 mM assuming equal amounts of D- or L-CAA in the preparation. Inorganic orthophosphate may alter the enzyme's susceptibility to mersalyl at levels much lower than 10 mM. Mersalyl is more inhibitory in expt II of Table III than in expt I. The major difference is that the final phosphate buffer concentration in the mersalyl incubation mixtures of expt I is 5 mM; the concentration in expt II is 0.5 mM. This difference in phosphate buffer concentration may account for the differences in mersalyl inhibition. The great differences in mersalyl inhibition between the mixtures containing no phosphate, 10 mM phosphate, and 50 mM phosphate in expt II also indicate that phosphate must protect at levels well below 10 mM.

The mercurial effects are quite different from those seen with the *E. coli* enzyme (Gerhart and Schachman, 1968; von Fellenberg *et al.*, 1968). With the *E. coli* enzyme, *p*-mercuribenzoate binds first to the sulfhydryl group of regulatory subunit and dissociates the enzymes into catalytic and regulatory subunits (Gerhart and Schachman, 1965). Since the free catalytic subunit is a more active enzyme than the holoenzyme, the activity of this enzyme initially increases on treatment with *p*-mercuribenzoate (Gerhart and Pardee, 1962). When the *p*-mercuribenzoate is raised, even further, the mercurial binds to the sulfhydryl groups of the catalytic subunit and the activity of the catalytic subunit is inhibited (Gerhart and Schachman, 1968). In contrast the initial effect of *p*-mercuribenzoate on the *S. faecalis* enzyme is a marked inhibition of enzyme activity which is then followed by an activation.

Bethell and Jones (1969) defined three classes of bacterial ATCases. Class C ATCase was defined: (1) as that group of ATCases emerging from Sephadex G-200 with a K_d between 0.44 and 0.51; and (2) as those enzymes which are not inhibited by nucleotides. Using these criteria the *E. coli* catalytic subunit (mol wt 100,000) and the *S. faecalis* ATCase (mol wt 120,000–140,000) are both members of class C. The data presented here indicate that on further study these two proteins bear little resemblance to one another in their kinetic properties or their sensitivity to mercurials. It is possible that their ultimate structure, *i.e.*, the size of the polypeptides (Weber, 1968) associated to yield the protein isolated, may also be distinct and we therefore plan to purify the *S. faecalis* enzyme to homogeneity.

Acknowledgments

The authors acknowledge the very capable technical assistance of Mr. Johnny F. Obie and Mr. Gregory Headen. The assistance of the New England Enzyme Center, Boston, Mass., in the growth and disruption of the bacteria is also greatly appreciated.

References

Allen, C. M., and Jones, M. E. (1964), *Biochemistry 3*, 1238.
Bethell, M. R., and Jones, M. E. (1969), *Arch. Biochem. Biophys. 134*, 352.
Bethell, M. R., Smith, K. E., White, J. S., and Jones, M. E. (1968), *Proc. Nat. Acad. Sci. U. S. 60*, 1442.
Bleiweis, A. S., Karakawa, W. W., and Krause, R. M. (1964), *J. Bacteriol. 88*, 1198.
Chrambach, A. (1966), *Anal. Biochem. 15*, 544.
Chrambach, A., Reisfeld, R. A., Wyckoff, M., and Zaccari, J. (1967), *Anal. Biochem. 20*, 150.
Collins, K., and Stark, G. R. (1969), *J. Biol. Chem. 244*, 1869.
Davis, B. J. (1964), *Ann. N. Y. Acad. Sci. 121*, 404.
Dixon, M., and Webb, E. C. (1964), Enzymes, New York, N. Y., Academic, pp 75–78.
Drenth, J., Jansonius, J. N., Koekoek, R., Swen, H. M., and Wolthers, B. G. (1968), *Nature (London) 218*, 929.
Gerhart, J. C., and Pardee, A. B. (1962), *J. Biol. Chem. 237*, 891.
Gerhart, J. C., and Schachman, H. K. (1965), *Biochemistry 4*, 1054.
Gerhart, J. C., and Schachman, H. K. (1968), *Biochemistry 7*, 538.
Jones, M. E. (1962), *Methods Enzymol. 5*, 903.
Jones, M. E., and Spector, L. (1960), *J. Biol. Chem. 235*, 2897.
Mahler, H. R., and Cordes, E. H. (1966), Biological Chemistry, New York, N. Y., Harper & Row, pp 237–240.
Neumann, J., and Jones, M. E. (1964), *Arch. Biochem. Biophys. 104*, 438.
Oyama, V. I., and Eagle, H. (1956), *Proc. Soc. Exp. Biol. Med. 91*, 305.
Owens, R. G. (1953), *Contrib. Boyce Thompson Inst. 17*, 221.
Peterson, E. A., and Sober, H. A. (1962), *Methods Enzymol. 5*, 3.
Porter, R. W., Modebe, M. O., and Stark, G. R. (1969), *J. Biol. Chem. 224*, 1846.
Prescott, L. M. (1969), Ph.D. Dissertation, Brandeis University, Waltham, Mass.
Prescott, L. M., and Jones, M. E. (1969a), *Fed. Proc., Fed. Amer. Soc. Exp. Biol. 28*, 889.
Prescott, L. M., and Jones, M. E. (1969b), *Anal. Biochem. 32*, 408.
Shapiro, A. L., Vinuela, E., and Maizer, J. V. (1967), *Biochem. Biophys. Res. Commun. 28*, 815.
Siegelman, H. W., Weiczorek, G. A., and Turner, B. C. (1965), *Anal. Biochem. 13*, 402.
Sizer, I. W., and Jenkins, W. T. (1962), *Methods Enzymol. 5*, 677.
von Fellenberg, R., Bethell, M. R., Jones, M. E., and Levine,

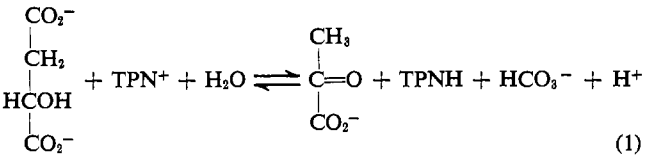
L. (1968), *Biochemistry 7*, 4322.
Weber, K. (1968), *Nature (London) 218*, 1116.
Weitzman, P. D. J., and Wilson, I. B. (1966), *J. Biol. Chem. 241*, 5481.

Purification and Properties of L-Malic Enzyme from *Escherichia coli**

Joseph Spina, Jr.,† Harold J. Bright,‡ and Joel Rosenbloom

ABSTRACT: A purification procedure for L-malic enzyme (EC 1.1.1.40) from *Escherichia coli*, which gives electrophoretically homogeneous enzyme, is described. The molecular weight of the native enzyme as determined by sedimentation equilibrium measurements was 550,000 g/mole. An $s_{20,w}$ value of 17.5 S was determined by a modified zone centrifugation technique. The subunit molecular weight, determined by sedimentation equilibrium measurements in 6.0 M guanidine hydrochloride and 0.01 M dithiothreitol, was 67,000 g/mole. This result, together with the tryptophan content from amino acid analysis, is consistent with an octameric structure for the native enzyme. The value of ϵ_{279} was $2.65 \times 10^5\ M^{-1}\ cm^{-1}$ and the maximum molar turnover number at 25° and pH 7.4 was about 1000 sec^{-1}. Electron paramagnetic resonance measurements showed that the inhibition of the enzyme by acetyl coenzyme A cannot be due to complexation of the essential activator Mn^{2+}, but rather to interaction of acetyl coenzyme A with the enzyme in a presumably allosteric fashion. There was an obligatory monovalent cation requirement for catalytic activity, which can be satisfied by K^+ or NH_4^+, and acetyl coenzyme A was a noncompetitive inhibitor with respect to K^+. At increasing concentrations in excess of its K_m value (8×10^{-3} M), K^+ progressively inhibited the enzyme and caused the malate saturation curve to become sigmoid. Since the allosteric properties of acetyl coenzyme A and other inhibitors appear to be consistent with a two-state enzyme conformational model (Sanwal, B. D., and Smando, R. (1969b), *J. Biol. Chem. 224*, 1824) in which malate binds preferentially to the conformational state which predominates in the absence of allosteric ligands, this behavior of K^+ at high concentrations may result from the fact that it has a greater affinity for that state of the enzyme which binds malate poorly.

L-Malic enzyme [L-malate:TPN oxidoreductase (decarboxylating), EC 1.1.1.40] catalyzes the oxidative decarboxylation of L-malate (eq 1). The enzyme appears to function in



(1)

both mammals and bacteria to provide pyruvate and TPNH for lipid synthesis, and not as a component of gluconeogenesis (Wise and Ball, 1964; Young *et al.*, 1964; Ashworth *et al.*, 1965; Jacobson *et al.*, 1966). In the case of *Escherichia coli*, the enzyme is induced by growth on L-malate as the sole carbon source (Stern and Hegre, 1966).

Sanwal and his colleagues have recently reported that L-malic enzyme from *E. coli* is inhibited by acetyl coenzyme A (Sanwal *et al.*, 1968), by oxalacetate, TPNH, DPNH (Sanwal and Smando, 1969a), and by adenosine 3′,5′-cyclic phosphate (Sanwal and Smando, 1969c). Sanwal and Smando (1969b) performed a steady-state kinetic analysis of the reaction, using partially (100-fold) purified enzyme, and concluded that an ordered mechanism, with isomerization of free enzyme, was consistent with their data. In the presence of certain inhibitors, such as acetyl coenzyme A, oxalacetate, and DPNH, the steady-state velocity patterns for malate became nonhyperbolic (sigmoidal) and, in the cases of acetyl coenzyme A and oxalacetate, the inhibition was competitive with respect to malate. The conclusion that acetyl coenzyme A, oxalacetate and DPNH are allosteric inhibitors was supported by the finding that the enzyme was desensitized to the action of these ligands by high concentrations of glycine. The allosteric effects were analyzed in terms of a two-state model in which malate has a high affinity for state R, whereas the inhibitors stabilize state T, with which malate binds poorly or not at all. Sanwal and Smando (1969a)

* From the Departments of Biochemistry (J. S., H. J. B., and J. R.), Ophthalmology (J. S.), and Medicine (J. R.), School of Medicine, University of Pennsylvania, Philadelphia, Pennsylvania 19104, and Philadelphia General Hospital (J. R.), Philadelphia, Pennsylvania 19104. *Received February 16, 1970.* This project was supported by Grants GM-11040 (H. J. B.) and FR-00107 (J. R.) from the National Institutes of Health, U. S. Public Health Service.

† Postdoctoral Fellow of the U. S. Public Health Service (Training Grant 5T01-GM-00957-08, Division of General Medical Sciences); Resident in Ophthalmology, Hospital of the University of Pennsylvania, Philadelphia, Pa. 19104.

‡ Research Career Development Awardee 5-K03-GM-34960 of the U. S. Public Health Service. To whom inquiries should be addressed.