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Aspartate Transcarbamylase from *Streptococcus faecalis*. Steady-State Kinetic Analysis†

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ABSTRACT: The kinetic mechanism of *Streptococcus faecalis* aspartate transcarbamylase has been studied using steady-state kinetic analysis in the absence or presence of moderate levels of acetate to partially saturate an anion activator site in the enzyme molecule (Chang, T. Y., and Jones, M. E. (1974), *Biochemistry 13*, 629). Initial velocity studies indicate that the enzyme catalyzes the reaction by a sequential mechanism. Product inhibition analysis yields three competitive patterns and one noncompetitive pattern, a result which is more consistent with a random than an ordered mechanism, and which also supports the existence of a dead-end complex: enzyme–carbamyl phosphate–carbamyl aspartate. Pyrophosphate and maleate were chosen as substrate analogs of carbamyl phosphate and L-aspartate, respectively, for dead-end inhibition analysis. Two competitive inhibition patterns and two noncompetitive inhibition patterns are obtained in this analysis; this result also indicates the absence of a compulsory binding order between the points or addition of carbamyl phosphate and aspartate to the enzyme. The lower limits for the dissociation constants are 1×10^{-4} M for K_d(carbamyl phosphate) and 1.7×10^{-3} M for K_d(L-aspartate). Binding of carbamyl phosphate to the enzyme first hinders the binding of aspartate, while binding of aspartate first facilitates the binding of carbamyl phosphate. An asymmetrical random mechanism has been constructed as a tentative scheme for the kinetic mechanism for this enzyme since it can qualitatively explain all of the data.

In a previous paper (Chang and Jones, 1974a) we reported the purification of aspartate transcarbamylase from *Streptococcus faecalis* to near homogeneity and some kinetic evidence for an anion activator site of this enzyme. The activator site is sensitive to many anions including the anionic substrates and products of the reaction. With kinetic techniques, it is not possible to be certain whether the activator site is absolutely essential for the catalytic reaction of this enzyme or not. Fortunately, normal hyperbolic plots for the aspartate and carbamyl phosphate saturation curves can be obtained when acetate concentrations are high, because when acetate has partially saturated the activator site the substrates bind only once to the catalytic sites of this enzyme at low substrate concentrations. Under these conditions, the concentrations of aspartate, carbamyl phosphate, phosphate, and carbamyl aspartate can be varied to a reasonable extent without perturbing the activator site so that conventional steady-state kinetic analysis of the initial velocity, as well as product inhibition and dead-end inhibition studies, can be used to elucidate the kinetic mechanism of the reaction including the possible role of the activator molecule during enzyme catalysis.

Experimental Section

Chemicals and Reagents. The aspartate transcarbamylase used was 90–95% pure and is the purest enzyme available (Chang and Jones, 1974a). It can be stored for 2 months at 4°, at a concentration near 0.1 mg/ml, in 5 mM potassium phosphate buffer (pH 6.6). The stock enzyme solutions (about 1 mg/ml, in 5 mM potassium phosphate buffer (pH 6.6)) are stored at −20° and are stable for at least 2 years.

[^{14}C]Carbamyl phosphate (dilithium salt) used here is the same as mentioned in the previous paper (Chang and Jones, 1974a). It contains a minor radioactive contaminant, which is not converted to $^{14}CO_2$ afer acidification and boiling, that

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amounts to about 0.15–0.20% of the total counts per minute used. The nonradioactive carbamyl phosphate was from Sigma Chemical Co. and was used without recrystallization. It was 99% pure, as determined by phosphate assays before and after hydrolysis in 10 mM NaOH at room temperature for 10 min (Jones and Spector, 1960; modification of Fiske–Subba-Row method). Dowex 50-X8 resins (200–400 mesh) purchased from Bio-Rad laboratories were washed extensively with NaOH, H_2O, HCl, and H_2O successively before use; the pH of the water suspensions of the resin was above 5. Dowex 1-X8 resins (200–400 mesh) were treated the same way except that HCOOH replaced HCl.

Potassium pyrophosphate was purchased from K&K Laboratories, Hollywood, Calif. Stock solutions of pyrophosphate contained less than 3% orthophosphate as determined by phosphate assay.

Synthesis of L-*Carbamyl Aspartate.* A procedure similar to the one used by Nyc and Mitchell (1947) and Reichard and Hanshoff (1956) was used. Potassium cyanate (25 mmol; solid crystals) was added quickly to 50 ml of a solution containing 0.5 M L-aspartate and 0.1 M *N*-2-hydroxyethylpiperazine-*N*-2′-ethanesulfonic acid buffer (pH 7.0). This solution was stirred and maintained at 60° for 2 hr in a 250-ml round-bottomed flask equipped with an air condenser. The reaction was terminated by adding enough glacial acetic acid (about 20 ml) so the final pH was at or below 3.7. The mixture was then concentrated to about 20 ml by lyophilization. The concentrated mixture was passed through a 2 × 40 cm column packed with Dowex 50-X8 beads (H^+ form), and the column was eluted with water. Fractions (2.0 ml/fraction) containing carbamyl aspartate (Prescott and Jones, 1969) were collected and pooled. The pooled fraction was lyophilized, redissolved in water, and lyophilized again several times to remove excess acetic acid. The final powders were redissolved in water, adjusted to pH 6 with NaOH, and then passed through a 2 × 12 cm Dowex 1-X8 formate column. The column was eluted with 30 ml of water, 30 ml of 0.1 M formic acid, and 0.5 M formic acid successively. The fractions containing carbamyl aspartate emerged during elution with 0.5 M formic acid. The pooled carbamyl aspartate fractions were lyophilized, redissolved, and lyophilized several times until the formic acid was removed. The solid obtained was stored. The amount of carbamyl aspartate determined colorimetrically (Prescott and Jones, 1969) agreed with the amount of aspartate released after alkaline hydrolysis. Moreover, the titration curve of 10 mM free L-carbamyl aspartic acid with 10 mM NaOH is identical with that of 10 mM free DL-carbamyl aspartic acid obtained commercially from Sigma Chemical Co., *i.e.*, a given quantity of added base gave the same pH with both samples over the entire titration curves. The final yield of L-carbamyl aspartic acid was about 60–70%.

All other reagents and chemicals were reagent grades and were used without recrystallization.

Aspartate Transcarbamylase Assay. The assay used for kinetic analysis was the [^{14}C]carbamyl phosphate assay of Bethell *et al.* (1968) unless otherwise stated. All incubations were performed at 25°, pH 8.52 ± 0.04. The 0.5- or 1-ml reaction mixture contained 5 mM Tris-HCl with varying levels of substrates, inhibitors, and aspartate transcarbamylase. Incubation time ranged from 10 to 20 min. The reaction was stopped by addition of 0.5 or 1 ml of perchloric acid. The amount of $HClO_4$ had to be increased when high concentrations of a buffering anion, *i.e.*, acetate, were used. Enough $HClO_4$ was added under these conditions to lower the pH below 4. After acidification the test tubes were capped with marbles and immersed in a 100° water bath for 6 min, and the solutions then bubbled with CO_2 for 30 min. The volume of each solution was adjusted to 2 ml with water, and the mixture was transferred to a glass scintillation vial to which 10 ml of dioxane–naphthalene scintillation fluid was added. The counting efficiency was around 78–81%, and the error for each sample was usually less than 3%. Controls without enzyme were routinely run and gave reproducible background values. The specific activity of [^{14}C]carbamyl phosphate used was selected so that the counts per minute in each experimental sample were greater than 500. The initial velocity was linear up to about 40% substrate consumption when carbamyl phosphate was limiting, or to about 20% substrate consumption when aspartate was the limiting substrate. All experiments were performed within these ranges.

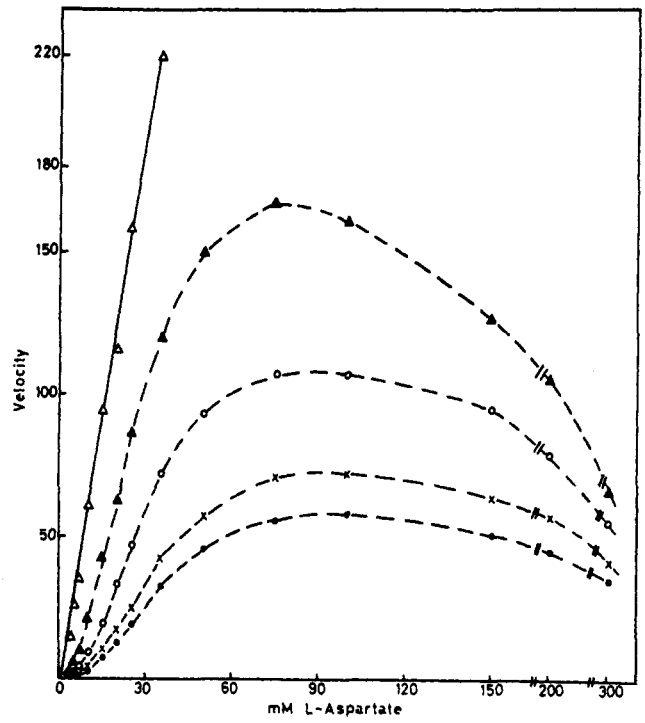


FIGURE 1: Velocity *vs.* L-aspartate at changing fixed concentrations of carbamyl phosphate in the absence of acetate. Carbamyl phosphate concentrations used: (●) 0.1 × 10^{-4} M; (×) 0.143 × 10^{-4} M; (○) 0.333 × 10^{-4} M; (▲) 1 × 10^{-4} M; (△) 5 × 10^{-4} M. The data at 0.2 × 10^{-4} M were not shown in order to keep the picture clear. The [^{14}C]carbamyl phosphate assay was used when carbamyl phosphate concentrations varied from 0.1 × 10^{-4} to 1 × 10^{-4} M. One milliliter of reaction mixture contained 5 mM Tris-HCl (pH 8.5), varying concentrations of [^{14}C]carbamyl phosphate (0.764 × 10^6 cpm/μmol), and 0.074 μg of aspartate transcarbamylase (for aspartate levels at 4–10 mM range) or 0.01 μg of aspartate transcarbamylase (for aspartate levels above 10 mM). The reaction time at 25° was 10 min. The colorimetric carbamyl aspartate assay was used when the carbamyl phosphate concentration was 5 × 10^{-4} M. The latter reaction mixture (1 ml) contained 5 mM Tris-HCl (pH 8.5), 5 × 10^{-4} M carbamyl phosphate, 0.333 μg of aspartate transcarbamylase when the L-aspartate concentration was 4–6 mM, 0.111 μg of aspartate transcarbamylase when the L-aspartate concentration was 10 or 15 mM, and 0.0185 μg of aspartate transcarbamylase when the L-aspartate concentration was 20–25 mM. The reaction time at 25° was 10 min. The reaction was terminated by 1 ml of color mix for the colorimetric carbamyl aspartate assay.

The terminology and nomenclature of all the kinetic terms and constants are those defined by Cleland (1963a). The velocity, V, is always expressed as micromoles of product formed per minute per milligram of protein unless stated otherwise.

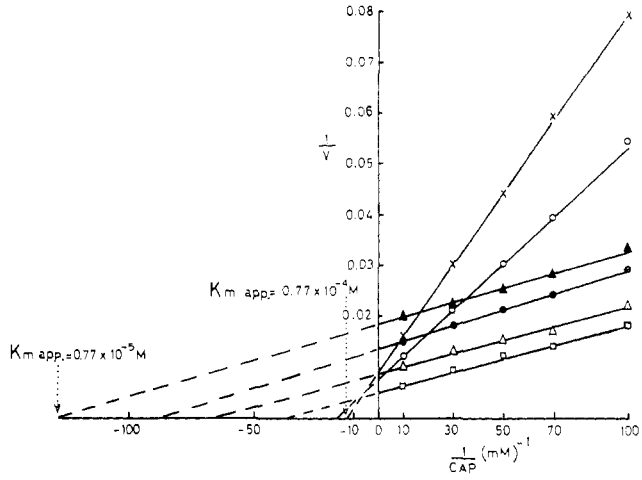


FIGURE 2: Double reciprocal plot using carbamyl phosphate as variable substrate at changing fixed concentrations of L-aspartate in the absence of acetate. The data are identical with part of the data shown in Figure 1. L-Aspartate concentrations used: (×) 20 mM; (○) 25 mM; (□) 75 mM; (△) 200 mM; (●) 300 mM; (▲) 350 mM. Abbreviations used in all figures are: CAP, carbamyl phosphate; L-Asp, L-aspartate; CAA, carbamyl aspartate.

Results

Initial Velocity Studies in the Absence of Added Acetate. Under this condition, a second molecule of aspartate may bind to the aspartate transcarbamylase activator site in addition to its normal binding to the substrate site (Chang and Jones, 1974a). Figure 1 shows the aspartate saturation curves at various concentrations of carbamyl phosphate. A series of sigmoidal curves is obtained before inhibition by aspartate intervenes. These curves become less sigmoidal as the carbamyl phosphate concentration is increased. Figure 2 shows part of the same set of data as in Figure 1, but the data are now plotted as $1/V$ *vs.* 1/[carbamyl phosphate] at different levels of aspartate. This replot allows one to perceive that the intercepts and slopes are complicated functions of the aspartate concentration and that the apparent K_m values for carbamyl phosphate always decrease as the aspartate level increases.

Initial Velocity Studies in the Presence of Acetate. To study only the substrate binding sites, one must fill the activator site with an anion that is not a substrate. Acetate was known to be a desirable anion (Chang and Jones, 1974a), and it was found that concentrations below 100 mM acetate produced no inhibition (Chang, 1973). Most of the experiments that follow were carried out with acetate concentrations ranging from 50 to 100 mM. The product inhibition studies were carried out in 400 mM sodium acetate.

There are two main types of initial velocity patterns: intersecting or parallel. If an intersecting pattern obtains the points of combination of the changing fixed substrate and the variable substrate in the reaction sequence are connected by reversible steps (Cleland, 1963b). Figures 3A and 3B represent the results of the initial velocity studies using carbamyl phosphate or L-aspartate as variable substrate; acetate, as activator, was constantly 50 mM. The aspartate concentrations in this and the following experiments were low in order to avoid substrate inhibition (see Chang and Jones, 1974a); as a result, the range of aspartate levels used had to be rather limited. Intersecting patterns were obtained in both plots. In ter-reactant mechanisms, intersecting initial velocity patterns may occur with ping-pong mechanisms (for discussion, see Cleland, 1970), but since the third reactant, in this case, is only an activator and not a substrate, the patterns shown in Figures 3A and 3B essentially rule out any chemically meaningful ping-pong mechanism.

The slope and intercept replots from Figures 3A and 3B are linear. Table I lists the kinetic constants derived from these data for the aspartate transcarbamylase of *S. faecalis*.

Product Inhibition Studies. The four possible pairings between substrates and products were studied (Chang, 1973), and two are shown in Figures 4 and 5, respectively. Acetate (0.4 M) was present in all experiments to partially saturate the enzyme activator site. As a result, the aspartate saturation curve is linear and most of the activation effects of the products, phosphate and L-carbamyl aspartate, are blocked. Competitive inhibition is seen when the pairs varied were carbamyl phosphate and phosphate (Figure 4), aspartate and phosphate, or aspartate and L-carbamyl aspartate (see microfilm version[1] Figures 1 and 2, respectively). The only noncompetitive effect is seen in the carbamyl phosphate and L-carbamyl aspartate pairing (Figure 5A). The slope replots of the pairs showing competitive inhibition are all linear. Figures 5B and 5C show the slope of intercept replot of Figure 5A. Both curves are nonlinear and concave down, presumably due to an antagonism between activation and inhibition by L-carbamyl aspartate, for L-carbamyl aspartate is a strong anion activator, and 0.4 M acetate probably cannot completely saturate the activator site when L-carbamyl aspartate is present. Table II lists the apparent inhibition constants of phosphate and L-carbamyl aspartate obtained from the replots of the four product inhibition pairs.

Phosphate Effect in the Absence of Acetate. Figure 6 shows slope and intercept replots obtained in the absence of acetate from $1/V$ *vs.* 1/[carbamyl phosphate] plots of the effect of phosphate (16–75 mM) against carbamyl phosphate (1–4 mM) as variable substrate at a fixed low level of aspartate (Chang, 1973). Under this condition phosphate serves both as an activator and a product inhibitor. It can be seen that the slope replot is affected by both the activation effect and the inhibition effect of phosphate, for this replot is definitely biphasic in nature. On the other hand, the intercept replot (Figure 6A) is strictly linear, indicating that the intercept effect comes only from activation; in other words, inhibition by phosphate only affects K_m and not V_{max}. This confirms the competitive nature of phosphate inhibition against carbamyl phosphate found in the product inhibition studies.

Dead-End Inhibition Studies. This is another powerful tool for kinetic analysis as emphasized by Fromm (1967). The two substrate analogs selected to be dead-end inhibitors were pyrophosphate as the carbamyl phosphate analog and maleate as the L-aspartate analog. It is known from the studies of many laboratories, but is best illustrated by the work of Porter *et al.* (1969), that essentially any compound with a phosphate moiety can inhibit the *Escherichia coli* aspartate transcarbamylase at the carbamyl phosphate site. Maleate has been known as a potent competitive analog of aspartate for many years (Jones, 1962; Gerhart and Pardee, 1964). With *S. faecalis* aspartate transcarbamylase (Chang, 1973) pyrophosphate is a linear competitive inhibitor of carbamyl phosphate (K_i(pyrophosphate) = 0.9 mM) and maleate is a linear competitive inhibitor of aspartate (K_i(maleate) = 1 mM), since the intercept values were not affected by these analogs but the slope values were. The levels (0.5–1.5 mM) of pyrophosphate

[1] See paragraph at end of paper regarding supplementary material.

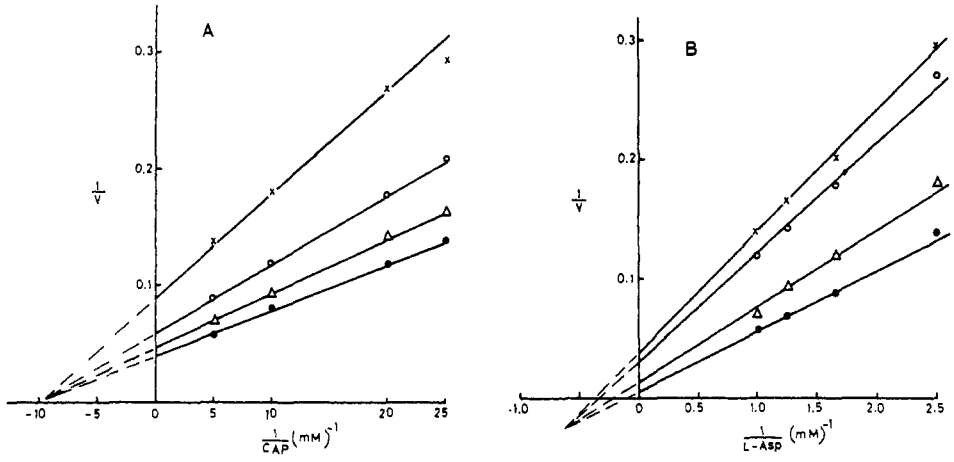


FIGURE 3: Initial velocity studies in the presence of acetate. (A) Double reciprocal plots with carbamyl phosphate as a variable substrate. L-Aspartate concentrations used: (×) 0.4 mM; (○) 0.6 mM; (△) 0.8 mM; (●) 1 mM. The reaction mixture (1 ml) contained 5 mM Tris-HCl (pH 8.5), 0.111 μg of aspartate transcarbamylase, 50 mM sodium acetate, varying concentrations of L-aspartate, and [^{14}C]carbamyl phosphate (0.573×10^6 cpm/μmol). The reaction time was 15 min. (B) Double reciprocal plots with L-aspartate as variable substrate. The data are the same as in A. Carbamyl phosphate concentrations used: (×) 0.4×10^{-4} M; (○) 0.5×10^{-4} M; (△) 1×10^{-4} M; (●) 2×10^{-4} M.

TABLE I: Kinetic Parameters for Aspartate Transcarbamylase.

Kinetic Constant	Estimated Value(s) from Kinetic Anal. (M)	Remarks
K_d(carbamyl phosphate)[a]	1.0×10^{-4}	Obtained from the point of intersection of lines in Figure 4A; lower limit for true K_d(carbamyl phosphate)
Apparent K_d(aspartate)[b]	1.7×10^{-3}	Obtained from the point of intersection of lines in Figure 4B; lower limits for true K_d(aspartate)
Apparent K_m(carbamyl phosphate)	0.77×10^{-5}	Obtained at 0.35 M aspartate without addition of acetate
Apparent K_m(aspartate)	10×10^{-3}	Obtained at 2×10^{-4} M carbamyl phosphate, 50 mM acetate

[a] K_d(carbamyl phosphate) is the dissociation constant for the equilibrium: enzyme–carbamyl phosphate $\rightleftharpoons$ enzyme + carbamyl phosphate. [b] K_d(aspartate) is the dissociation constant for the equilibrium: enzyme–aspartate $\rightleftharpoons$ enzyme + aspartate.

TABLE II: Apparent Inhibition Constants (mM) of Phosphate or L-Carbamyl Aspartate.

Variable Substrate	K_{is}(Phosphate)[a]	K_{ii}(L-Carbamyl Aspartate)[b]	K_{is}(L-Carbamyl Aspartate)
Carbamyl phosphate	5	13[c]	7[c]
L-Aspartate	14		15

[a] K_{is} designates apparent slope inhibition constant. [b] K_{ii} designates apparent intercept inhibition constant. [c] Obtained by extrapolating the tangent lines of the curves in Figures 8A and 8B to the horizontal axis.

TABLE III: Expected Product Inhibitions and Dead-End Inhibition Patterns in Ordered "Ter–Ter" [a] Reaction.

Variable Reactant[e]	Inhibition[b] by Analog of A	B	C	Inhibition[c] by Products P	Q	R
A	C[d]	UC	UC	NC	UC	C
B	NC	C	UC	NC	UC	NC
C	NC	NC	C	NC	UC	NC

[a] The term "ter–ter" refers to a reaction which involves three reactants in both directions. [b] Assuming an analog of A binds to the same site where A binds; the same assumptions apply as to an analog of B or an analog of C. [c] Assuming $A + B + C \rightleftharpoons P + Q + R$, where A, B, and C refer to the first, second, and third reactants added to the enzyme, respectively, and P, Q, and R are the products to leave the enzyme subsequently in that order. [d] C, competitive; NC, noncompetitive; UC, uncompetitive. [e] Changing fixed reactants kept at nonsaturating levels.

and maleate used were low enough so they probably did not appear to cause any other effect than binding to either the carbamyl phosphate or aspartate catalytic sites, respectively.

Figure 7 shows the inhibitory effect of pyrophosphate against aspartate, and Figure 8 shows the inhibitory effect of maleate against carbamyl phosphate. Both the slope and intercept values vary with the analog concentration; the nonparallel lines are indicative of a random binding of carbamyl phosphate and aspartate to the enzyme. Table III lists the predicted product inhibition patterns and dead-end inhibition patterns for an ordered ter–ter reaction. Table IV summarizes the observed product inhibition patterns and dead-end inhibition patterns for aspartate transcarbamylase.

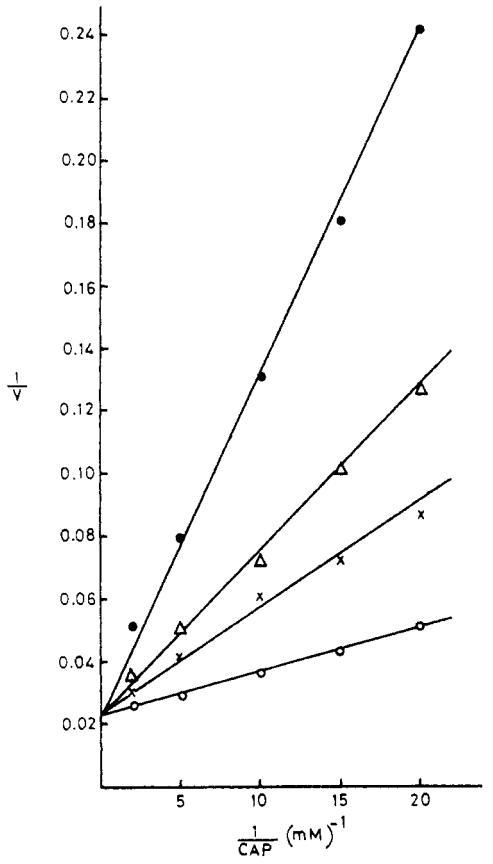


FIGURE 4: Product inhibition by phosphate against carbamyl phosphate as variable substrate. Phosphate concentrations used: (○) 0 mM; (×) 5 mM; (△) 12.5 mM; (●) 30 mM. The reaction mixture (1 ml) contained 5 mM Tris-HCl with 0.4 M NaOAc (pH 8.5), 1 mM L-aspartate, 2.47×10^{-2} of μg of aspartate transcarbamylase, and varying concentrations of [^{14}C]carbamyl phosphate (0.38×10^6 cpm/μmol). The reaction time was 15 min.

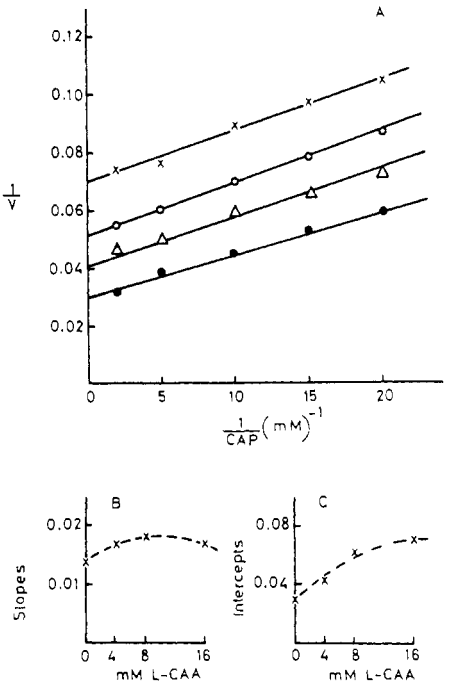


FIGURE 5: Product inhibition of L-carbamyl aspartate against carbamyl phosphate as variable substrate. (A) L-Carbamyl aspartate concentrations used: (●) 0 mM; (△) 4 mM, (○) 8 mM; (×) 16 mM. The reaction mixture (1 ml) contained 5 mM Tris-HCl with 0.4 M sodium acetate (pH 8.5), 1 mM L-aspartate, 2.47×10^{-2} μg of aspartate transcarbamylase, and varying concentrations of [^{14}C]carbamyl phosphate (0.38×10^6 cpm/μmol). The reaction time was 15 min: (B) the slope replot; (C) the intercept replot.

TABLE IV: Inhibition Patterns Found in Aspartate Transcarbamylase Reaction.

Variable[a] Reactant	Dead-End Inhibition by		Product Inhibition by	
	PP_i	Maleate	P_i	L-Carbamyl Aspartate
Carbamyl phosphate	C[b]	NC	C	NC
L-Aspartate	NC	C	C	C

[a] Changing fixed reactants kept at nonsaturating levels. [b] C, competitive; NC, noncompetitive.

Discussion

Product inhibition and dead-end inhibition studies are powerful tools for distinguishing between different sequential mechanisms of multi-reactant enzyme-catalyzed reactions. In the present case, the situation is complicated by the existence of an activator site within the enzyme molecule which is sensitive to all anions tested (Prescott, 1969a,b; Chang and Jones, 1974a,b). To eliminate activation by the substrates, products, or dead-end inhibitors, we have carried out the product and dead-end inhibition studies in the presence of acetate to fill the activator site. The large amount of acetate (0.4 M) present in the product inhibition experiments cannot completely prevent L-carbamyl aspartate from binding to the activator site; despite this complication, the inhibition patterns are qualitatively quite clear. Maleate and pyrophosphate, chosen as substrate analogs of aspartate and carbamyl phosphate for the dead-end inhibition analysis, were kept very low (about 1 mM), so that the prerequisite for using substrate analogs in dead-end inhibition analysis (Fromm, 1967), namely, that the analog binds only to the substrate site of aspartate transcarbamylase, was probably met.

Several important conclusions which result from the product and dead-end inhibition studies can be summarized as follows. (a) The sequence of addition of carbamyl phosphate and aspartate or the release of carbamyl aspartate and phosphate is probably random. Three competitive product-inhibition patterns and only one noncompetitive pattern are obtained which fit the random mechanism better than the ordered one (compare Tables III and IV). The two noncompetitive dead-end inhibition patterns obtained also indicate that carbamyl phosphate can bind to aspartate transcarbamylase either before or after binding of aspartate, and *vice versa*. (b) A dead-end complex, enzyme–carbamyl phosphate–carbamyl aspartate, may exist, and this presumably explains the noncompetitive nature of carbamyl aspartate inhibition against carbamyl phosphate as variable substrate. As pointed out by Cleland (1970), a dead-end complex is more likely to be formed between the smaller pair of reactants (*i.e.*, aspartate and P_i[2]), which do not contain the group transferred, than between the larger pair of reactants (*i.e.*, carbamyl phosphate and

[2] Abbreviations used are: P_i, inorganic phosphate; PP_i, inorganic pyrophosphate.

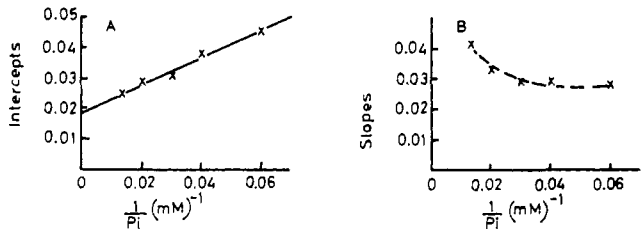


FIGURE 6: Intercept (A) and slope (B) replot of carbamyl phosphate as a variable substrate at different phosphate concentrations. Phosphate concentrations used were: 16.67, 25, 33.33, 50, and 75 mM. The reaction mixture (1 ml) contained 5 mM Tris-HCl (pH 8.5), 0.296 μg of aspartate transcarbamylase, and [^{14}C]carbamyl phosphate concentrations were 0.25, 0.33, 0.5, and 1 mM. The reaction time was 20 min. Aspartate was 1 mM.

carbamyl aspartate). In our case, the existence of the activator site, which presumably influences the conformation of the substrate sites, may favor the unusual binding suggested by the more complicated dead-end pattern. A second possibility, which can be envisioned if the activator and substrate sites are located near one another, is that when carbamyl aspartate binds to the enzyme–carbamyl phosphate complex, a part of the carbamyl aspartate molecule binds to the aspartate catalytic site and the other part of the same molecule binds to the activator site, thereby forming the unusual enzyme–carbamyl phosphate–carbamyl aspartate dead-end complex. The possible dead-end complex, enzyme–aspartate–P_i, is not seen by the product-inhibition analysis. It may be that when there is a high level of acetate (0.4 M), acetate can compete with phosphate for the enzyme–aspartate complex to produce an active enzyme complex (enzyme–aspartate–OAc) and reduce the accumulation of the dead-end enzyme–aspartate–P_i complex. (c) Although the sequence of addition of carbamyl phosphate and aspartate is most probably random, there is no definitive evidence that either one or both of them binds first; rather, the activator (acetate) could bind first, followed by random additions of carbamyl phosphate and aspartate. Product-inhibition analysis cannot determine between these possibilities.

Dalziel (1969) has presented a systematic and comprehensive treatment of the analysis of initial rate data for ter-reactant enzymes. We find the use of this analysis difficult since the third reactant in this instance is an activator and not a substrate (it is uncertain whether this activator molecule is auxiliary or if it is absolutely essential for enzyme catalysis to occur). Moreover, the substrate inhibition by aspartate prevents one from designing initial velocity experiments over as extensive a range of aspartate concentrations as one would wish for such a study.

Initial velocity studies reported here were carried out *without* or *with* acetate (50 mM). The intersecting patterns obtained in the presence of acetate (Figures 3A and 3B) effectively rule out any meaningful ping-pong mechanism. Important consequences which result from the initial velocity studies can be summarized as follows. (a) The extent of sigmoidicity of aspartate saturation curves decreased in response to increasing levels of carbamyl phosphate (Figure 1). Since it is known that the main cause of sigmoidicity in the *V vs.* [aspartate] plots is probably the binding of aspartate to the aspartate transcarbamylase activator site (Chang and Jones, 1974a), this datum would suggest that binding of an activator by aspartate transcarbamylase may not be necessary for catalysis to occur at higher concentrations of carbamyl phosphate. The apparent K_m(carbamyl phosphate) (Figure 2)

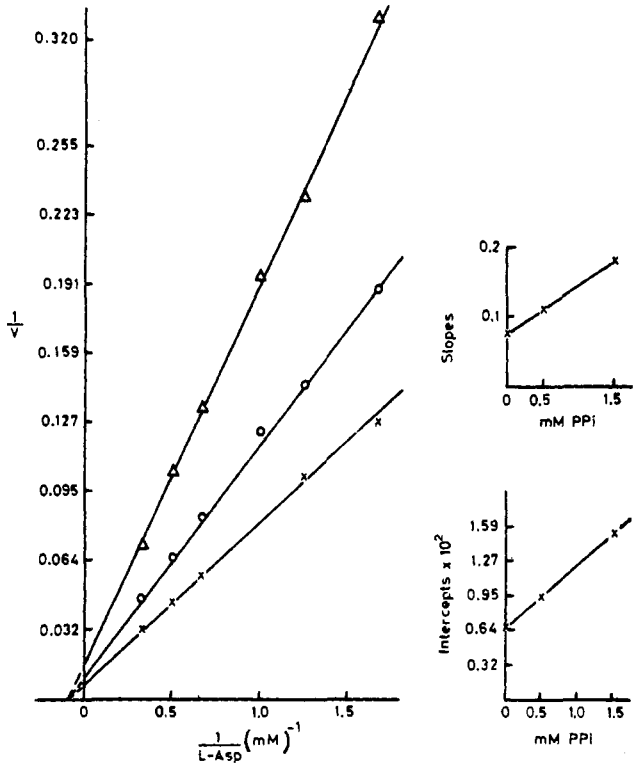


FIGURE 7: Dead-end inhibition by pyrophosphate against L-aspartate as a variable substrate. Pyrophosphate concentrations used: (×) 0 mM; (○) 0.5 mM; (△) 1.5 mM. The reaction mixture (1 ml) contained 5 mM Tris-HCl, 0.04 mM [^{14}C]carbamyl phosphate (0.573 × 10^6 cpm/μmol), 66.67 mM sodium acetate, 1.59 × 10^{-2} μg of aspartate transcarbamylase, and varying concentrations of L-aspartate (pH 8.5). The reaction time was 15 min. The slope replot and intercept replot of this figure are shown in the offset.

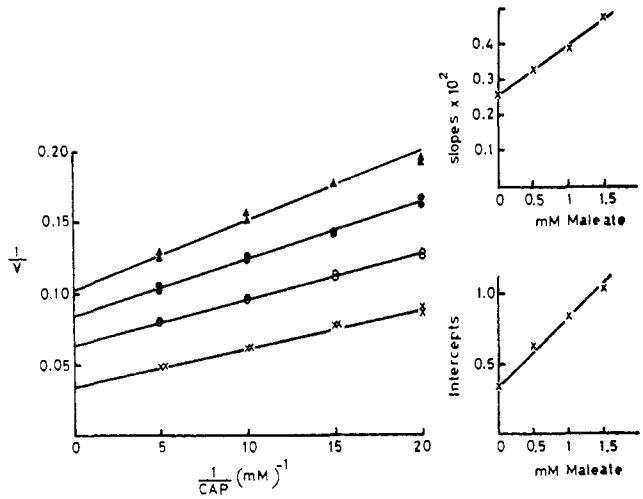
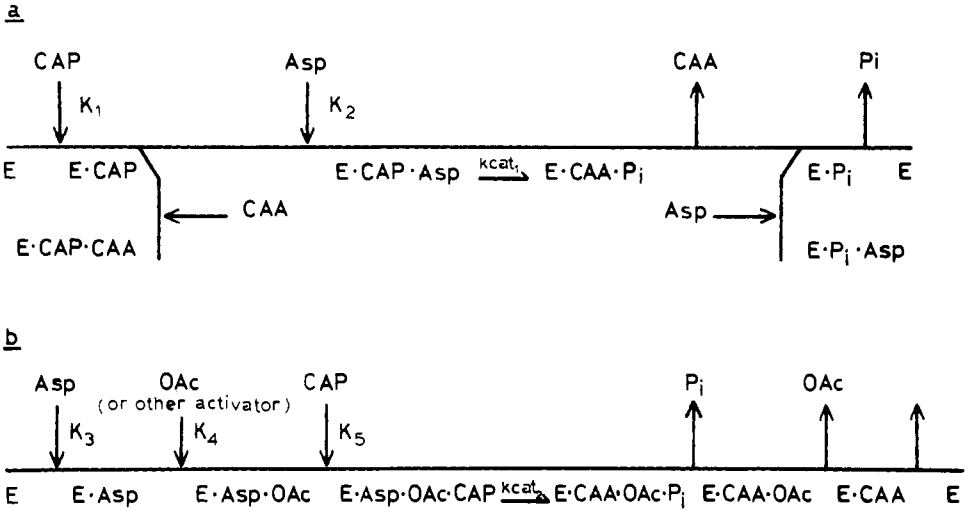


FIGURE 8: Dead-end inhibition by maleate against carbamyl phosphate as a variable substrate. Maleate concentrations used: (×) 0 mM; (○) 0.5 mM; (●) 1.0 mM; (▲) 1.5 mM. The reaction mixture (1 ml) contained 20 mM Tris-HCl, 0.6 mM L-aspartate, varying concentrations of [^{14}C]carbamyl phosphate (0.573 × 10^6 cpm/μmol), 66.67 mM sodium acetate, 5.55 × 10^{-2} μg of aspartate transcarbamylase (pH 8.5). The reaction time was 16 min.

decreased from 0.77 × 10^{-4} to 0.77 × 10^{-5} M with increasing levels of aspartate (from 20 to 350 mM). The apparent K_m values of carbamyl phosphate can also be reduced from 13 to 0.2 mM when 0.2 M sodium acetate is added to the medium (while aspartate was kept at 1 mM (Chang and Jones, 1974a)).

SCHEME I[a]



[a] K represents the dissociation constant between reactant and enzyme complex.

Thus, it would appear that part of the function of the activator molecule is to facilitate carbamyl phosphate binding. (b) The point of intersection of lines in Figure 4A lies above the horizontal axis, while that in Figure 4B lies below the horizontal axis. It has been pointed out by Cleland (1970) that the position of the intersecting point relative to the horizontal axis has no diagnostic value as far as distinguishing the overall kinetic mechanism, though it can be related to the degree of binding. When the binding of the first substrate can facilitate the binding of the other substrate, the points of intersection in both $1/V$ *vs.* $1/A$ and $1/V$ *vs.* $1/B$ plots are above the horizontal axis. When the binding of the first substrate can hinder the binding of the other substrate, the points of intersection are below the axis. When the binding of the first substrate does not affect binding of the other substrate, the points of intersection are on the axis. Numerous examples of this sort are seen in literature (for a review and discussion, see Cleland, 1967). The unusual patterns seen in Figures 3A and 3B suggest that, although the additions of carbamyl phosphate and aspartate are random in nature (a result also confirmed by product and dead-end inhibition analysis), binding of carbamyl phosphate first followed by aspartate binding may not yield the identical complex formed by binding aspartate first followed by carbamyl phosphate binding. The data actually suggest that binding of aspartate first facilitates binding of carbamyl phosphate, while binding of carbamyl phosphate first hinders binding of aspartate. The possibility that aspartate transcarbamylase binds activator as the first obligatory reactant, followed by random additions of carbamyl phosphate and aspartate, fits the product inhibition and dead-end inhibition data well; however, it would lead to a symmetrical model which can be ruled out by initial velocity data. (c) The horizontal coordinate of the cross-over point in Figure 3A is around $-10(\text{mM})^{-1}$. In bi-reactant mechanisms, this point corresponds to $-1/K_d$, where K_d is the dissociation constant for binding between free enzyme and the variable substrate (Frieden, 1956). Analogously, in ter-reactant mechanisms, it represents the apparent dissociation constant of binding between free enzyme and the variable substrate (in this case, aspartate transcarbamylase and carbamyl phosphate) which usually will be function of the third, unvaried reactant, in this case, the activator (acetate). The true K_d(carbamyl phosphate) can only be obtained at zero activator (acetate) concentration; however, the value of apparent K_d(carbamyl phosphate) (0.1 mM) obtained in Figure 3A does set a lower limit for true K_d(carbamyl phosphate). By the same reasoning, the value of the apparent K_d(aspartate) (1.7 mM) obtained in Figure 3B sets a lower limit for the true K_d(aspartate). The true V_{max} of the reaction cannot be obtained accurately due to the inhibitory effects of aspartate and acetate. Whether the activator molecule can bind to the enzyme as the first, second, or third reactant in an ordered or a random fashion cannot be answered by the present study.

In view of the above discussion, at least two pathways are necessary to describe the kinetic mechanism of this enzyme and to explain all of the present experimental data qualitatively. These are shown in Scheme I.

It is unknown whether the complex enzyme–aspartate–OAc–carbamyl phosphate (formed in pathway b) can be converted to the complex enzyme–carbamyl phosphate–aspartate (formed in pathway a) by dissociation of OAc (or *vice versa*); it is also unknown whether $k_{cat\,1}$ equals $k_{cat\,2}$. However, one can estimate that $K_1 \geq 0.1$ mM, $K_3 \geq 1.7$ mM, and $K_5 < K_1$, because binding of aspartate first markedly facilitates binding of carbamyl phosphate. In addition, K_2 may be greater than K_3, since binding of carbamyl phosphate first may hinder slightly the binding of aspartate. The rate-limiting step of the reaction may be the step of chemical transformation while all the enzyme complexes are in rapid equilibrium with one another; however, this needs to be reexamined carefully because, as pointed out by Cleland (1970), most of the random mechanisms resemble rapid equilibrium mechanisms in their initial velocity and product-inhibition patterns, even though the rate-limiting step is not solely the interconversion of two central complexes (a typical example is the study of formyltetrahydrofolate synthetase by Joyce and Himes (1966a,b), in which the chemical transformation step was found to be only partially rate limiting in a random ter–ter reaction).

In summary, the above scheme states that either carbamyl phosphate (mechanism a) or aspartate (mechanism b) can be the first substrate added, but the major pathway observed will depend on the relative concentrations of carbamyl phosphate and aspartate. When carbamyl phosphate is high and aspartate is low, then mechanism a is preferred unless an activating anion (acetate, lactate, etc.) is present in large enough amounts to improve the binding of aspartate significantly, in which case both a and b can occur. When aspartate is high, the major pathway would be b. Mechanism a does not

necessarily exclude binding of an activating anion to the enzyme; however, such anion binding occurring after carbamyl phosphate is bound would have little significant kinetic effect on the reaction if the major effect of the anion is to aid carbamyl phosphate binding. At least two different dead-end enzyme complexes can be observed. They are enzyme–carbamyl phosphate–carbamyl aspartate and enzyme–P_i–aspartate.

Scheme I is only tentative in nature; it may be viewed as a "minimal" kinetic mechanism for this enzyme. It is probable that the structural integrity around the active sites of this enzyme is rather flexible; therefore, it may catalyze the reaction *via* some other minor pathways which are not observed in the present study. More sophisticated kinetic studies are needed to examine or improve this scheme; the use of the technique of isotope exchange at equilibrium (Boyer, 1959) would be especially valuable. Binding studies between enzyme and substrates would also yield a great deal of useful information (see Chang and Jones, 1974b).

The only well-studied aspartate transcarbamylase in the literature is the one isolated and purified from *E. coli*. The catalytic subunit of *E. coli*, studied by Collins and Stark (1969), at pH 7.0 and 28°, probably proceeds with an ordered mechanism with carbamyl phosphate binding first. At pH 7.8 and 28°, the data from Stark's laboratory (Porter *et al.*, 1969) did not as clearly support the ordered mechanism, and Collins and Stark (1971) point out that the carbamyl phosphate concentrations used in most of the experiments of Porter *et al.* (1969) were at a saturating level which would prevent one from differentiating an ordered mechanism from a random mechanism. Silverstein (1969), in an abstract, claimed that the native *E. coli* aspartate transcarbamylase has a rapid-equilibrium random mechanism at 0° and pH 7.8.[3] The existence of a carbamyl–enzyme intermediate necessary in a ping-pong mechanism was ruled out for the catalytic subunit of *E. coli* aspartate transcarbamylase (Schaffer and Stark, 1972). Although it is still possible that a carbamyl–enzyme intermediate is formed during these reactions, the present report and others mentioned above do not support it and suggest that, if such an enzyme-bound intermediate is involved, the products of the aspartate transcarbamylase reaction (L-carbamyl aspartate and phosphate) cannot be released from the enzyme before both substrates are added to the enzyme (for a discusion of reactions with a covalent enzyme–substrate intermediate not exhibiting "ping-pong" kinetics, see Moffet and Bridger, 1970).

Supplementary Material Available

Supplementary material describing this experiment will appear following these pages in the microfilm edition of this volume of the journal. Photocopies of the supplementary material from this paper only or microfiche (105 × 148 mm, 24× reduction, negatives) containing all of the supplementary material for the papers in this issue may be obtained from the Journals Department, American Chemical Society, 1155 16th St., N.W., Washington, D. C. 20036. Remit check or money order for $3.00 for photocopy or $2.00 for microfiche, referring to code number BIO-74-638.

[3] Ong and Jackson (1972) studied the aspartate transcarbamylase isolated from *Phaseolus aureus*; the set of parallel reciprocal plots obtained in the initial velocity studies as well as the ambiguity in one of the product inhibition patterns (the carbamyl phosphate *vs.* carbamyl aspartate pair) make this study of the kinetic mechanism for this aspartate transcarbamylase inconclusive.

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