Aspartate Transcarbamylase from *Streptococcus faecalis*. Reverse Reaction and Binding Studies†

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ABSTRACT: The reverse reaction of aspartate transcarbamylase has been demonstrated using a coupled enzymatic assay in which one product, carbamyl phosphate, is removed and transformed as it is formed by pure ornithine transcarbamylase and L-ornithine into L-citrulline. Using L-[^{14}C]carbamyl aspartate and phosphate as substrates, a very sensitive and reproducible assay was developed. In steady-state kinetic analysis of the reverse reaction the double reciprocal plot for phosphate is linear, but the plot for L-carbamyl aspartate is concave upward; the data on anion activating effects are consistent with the existence of an allosteric activator site in the enzyme molecule. With the results obtained previously from studies of the forward reaction (Chang, T. Y., and Jones, M. E. (1974), *Biochemistry* 13, 629, 638), the combined data suggest that the activator molecule may not be the first reactant bound to aspartate transcarbamylase during catalysis. Aspartate is shown to give a noncompetitive inhibition pattern against phosphate as the variable substrate, a result consistent with the existence of a dead-end enzyme–P_i–aspartate complex. The equilibrium of the reaction was also studied and $K_{eq} = 1.1 \times 10^5$ (pH 8.56, 25°). Binding between aspartate transcarbamylase and radioactive substrates or substrate analogs was examined by using an ultrafiltration cell developed by H. Paulus (1969, *Anal. Biochem. 32*, 91). In the absence of succinate, the dissociation constant for carbamyl phosphate is 1.7×10^{-4} M, 1 mol of aspartate transcarbamylase binds 3.7 mol of carbamyl phosphate. The binding of carbamyl phosphate is facilitated in the presence of a saturating level of succinate (0.2 M); under this condition, the dissociation constant for carbamyl phosphate becomes 3.8×10^{-6} M. Dissociation constants for L-aspartate and succinate are within the millimimolar range; the dissociation constant for succinate is not decreased in the presence of carbamyl phosphate (2 mM). The effect of acetate on the binding of substrates or the aspartate analog, succinate, to aspartate transcarbamylase was also investigated. All the data obtained from studies are qualitatively in agreement with the tentative kinetic mechanism of this enzyme proposed in the previous paper (Chang, T. Y., and Jones, M. E. (1974), *Biochemistry 13*, 638).

In the previous two papers (Chang and Jones, 1974a,b), we reported on the purification and characterization of aspartate transcarbamylase from *Streptococcus faecalis*. The native enzyme has a mol wt of 1.30×10^5, possessing four subunits of apparently identical size. An allosteric activator site present in the enzyme molecule can be demonstrated kinetically and is responsible for the unusual carbamyl phosphate and aspartate saturation curves at low buffer concentrations. Steady-state kinetic analysis suggests that the enzyme catalyzes the reaction by a random sequential mechanism, with either carbamyl phosphate or aspartate being able to serve as the first reactant. However, if carbamyl phosphate binds to aspartate transcarbamylase first, the subsequent binding of aspartate is hindered, while, if the binding of aspartate is first, it leads to binding of an activator molecule, which in turn strongly facilitates the binding of carbamyl phosphate. Substrate inhibition by aspartate is thought to be due to the formation of an abortive enzyme–phosphate–aspartate complex. It is uncertain whether the activator molecule is necessary for substrate(s) to bind to aspartate transcarbamylase, or whether this activator can bind to aspartate transcarbamylase as the first, second, or third reactant in an ordered or random fashion (Chang and Jones, 1974b).

An analysis of the reaction from the reverse direction, *i.e.*, the formation of carbamyl phosphate and aspartate from carbamyl aspartate and phosphate, should provide additional data to test the proposed mechanism. This reverse reaction is thermodynamically very unfavorable. In 1956, Reichard and Hanshoff (1956), using aspartate transcarbamylase from crude extracts of *Escherichia coli* and chromatographic separation of [^{14}C]carbamyl aspartate and [^{14}C]aspartate, obtained some evidence to indicate the reaction might be reversed. Silverstein (1969) studied the pure aspartate transcarbamylase from *E. coli* and claimed to have demonstrated reversibility by coupling the reaction with the aspartate aminotransferase and maleate dehydrogenase reactions in order to remove one product, aspartic acid.

In this work we have developed a very sensitive and reproducible coupled enzymatic assay, but have selected pure ornithine transcarbamylase to remove the product, carbamyl phosphate. In addition to initial velocity studies with L-carbamyl aspartate and phosphate as substrates, binding studies between aspartate transcarbamylase and substrates or substrate analogs are reported and discussed in relation to the kinetic results for both the forward (Chang and Jones, 1974b) and reverse reactions.

Materials and Methods

Enzymes. The aspartate transcarbamylase used in this work was the purest fraction (93% pure) obtained in the first paper of this series (Chang and Jones, 1974a); the enzyme solutions

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were stored as described in the second paper (Chang and Jones, 1974b).

The ornithine transcarbamylase was 80–90% pure, judging from analytical polyacrylamide gel electrophoresis. It was prepared by a modification of the procedure of Nakamura and Jones (1970). The specific activity of this preparation is 2700 μmol of citrulline/min per mg of protein (assay condition: 0.1 M Tris-HCl, 50 mM KCl, 5 mM carbamyl phosphate, 5 mM ornithine (pH 8.5), at 37°). The enzyme is devoid of aspartate transcarbamylase activity and is very stable when stored at −20 or 4°, at a protein concentration of 1 mg/ml, in 20 mM Tris-HCl buffer (pH 8.3). Ornithine transcarbamylase (42 units) can quantitatively transform all of the carbamyl phosphate (0.1 μmol) present in 0.5 ml into citrulline in 6 min: 10 mM L-carbamyl aspartate, 0.1 M sodium phosphate, 7 mM ornithine-HCl, 0.2 mM carbamyl phosphate (pH 8.5), 25°. Citrulline formed was determined by colorimetric citrulline assay (Guthöhrlein and Knappe, 1968).

Chemicals and Reagents. Dowex 1-X8 resins, 200–400 mesh, were purchased from Bio-Rad laboratories (control no. 6385-28-29) and washed with 1 M NaOH, H_2O, 1 M HCOOH, and H_2O successively before use. The final pH of the H_2O suspension was higher than 5.

L-Malic acid and malonic acid were recrystallized from an H_2O–acetone mixture once before use. The DEAE-cellulose (specification: MN 300) used in thin layer chromatography was purchased from Brinkmann Instruments, Westbury, N. Y.

Radioactive substrates or substrate analogs used as ligands in the binding studies were all purchased from New England Nuclear. They were: [^{14}C]carbamyl phosphate (1.146×10^7 cpm/μmol, purified as described in Chang and Jones, 1974a,-b); [2,3-^{14}C]succinic acid (Catalog no. NEC-201, 0.32 mg, containing 0.05 mCi; it was diluted with 0.5 ml of nonradioactive sodium succinate in 5 mM Tris-HCl (pH 8.5) to give a final specific activity of 3.78×10^6 cpm/μmol); L-[U-^{14}C]-aspartic acid (Catalog no. NEC-201, 0.16 mg, containing 0.25 mCi; it was diluted with 1.2 ml of nonradioactive 0.5 M L-sodium aspartate (pH 8.5) to give a final specific activity of 2.87×10^6 cpm/μmol). [^{14}C]Urea (Catalog no. NEC-108, 3.5 mCi, lot No. 453-13) was used in the synthesis of [^{14}C]KCNO; D,L-[^{14}C]carbamyl aspartate was Catalog no. NEC-159, 2.75 mCi/mmol.

L-Malic acid and malonic acid were recrystallized from an H_2O–acetone mixture before use. All other chemicals used were of reagent grade, purchased commercially. All stock solutions of salts were neutralized to pH 8.5 at room temperature before use.

Synthesis of Potassium [^{14}C]Cyanate. This was synthesized according to the procedure of Scattergood (1946). [^{14}C]Urea (420 mg) and 507 mg of potassium carbonate were mixed in a 1.5 × 15 cm Pyrex tube. The mixture was heated with a Meeker burner; partial melting occurred, with evolution of gas (ammonia). The mixture then became nearly solid and finally remelted with increased heat. As soon as the melt was quiescent and the surface free of bubbles, the flame was taken away. The [^{14}C]KCNO was immediately put in a desiccator and kept under vacuum for several hours before using it for the synthesis of L-[^{14}C]carbamyl aspartate.

Synthesis of L-[^{14}C]Carbamyl Aspartate. [^{14}C]KCNO was diluted with 18 mmol of [^{12}C]KCNO (solid) to serve as the starting material for the synthesis of L-[^{14}C]carbamyl aspartate. The rest of the materials and procedures used for synthesis and purification were the same as those used for the synthesis of L-[^{12}C]carbamyl aspartate described previously (Chang and Jones, 1974b).

Thin-Layer Chromatography of L-[^{14}C]Carbamyl Aspartate on DEAE-Cellulose. $CaSO_4 \cdot 2H_2O$ (1.58 g), 225 ml of H_2O, and 25 g of DEAE-cellulose were thoroughly mixed with a Waring Blendor to serve as ingredients to prepare thin layers on glass plates. The solvent used was methanol–glacial acetic acid–water in a ratio of 10:3:27. When a spot of L-[^{14}C]carbamyl aspartate solution was applied on the thin-layer plate (20 × 5 × 0.4 cm) and chromatographed overnight, it gave, on radioactive scanning, a single symmetrical peak, with an R_F value of 0.806, which was identical with the R_F value given by a sample of DL-[^{14}C]carbamyl aspartate purchased commercially from New England Nuclear.

Radioactive Specific Activity of L-[^{14}C]Carbamyl Aspartate. L-[^{14}C]Carbamyl aspartate (1 μmol) gave 1.50×10^5 cpm in a naphthalene–dioxane liquid scintillation system, the efficiency of which was 80%.

Assay of the Reverse Reaction. Small glass tubes (1 × 8 cm) were used for this assay. The reaction volume usually was 0.2 ml. The assay mixture contained 10 or 20 mM Tris-HCl buffer (pH 8.5), varying amounts of sodium phosphate (2–100 mM), and L-[^{14}C]carbamyl aspartate (5–400 mM) both neutralized to pH 8.5, 7 mM ornithine-HCl (pH 8.5), 15–40 units of ornithine transcarbamylase (the amount used depended on the amounts of sodium phosphate or L-carbamyl aspartate used), and 0.74–2.22 μg of aspartate transcarbamylase. The reaction was started at 25° with the addition of aspartate transcarbamylase. During the reaction, usually 2 hr, each test tube was capped tightly with a small cork to prevent a pH change; the incubation mixture always remained at pH 8.5. Reactions were terminated by partially immersing the test tubes in a boiling water bath for 1 min. The reaction mixture was then diluted with 0.8 ml of a solution containing 100 mM sodium phosphate and 10 mM citrulline at pH 7.0. The diluted mixture was transferred to a 0.8 × 2 cm Dowex 1-X8 formate column. Each test tube was rinsed twice with 0.8 ml of H_2O and the rinsing solution was applied on the column and used as an eluent in the elution of citrulline. The 2.5-ml eluent was collected in a scintillation vial into which 10 ml of naphthalene–dioxane scintillation fluid was added. The counting efficiency was 80%. Controls without aspartate transcarbamylase were run in each experiment; the blank values were constant and very low, about 0.03% of the total counts given by the amount of L-[^{14}C]carbamyl aspartate used as substrate.

Binding Experiments. The ultrafiltration apparatus and technique for measuring ligand binding were developed by Paulus (1969). The apparatus is manufactured by MRA Co. (Boston, Mass.). Membranes (PM-10) manufactured by Amicon Co. were used as filters. Each value presented is the average of either duplicate or triplicate samples which did not deviate from each other by more than 15%. The apparatus was kept in a 25° chamber; the variation of temperature was less than 1°. The samples, usually in 0.2 or 0.3 ml, were preincubated at 25° for 1 min and then promptly transferred into a channel of the Paulus binding apparatus. Sample ports were then quickly sealed. Pressure was applied to the apparatus (about 40 psi) from a pressurized nitrogen source, until the solutions were completely forced out through the PM-10 membranes in all channels. Ethylene glycol was injected through the rinse channels to wash the bottom side of the membranes. The pressure was released and membranes were transferred to vials containing 2 ml of water, to be counted in a liquid scintillation counter. Before counting, contents in the vials were shaken vigorously. From the preincubation of samples (as many as eight) until the solution in each channel was forced out by pressurized nitrogen, the time span was

about 10–15 min. If only one or two samples were used in the experiment, the time span could be reduced to 3–5 min.

The blank values given by [^{14}C]carbamyl phosphate and [^{14}C]succinate used as ligands were consistent, corresponding to about 2.5–3.0 μl of the radioactive solutions applied to the ultrafiltration apparatus, as claimed by Paulus (1969). A higher background equal to about 5 μl of the applied solution was obtained with L-[^{14}C]aspartate.

[^{14}C]Carbamyl Phosphate Assay. This assay was described in detail in the preceding papers (Chang and Jones, 1974a,b).

[^{14}C]Citrulline Assay. The assay was essentially the same as the [^{14}C]carbamyl phosphate assay described in detail in the previous paper (Chang and Jones, 1974a,b), except that the enzyme used was ornithine transcarbamylase, and the substrate was changed from L-aspartate to L-ornithine.

Results

Demonstration of the Reverse Reaction. A coupled enzymatic assay using pure ornithine transcarbamylase was developed in order to demonstrate and study the reverse aspartate transcarbamylase reaction. The entire system can be summarized with the following scheme.

$$P_i + \text{L-[}^{14}\text{C]carbamyl aspartate} \xrightleftharpoons{\text{aspartate transcarbamylase}} \text{L-aspartate} + \text{[}^{14}\text{C]carbamyl phosphate}$$

$$\text{[}^{14}\text{C]carbamyl phosphate} \xrightleftharpoons{\text{L-ornithine, ornithine transcarbamylase}} P_i + \text{L-[}^{14}\text{C]citrulline}$$

Using L-[*carbamyl*-^{14}C]carbamyl aspartate and P_i as the substrate, the products of the aspartate transcarbamylase reaction are L-aspartate and [^{14}C]carbamyl phosphate. [^{14}C]-Carbamyl phosphate does not accumulate in the test tube because it is transformed immediately into L-[*carbamyl*-^{14}C]-citrulline and P_i by the ornithine transcarbamylase present in the incubation mixture in the presence of a nearly saturating level of L-ornithine (7 mM). L-[*carbamyl*-^{14}C]Citrulline can be separated easily from the starting material, L-[*carbamyl*-^{14}C]-carbamyl aspartate on Dowex 1 columns because of the charge difference between these two compounds at neutral pH (see the Materials and Methods section). Table I gives the results of a typical experiment.

Arsenolysis. Final proof that the ^{14}C-labeled material isolated from the Dowex column is L-[*carbamyl*-^{14}C]citrulline relies on enzymatic arsenolysis catalyzed by ornithine transcarbamylase, which transforms L-citrulline into ammonia, carbon dioxide, and L-ornithine (Krebs *et al.*, 1955). The result of such an experiment using a mixture of L-[^{14}C]citrulline isolated from the Dowex column and carrier nonradioactive L-citrulline as substrate demonstrated that the curve for the decrease in the amount of citrulline, as judged by the color assay, coincided precisely with the curve for the decrease in radioactivity at different times.[1]

Initial Velocity Studies of the Reverse Reaction. Using the coupled enzymatic assay described above, one can measure the initial rates of the reverse reaction catalyzed by aspartate transcarbamylase, as long as enough ornithine transcarbamylase is added to each test tube that the transformation of [^{14}C]carbamyl phosphate into [^{14}C]citrulline is never rate limiting. When L-[^{14}C]carbamyl aspartate is the limiting substrate, the initial rate is linear with time until about 3.5% of the substrate is consumed (Chang, 1973).

TABLE I: Demonstration of Reverse Reaction of Aspartate Transcarbamylase.[a]

Exptl Condns	Total μmol of Citrulline Formed[b]	Total Counts Obtained
Complete	0.06	9100
No aspartate transcarbamylase	0.00	110
No ornithine transcarbamylase	0.00	120

[a] The complete experiment was done with 4.5 mM L-[^{14}C]-carbamyl aspartate (1.5×10^5 cpm/μmol), 100 mM sodium phosphate buffer (pH 7.5), 7 mM L-ornithine-HCl (pH 7.5), 42 units of ornithine transcarbamylase, and 2.45 μg of aspartate transcarbamylase. The final volume was 0.5 ml. The reaction mixtures were deproteinized and then analyzed with the Dowex 1 assay given under Materials and Methods. Only 0.6 ml of the 2-ml water eluents from each column was used for citrulline colorimetric assay and ^{14}C counting. The remaining material was used for the arsenolysis experiment of Figure 1 of the microfiche version.[1] [b] By colorimetric citrulline assay.

When P_i is the limiting substrate, the rate is still within linear range, when the amount of L-[^{14}C]citrulline (and of L-aspartate) produced is about 11% of the phosphate added as substrate (Chang, 1973). This experiment also indicated that aspartate transcarbamylase and ornithine transcarbamylase are stable at 25° for more than 2 hr. All of the steady-state kinetic experiments reported in this paper were carried out under conditions where initial rates pertained.[2]

Figure 1A shows the double reciprocal plot of the L-carbamyl aspartate saturation curve at 5.15 mM sodium phosphate. It is concave upward, which suggests that more than 1 equiv of L-carbamyl aspartate is bound to the enzyme during the catalytic reaction. The double reciprocal plot of the phosphate saturation curve, shown in Figure 1B, is strictly linear. Neither substrate activation nor inhibition is seen in the range from 2.5 to 100 mM. The apparent K_m of phosphate at 9 mM L-carbamyl aspartate is 13.5 mM. This figure suggests that P_i cannot bind to the enzyme activator site during the reverse reaction. The apparent V_{max} of the reverse reaction, calculated from this figure, is 0.62 μmol of citrulline/min per mg of protein.

Activation Effects by Anions. The activation effects by several organic and inorganic salts were studied.[1] Only lactate and acetate showed appreciable activation. Moreover, the activation by these anions is not nearly as dramatic being only about twofold at 50 mM concentrations, whereas their activation of the forward reaction at the same concentration was nearly 100-fold (Chang and Jones, 1974a). Sulfate and nitrate have no effect. None of these anions are inhibitory to the *S. faecalis* ornithine transcarbamylase (Nakamura and Jones, 1970). This and the previous data (Figures 1A and 1B) suggest that the activator site of the aspartate transcarbamylase molecule is more specific and restricted in the reverse reaction.

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

[2] In a given experiment, selected points were repeated with ornithine transcarbamylase added in two- or threefold quantities to recheck that the transformation of [^{14}C]carbamyl phosphate into [^{14}C]citrulline was not rate limiting in the system.

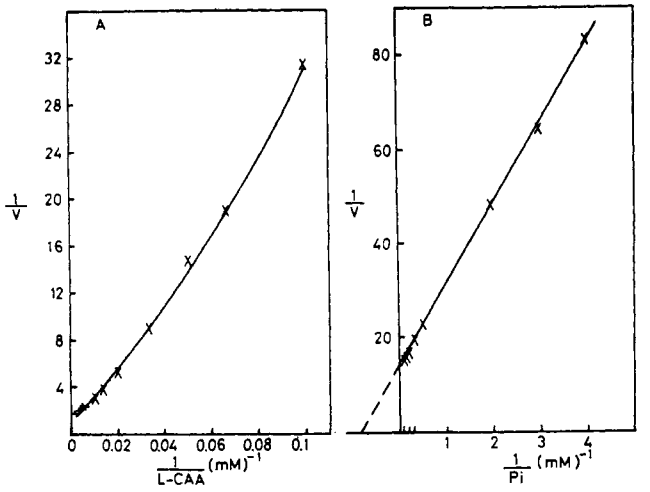

FIGURE 1: Substrate curves for the reverse reaction. Velocity (V) expressed as micromoles of citrulline/minute per milligram of protein. (A) Double reciprocal plot with L-carbamyl aspartate (L-CAA) as the variable substrate. The reaction mixture contained 5 mM sodium phosphate, 20 mM Tris-HCl (pH 8.5), 7 mM ornithine-HCl, 45 units of ornithine transcarbamylase, and varying levels of L-[^{14}C]carbamyl aspartate. The final volume was 0.2 ml. The reaction was started by the addition of 3.75 μg of aspartate transcarbamylase. The incubation time was 2 hr at 25°. The two points with the two highest L-carbamyl aspartate concentrations (300 and 400 mM) were repeated under identical conditions except 135 units of ornithine transcarbamylase were used; the data obtained were identical with the original data. (B) Double reciprocal plot with phosphate as the variable substrate. The reaction mixture contained 9 mM L-[^{14}C]carbamyl aspartate, 20 mM Tris-HCl (pH 8.5), 7 mM ornithine-HCl, 15 units of ornithine transcarbamylase, and varying levels of sodium phosphate. The final volume was 0.2 ml. The reaction was started by the addition of 6.25 μg of aspartate transcarbamylase. The incubation time was 2 hr at 25°.

Inhibition by Analogs of L-Aspartate. Analogs of L-aspartate were studied as inhibitors rather extensively in the forward aspartate transcarbamylase reaction by many workers (Jones, 1962; Gerhart and Pardee, 1964; Davies *et al.*, 1970). For the *S. faecalis* enzyme, maleate and succinate appear to be the best competitive inhibitors, binding more tightly to the enzyme than L-aspartate itself (Jones, 1962; Chang and Jones, 1974b). Figure 2 shows the inhibition of the reverse reaction by L-aspartate and analogs of aspartate. It can be seen that L-aspartate, the best inhibitor, is much more potent than any other compound. This result is consistent with the existence of a dead-end enzyme–P_i–aspartate complex proposed in the previous paper (Chang and Jones, 1974b). Maleate, succinate, and others probably inhibit the reaction principally by binding to the aspartate substrate site of the free enzyme molecule. A slight activation of the reaction is produced by β-alanine. In the forward reaction, P_i was shown to be competitive against L-aspartate as a variable substrate (Chang and Jones, 1974b, Figure 4; in this figure, aspartate levels were kept below inhibitory range). Figure 3 shows that when P_i is the variable substrate in the reverse reaction, aspartate gives apparent linear noncompetitive inhibition, a finding that adds further support to the existence of a dead-end enzyme–P_i–aspartate complex. The K_i(intercept) and K_i(slope) of L-aspartate found from intercept replot and slope replot of Figure 3 are 1 and 3 mM, respectively.

Equilibrium of the Aspartate Transcarbamylase Reaction. Chemical equilibrium of the aspartate transcarbamylase reaction can be reached from the forward direction within 10 min (Chang, 1973) by adding a large amount (>18 μg) of aspartate transcarbamylase under the conditions used for Figure 4. Figure 4 shows that, using 22.2 μg of aspartate transcarbamylase, equilibrium was essentially established within 10 min. By measuring the carbamyl phosphate concentration and L-carbamyl aspartate concentration at equilibrium, the apparent equilibrium constant of the reaction at 25°, pH 8.56, is 1.1×10^5 (K_{eq} = [L-carbamyl aspartate][P_i]/[carbamyl phosphate]·[aspartate]. Since the half-life of carbamyl phosphate for hydrolysis at 25°, pH 8.56, is 260 min (T. Y. Chang and M. E. Jones, unpublished data), the very small percentage of carbamyl phosphate which is hydrolyzed was neglected in the calculation.

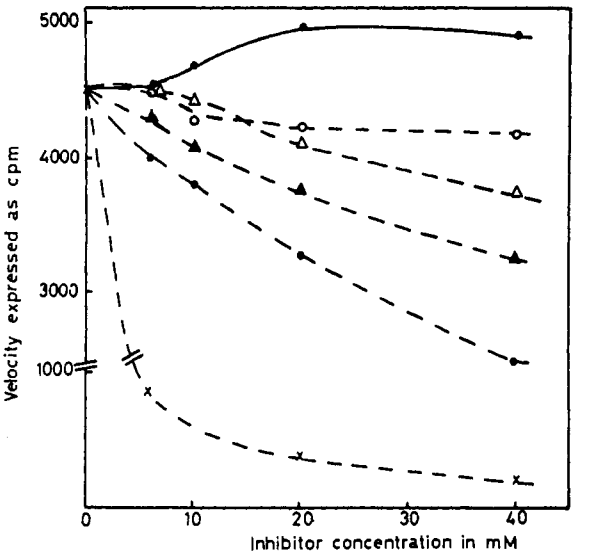

FIGURE 2: Inhibition by L-aspartate and analogs of L-aspartate. The reaction mixture contained 20 mM Tris-HCl (pH 8.5), 15 units of ornithine transcarbamylase, 7 mM ornithine-HCl, 100 mM L-[^{14}C]-carbamyl aspartate, 5 mM sodium phosphate, and 1.48 μg of aspartate transcarbamylase. The total volume was 0.2 ml. Incubation time was 2 hr at 25°: (●) β-alanine; (○) malonate; (△) L-malate; (▲) succinate; (●) maleate; (×) L-aspartate. The point with no inhibitor (0 mM) added and the points with 40 mM of each of the six inhibitors added were repeated under identical conditions except that 30 units of ornithine transcarbamylase were used; the data obtained differed no more than 5% from the values shown.

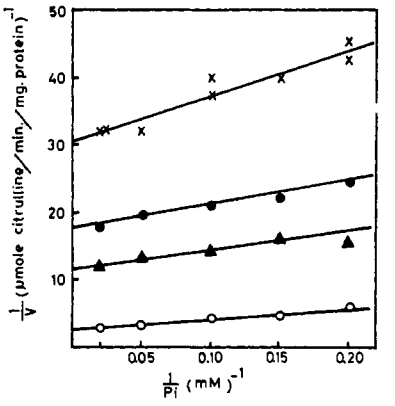

FIGURE 3: Inhibition by L-aspartate against phosphate as a variable substrate. The reaction mixture contained 20 mM Tris-HCl (pH 8.5), 15 units of ornithine transcarbamylase, 7 mM ornithine-HCl, 50 mM L-[^{14}C]carbamyl aspartate, varying levels of sodium phosphate, and 1.48 μg of aspartate transcarbamylase. The final volume was 0.2 ml. Incubation time at 25° was 2 hr. Aspartate concentrations: (○) 0 mM; (▲) 3 mM; (●) 5 mM; (×) 10 mM.

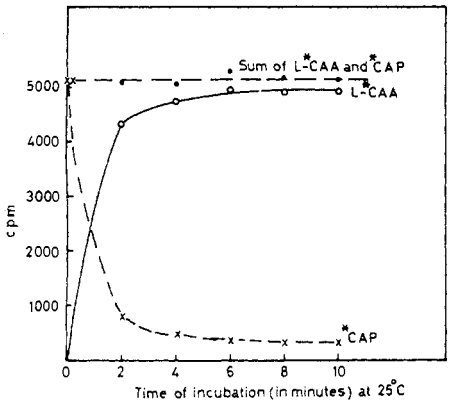

FIGURE 4: Demonstration of equilibrium. The equilibrium was reached by incubating reaction mixtures for increasing time intervals. The reaction mixture contained 100 mM Tris-acetate buffer (pH 8.54), 0.1 mM L-aspartate (pH 8.5), 102 mM sodium phosphate (pH 8.56), 0.090 mM [^{14}C]carbamyl phosphate (CAP) (1.146×10^7 cpm/μmol), and 22.2 μg of aspartate transcarbamylase. The final volume was 50 μl. Two identical reaction mixtures were prepared this way. The preincubation time was 1 min. Reactions were started by pipetting 5 μl of 1 mM [^{14}C]carbamyl phosphate into the test tubes. At appropriate time intervals, 5-μl portions of the aliquots were withdrawn from one of the two identical reaction mixtures and transferred into test tubes containing 0.1 ml of 2 M $HClO_4$. This set of tubes (five tubes) was then analyzed by [^{14}C]-carbamyl phosphate assay; the radioactive counts obtained this way represented the amount of L-[^{14}C]carbamyl aspartate (L-CAA) formed at different time intervals. On the other hand, at appropriate time intervals, 5-μl portions of the aliquots were withdrawn from the other reaction mixture and transferred into test tubes containing 0.495 ml of an ornithine transcarbamylase reaction mixture. This ornithine transcarbamylase assay mixture contained 7 mM ornithine-HCl, 10 mM Tris-HCl (pH 8.5), and 42 units of orinithine transcarbamylase. The final volume was 0.5 ml. This set of tubes (five tubes) was incubated for 10 min to transform the [^{14}C]carbamyl phosphate into [^{14}C]citrulline quantitatively. The reaction was terminated by immersing the test tubes in a 100° water bath for 1 min. The citrulline was measured by the Dowex 1 assay. H_2O eluent (2 ml) from each column elution is collected and counted. The radioactive counts obtained this way represented the amount of [^{14}C]carbamyl phosphate still present as such at each time interval. The backgrounds counts obtained after [^{14}C]citrulline assay and Dowex 1 assay were nearly identical, differing from each other by no more than 5%. One micromole of [^{14}C]citrulline corresponds to 1.146×10^7 cpm, measured by colorimetric assay of citrulline and scintillation counting after [^{14}C]citrulline assay.

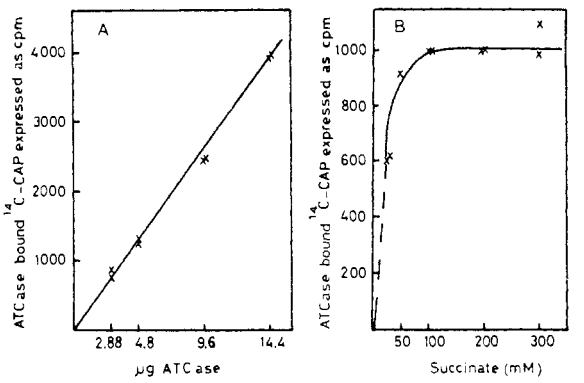

FIGURE 5: Carbamyl phosphate binding experiments. (A) Linear proportionality between aspartate transcarbamylase bound [^{14}C]-carbamyl phosphate and an amount of aspartate transcarbamylase The reaction mixture contained 5 mM Tris-HCl (pH 8.5), 0.2 M succinate, 2×10^{-5} M [^{14}C]carbamyl phosphate (1.146×10^7 cpm/μmol), and aspartate transcarbamylase (93% pure) as indicated. The final volume was 0.2 ml. (B) Extent of [^{14}C]carbamyl phosphate binding to aspartate transcarbamylase as a function of succinate. The reaction mixture contained 5 mM Tris-HCl (pH 8.5), 1×10^{-5} M [^{14}C]carbamyl phosphate (1.146×10^7 cpm/μmol), 4.72 μg of aspartate transcarbamylase (93% pure), and varying levels of succinate. The final volume was 0.3 ml.

Binding Studies. The ultrafiltration method for measuring ligand binding developed by Paulus (1969) is sensitive and rapid. Binding studies can be carried out with microgram quantities of protein when ligand concentrations exceed the concentration of protein by several orders of magnitude. Since the enzyme-bound substrate is negligible with respect to the free substrate, the substrate concentration is essentially constant throughout the filtration process. Figure 5A shows that the amount of [^{14}C]carbamyl phosphate bound to aspartate transcarbamylase was linearly proportional to the amount of aspartate transcarbamylase when the fraction of total [^{14}C]-carbamyl phosphate bound to asparate transcarbamylase varied from 1.7 to 8.6% at 2×10^{-5} M [^{14}C]carbamyl phosphate. All the studies reported here are well within this range. Figure 6A shows the binding curve between [^{14}C]carbamyl phosphate and aspartate transcarbamylase in double reciprocal form. The dissociation constant for carbamyl phosphate obtained by extrapolating the curve to the abscissa is 1.7×10^{-4} M. The maximum amount of substrate bound per aspartate transcarbamylase was obtained by extrapolating the curve to the ordinate. Assuming the asparate transcarbamylase was 93% pure, 1 mol of aspartate transcarbamylase bound 3.7 mol of carbamyl phosphate.

The binding of carbamyl phosphate is much stronger in the presence of succinate. Figure 5B shows the amount of carbamyl phosphate bound to enzyme as a function of increasing concentrations of succinate. A similar result is obtained if maleate is used instead of succinate. The double reciprocal plot of carbamyl phosphate binding in the presence of 200 mM succinate (Figure 6B) gives a dissociation constant for carbamyl phosphate of 3.8×10^{-6} M. Assuming the aspartate transcarbamylase was 93% pure, 1 mol of aspartate transcarbamylase bound 3.8 mol of carbamyl phosphate in the presence of saturating levels of succinate. As can be seen from Table II, sodium acetate has a minimal effect on the binding of carbamyl phosphate when succinate is absent. However, if 2 mM succinate is added, sodium acetate does stimulate the

TABLE II: Effects of Sodium Acetate on Binding of [^{14}C]-Carbamyl Phosphate to Aspartate Transcarbamylase.[a]

Aspartate Transcarbamylase (μg)	Sodium Acetate Added (mM)	Succinate Added (mM)	Net cpm Bound
21	0	0	390 ± 40
21	120	0	300 ± 70
21	120	2	1200 ± 100
21	0	2	700 ± 100
5.25	0	48	743 ± 50
5.25	120	48	750 ± 30

[a] The reaction mixture contained 5 mM Tris-HCl (pH 8.5), 21 μg or 5.25 μg of aspartate transcarbamylase (93% pure), with or without sodium acetate and/or succinate, 1×10^{-5} M [^{14}C]carbamyl phosphate (1.146×10^7 cpm/μmol). Controls were run without aspartate transcarbamylase. The final volume was 0.2 ml. The blank value is 300 ± 20.

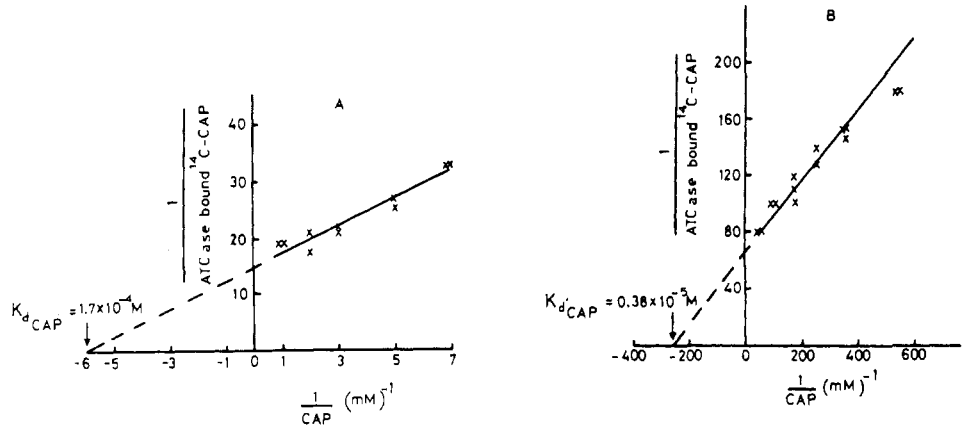

FIGURE 6: Binding of [^{14}C]carbamyl phosphate by aspartate transcarbamylase. The datum was expressed in (1/enzyme-bound carbamyl phosphate) [(1/cpm) $\times$ 10^5] *vs.* 1/[carbamyl phosphate] mM^{-1}. (A) Binding in the absence of succinate. The reaction mixture contained 5 mM Tris-HCl (pH 8.5), 107 μg of aspartate transcarbamylase, varying levels of [^{14}C]carbamyl phosphate (2.29 $\times$ 10^6 cpm/μmol). The final volume was 0.2 ml. The maximum amount of [^{14}C]carbamyl phosphate bound to aspartate transcarbamylase, obtained by extrapolating the linear line to the ordinate, is 6666 cpm/(2.29 $\times$ 10^6 cpm/μmol) = 2.909 $\times$ 10^{-3} μmol. Assuming the mol wt of aspartate transcarbamylase is 1.30 $\times$ 10^5, and the enzyme is 93% pure, 107 μg of aspartate transcarbamylase should be 0.84 $\times$ 10^{-3} μmol. The maximum number of moles of [^{14}C]carbamyl phosphate bound per mole of aspartate transcarbamylase is thus (2.909 $\times$ 10^{-3})/(0.84 $\times$ 10^{-3}) = 3.7. (B) Binding in the presence of 0.2 M succinate. The reaction mixture contained 5 mM Tris-HCl (pH 8.5), 0.2 M succinate, 4.8 μg of aspartate transcarbamylase (93% pure), and varying levels of [^{14}C]carbamyl phosphate (1.146 $\times$ 10^7 cpm/μmol). The final volume was 0.2 ml. The maximum amount of [^{14}C]-carbamyl phosphate bound to aspartate transcarbamylase obtained by extrapolating the linear line to the ordinate is 1500 cpm/(1.146 $\times$ 10^7 cpm/μmol) = 1.31 $\times$ 10^{-4} μmol. Assuming aspartate transcarbamylase is 93% pure, 4.8 μg of aspartate transcarbamylase should be 0.344 $\times$ 10^{-4} μmol. The maximum number of moles of [^{14}C]carbamyl phosphate bound per mole of aspartate transcarbamylase is thus, (1.31 $\times$ 10^{-4})/(0.344 $\times$ 10^{-4}) = 3.8.

binding of carbamyl phosphate. If 48 mM succinate is present, addition of sodium acetate has no effect again. This is consistent with one of the proposed kinetic pathways reported in the previous paper (Chang and Jones, 1974b) where succinate (an analog of L-aspartate) binds first to the aspartate catalytic site of aspartate transcarbamylase, then binds to the activator site, followed by tight carbamyl phosphate binding.

Test of Carbamylation of Aspartate Transcarbamylase. Since the transient product after carbamyl phosphate hydrolysis is cyanate, which is potent carbamylation agent for amino, sulfhydryl, and imidazole groups of proteins (Stark, 1964, 1965), it was necessary to examine whether the aspartate transcarbamylase bound ^{14}C-labeled product(s) was indeed carbamyl phosphate at the end of the ultrafiltration studies. With or without succinate, at least 93% of the ^{14}C-labeled material bound by aspartate transcarbamylase during carbamyl phosphate binding studies can be transformed into citrulline (an acid-stable material) by ornithine transcarbamylase in the presence of L-ornithine.[1] Without addition of ornithine transcarbamylase, no radioactivity remains after the acidification, heating, and CO_2 bubbling employed in the [^{14}C]citrulline assay. Since carbamyl phosphate is a substrate for ornithine transcarbamylase, while cyanate is not (Allen and Jones, 1964), this indicates that essentially all of the enzyme-bound ^{14}C-labeled material is intact [^{14}C]carbamyl phosphate; therefore, no carbamyl–enzyme intermediate was formed during the binding between carbamyl phosphate and aspartate transcarbamylase with or without succinate. (See Table I of microfilm edition).[1]

Binding of L-Aspartate and Succinate. Table III lists the binding of L-aspartate or succinate as ligand in the absence or presence of sodium acetate. This table is only qualitative in nature; quantitative data cannot be obtained by this ultrafiltration technique since it is not suitable for measuring dissociation constants weaker than 5 or 10 mM; moreover, for binding constants weaker than 5 or 10 mM, large quantities of enzyme are required for this and other techniques to measure ligand binding. Qualitatively, it can be seen that the binding constants between aspartate transcarbamylase and succinate (or L-aspartate) are in the millimolar range, and that succinate binds more tightly to aspartate transcarbamylase than L-aspartate. This is consistent with the kinetic data (Jones, 1962). One possibility to explain this phenomenon is that part of the L-aspartate binding energy is utilized to force the substrates together during catalysis (Collins and Stark, 1969; for review and discussion, see Jencks, 1969). Since aspartate or other anions presumably cannot bind to the activator site until the aspartate substrate site is filled, one can make a preliminary estimate from the data of Table III that the binding constant between L-aspartate and the aspartate transcarbamylase activator site is probably lower than 0.5 mM, because the amount of L-aspartate bound to the aspartate transcarbamylase activator site (1.40 $\times$ 10^{-4} μmol) is more than half of the amount of L-aspartate bound to the aspartate transcarbamylase substrate site (2.44 $\times$ 10^{-4} μmol). By the same reasoning, the binding constant between succinate and the aspartate transcarbamylase activator site is probably higher than 0.5 mM. This is consistent with the idea reported in the previous paper (Chang and Jones, 1974a) that L-aspartate is probably the "preferred" activator for this enzyme.

Discussion

Several important consequences result from these studies of the reverse reaction. (a) The proposed mechanism that the substrate binds twice to aspartate transcarbamylase during the catalytic reaction (Chang and Jones, 1974a,b) is strengthened. (b) The activator site of aspartate transcarbamylase is much more specific and restricted in the reverse reaction (phosphate, sulfate, and nitrate, etc., do not activate the enzyme as they do in the forward reaction); therefore, it is extremely unlikely that the activator molecule can be the first reactant bound to aspartate transcarbamylase during either the forward or backward reaction. (c) In the forward reaction (Chang and

TABLE III: Binding of Succinate or L-Aspartate at Different Conditions.[a]

[^{14}C]Ligand Used	Sodium Acetate Added (mM)	Carbamyl Phosphate Added (mM)	Net cpm Bound to Aspartate Transcarbamylase	μmol of Ligand Bound to Aspartate Transcarbamylase	μmol of Ligand Bound to the Aspartate Transcarbamylase Activator Site[b]
0.5 mM succinate	0	0	1953 ± 111	5.16×10^{-4}	
0.5 mM succinate	100	0	1334 ± 100	3.53×10^{-4}	1.63×10^{-4}
0.5 mM succinate	0	2	1350 ± 200	3.53×10^{-4}	
0.5 mM L-aspartate	0	0	1100 ± 300	3.83×10^{-4}	
0.5 mM L-aspartate	100	0	700 ± 62	2.44×10^{-4}	1.40×10^{-4}

[a] The reaction mixture for the binding experiment contained 5 mM Tris-HCl (pH 8.5), 96 μg of aspartate transcarbamylase (93% pure), with or without sodium acetate or carbamyl phosphate, 0.5 mM [2,3-^{14}C]succinate (3.78×10^6 cpm/μmol), or 0.5 mM L-[U-^{14}C]aspartate (2.87×10^6 cpm/μmol). Controls were run without aspartate transcarbamylase. The final volume was 0.2 ml. The blank value obtained during 0.5 mM [^{14}C]succinate binding was 4451 ± 82. For 0.5 mM L-[^{14}C]aspartate binding, the blank value obtained was 8200 ± 100. [b] Assuming that succinate binds to the aspartate transcarbamylase catalytic site (of L-aspartate) first, then binds to the activator site. When 100 mM sodium acetate is added, the [^{14}C]succinate bound to the activator site is assumed to be "chased out." The micromoles of succinate bound to aspartate transcarbamylase activator site are thus the difference between the micromoles of succinate bound to aspartate transcarbamylase in the presence and in the absence of 100 mM sodium acetate. The same assumptions are applied for L-[^{14}C]aspartate binding.

Jones, 1974b), phosphate was shown to be a competitive product inhibitor against aspartate as variable substrate when aspartate levels were below inhibitory levels, while in the reverse reaction aspartate is a noncompetitive product inhibitor against phosphate as variable substrate, suggesting that the dead-end inhibitory complex enzyme–P_i–aspartate, which was proposed (Chang and Jones, 1974a) in order to explain the aspartate substrate inhibition, does exist. Potent substrate analogs of aspartate such as maleate and succinate do not seem to form such a complex as readily as aspartate does, although maleate and succinate do bind to the free enzyme molecular better than aspartate itself (Jones, 1962; Chang and Jones, 1974b, and Table III of this paper).

The equilibrium constant determined in this report (1.1×10^5, pH 8.56, 25°) is of the same order as that (7.5×10^4, pH 7.8, 0°) reported by Silverstein (1969) in an abstract.

Since we only obtained about 8 mg of pure aspartate transcarbamylase from 5.2 kg of wet *S. faecalis* paste (Chang and Jones, 1974a), the limited amount of pure enzyme available constituted a major problem in binding studies between enzyme and substrates. Fortunately, the ingenious ultrafiltration method developed by Paulus partially solved this problem. Several important consequences result from the present binding studies. (a) Since both carbamyl phosphate and L-aspartate (or succinate) can bind to aspartate transcarbamylase alone and since essentially all of the carbamyl phosphate bound to aspartate transcarbamylase in the presence or absence of succinate (analog of aspartate) remains as intact carbamyl phosphate, the random sequential mechanism proposed earlier (Chang and Jones, 1974b) is confirmed. (b) Since acetate, kinetically shown to be one of the better activators (Chang and Jones, 1974a), does not have any effect on carbamyl phosphate binding, but does facilitate carbamyl phosphate binding when very low succinate (2 mM) is added, there must exist an activator site different from the aspartate catalytic binding site in the aspartate transcarbamylase, a result consistent with the kinetic data (Chang and Jones, 1974a). Also, since acetate has no effect on carbamyl phosphate binding when the added succinate concentration is high (48 mM), this suggests that acetate and succinate can bind to the same activator site. (c) Since acetate alone has no effect on carbamyl phosphate binding, this suggests that the activator molecule cannot be the first reactant added to aspartate transcarbamylase during catalysis. The amount of [^{14}C]-aspartate (or [^{14}C]succinate) bound to aspartate transcarbamylase when the concentration of either ligand is 0.5 mM decreases appreciably in the presence of 100 mM acetate. This is consistent with the idea that, in the absence of acetate, aspartate binds twice to aspartate transcarbamylase, and that the binding is sequential. The first mole of aspartate binds to the catalytic site which cannot bind acetate, while the second mole of aspartate binds to the activator site which can bind acetate. In the presence of 100 mM acetate, aspartate (or succinate) bound to the enzyme activator site is "chased out" by acetate, a result consistent with the kinetic observation that aspartate and acetate compete for the activator site. The binding constant of aspartate as substrate, calculated from the data in Table III, should be around 5 mM (estimated per cent error: ±50%). (d) The dissociation constant of carbamyl phosphate in the absence of succinate is 1.7×10^{-4} M, in reasonable agreement with the value determined kinetically (Chang and Jones, 1974b). The binding affinity of carbamyl phosphate becomes about 50-fold greater in the presence of saturating levels of succinate, which probably binds twice to the enzyme in an ordered fashion, indicating that, at a minimum, one major function of the activator site is to greatly enhance the binding affinity of aspartate transcarbamylase for carbamyl phosphate. (e) The binding of succinate in the presence of carbamyl phosphate (2 mM) does not seem to be any better than its binding in the absence of carbamyl phosphate, *i.e.*, binding of succinate to the enzyme first facilitates the binding of carbamyl phosphate, but binding of carbamyl phosphate to the enzyme first does not facilitate the binding of succinate. This supports the suggestion that aspartate transcarbamylase catalyzes the reaction in an asymmetrical random sequential mechanism (see Scheme I in Chang and Jones, 1974b). When carbamyl phosphate is bound first followed by aspartate as the second substrate, the enzyme complex formed is apparently not the same as the enzyme complex formed when the order of the substrate addition is to initially bind aspartate at the substrate site and then to bind aspartate a second time at the activator site followed finally by the binding

of carbamyl phosphate. Consequently, in the presence of high levels of carbamyl phosphate, aspartate may bind to aspartate transcarbamylase only once, *i.e.*, at the substrate site. All of the consequences derived from the binding data are consistent with the kinetic data and the proposed tentative kinetic mechanism (Chang and Jones, 1974b); however, only two major pathways can be deduced from the kinetic and binding studies. It is not clear whether or not this enzyme can catalyze the reaction *via* some other pathways; for example: enzyme → enzyme–aspartate → enzyme–aspartate–carbamyl phosphate- → enzyme + products; or enzyme → enzyme–carbamyl phosphate → enzyme–carbamyl phosphate–aspartate → enzyme–carbamyl phosphate–aspartate–activator → enzyme + products may still exist as minor pathways during the reaction.

There certainly appear to be no changes in subunit interactions during carbamyl phosphate binding in the absence or presence of succinate, for the double reciprocal plots of the carbamyl phosphate binding curves are linear (Figures 6A and 6B). Whether there are changes in subunit interactions or not during aspartate binding as substrate is not certain, since the binding of aspartate could not be demonstrated quantitatively; the kinetic data certainly do not support this possibility. The binding technique developed by Gray and Chamberlin (1971), which utilizes the differential distribution of enzyme and substrate in two different aqueous phases, might be a desirable system for studying the binding between aspartate and aspartate transcarbamylase quantitatively. The fact that nearly 4 mol of carbamyl phosphate appears to be the maximum amount of carbamyl phosphate bound as well as the fact that the enzyme has four subunits of a similar size suggests, but is not proof, that each subunit is identical. Chemical analysis of the protein is necessary before this can be stated as a fact.

The asymmetric mechanism proposed for the *S. faecalis* aspartate transcarbamylase reaction, where the activator site only expresses itself when the concentration of one (carbamyl phosphate in this case) of the two substrates required for product formation is low, may exist more generally. It may serve, as it does here, to fix an unstable intermediate effectively because the other substrate (in this case aspartate, a stable substrate that is generally present *in vivo* in a reasonable concentration) can activate the enzyme to facilitate binding of the limiting or unstable substrate.

Supplementary Material Available

Supplementary material describing this experiment, including two figures and Table I, 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-74-646.

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