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Ornithine Transcarbamylase from *Salmonella typhimurium*: Purification, Subunit Composition, Kinetic Analysis, and Immunological Cross-Reactivity

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Ornithine transcarbamylase (OTCase) was purified to homogeneity from a derepressed strain of *Salmonella typhimurium*. The optimal pH for enzyme activity is 8.0. The molecular weight of the enzyme was calculated to be 116,000, based on measurements of the sedimentation coefficient by sucrose gradient ultracentrifugation and the Stokes radius by gel filtration. Polyacrylamide gel electrophoresis of cross-linked OTCase in the presence of sodium dodecyl sulfate showed that the enzyme is composed of three identical subunits. The molecular weight of the monomer was determined to be 39,000. Steady-state kinetics indicate that the reaction mechanism is sequential. The limiting Michaelis constants for carbamylphosphate and ornithine were determined to be 0.06 and 0.2 mM, respectively. The dissociation constant for carbamylphosphate was 0.02 mM. Product and dead-end inhibition patterns are consistent with an ordered Bi Bi mechanism, in which carbamylphosphate is the first substrate added and phosphate is the last product released. OTCase activity was inhibited by arginine, but relatively high concentrations were required for significant inhibition. The inhibition by arginine might be physiologically significant in the regulation of carbamylphosphate utilization; a single carbamylphosphate synthetase is responsible for the synthesis of carbamylphosphate for both arginine and pyrimidines in *S. typhimurium* and the inhibition by arginine might serve to divert carbamylphosphate to the synthesis of pyrimidines when arginine is present at high concentrations. The cross-reaction of OTCases from different microorganisms with purified antibodies raised against the homogeneous OTCase from *S. typhimurium* was investigated. The results of immunotitration and immunodiffusion experiments revealed a high degree of identity between the enzymes from *S. typhimurium* and *Escherichia coli* B and W. In these three cases, a single gene (*argI*) encodes OTCase. Wild-type *E. coli* K-12 and strain 3000 X 111, which carry two OTCase genes (*argI*, *argF*), also revealed similar cross-reactivity, supporting the hypothesis that *argF* is the product of a relatively recent duplication. The activity of OTCase from *Bacillus subtilis* was partially inhibited by antibodies against the enzyme from *S. typhimurium*, indicating unusual conservation of primary structure among widely different taxonomic groups. OTCase from *Saccharomyces cerevisiae*, whose molecular weight and primary structure are similar to those of the enzyme from *S. typhimurium*, was without detectable cross-reactivity.

Ornithine transcarbamylase (OTCase; carbamylphosphate:L-ornithine carbamyl transferase, EC 2.1.3.3.) catalyzes the transfer of the carbamyl group from carbamylphosphate to the δ-amino group of ornithine, forming citrulline, an intermediate of the arginine biosynthetic pathway in *Salmonella typhimurium* (34) and many other organisms. It has been well established for *Escherichia coli* (7, 36) that expression of the structural genes encoding enzymes of this pathway are negatively controlled by the product of *argR*. Control of the synthesis of OTCase by the *argR* product was also recently shown for *S. typhimurium* (14).

The recent finding that OTCase is required for the assembly of the unequal subunits of carbamylphosphate synthetase (CPSase) in certain mutants of *S. typhimurium* (1) suggests an intimate in vivo association between

OTCase and CPSase. The present work was initiated to increase our knowledge of OTCase and thereby further understand its effect on the regulation of synthesis and utilization of carbamylphosphate, which is required for the synthesis of both arginine and pyrimidines in *S. typhimurium* (2, 3).

MATERIALS AND METHODS

Chemicals. Ultrapure grades of ammonium sulfate (Mann) and acrylamide (Eastman) were used. Arginine was obtained from Calbiochem and found to be chromatographically homogeneous. Yeast alcohol dehydrogenase and beef liver catalase were purchased in ammonium sulfate suspension from Boehringer-Mannheim. Ornithine, lithium carbamylphosphate, norvaline, and protamine sulfate were obtained from Sigma. Dimethyl suberimidate was prepared from suberonitrile (Aldrich Chemical Co.) as described by Davies and Stark (8).

Strains. *S. typhimurium* LT2 and *E. coli* B were obtained from J. L. Ingraham, University of California, Davis. The *argR* derivative of *S. typhimurium* was a gift from G. O'Donovan of Texas A & M University. *E. coli* K-12 (wild type; CGSC 5073), and 3000 X 111 (Paris strain; CGSC 5263) were obtained from the *E. coli* Genetic Stock Center (CGSC). *E. coli* K-12 (C600) was obtained from D. Vapnek, University of Georgia, Athens. *E. coli* W ATCC 25208 and *B. subtilis* ATCC 6633 were obtained from the American Type Culture Collection. *Saccharomyces cerevisiae* was obtained from D. G. Ahearn, Georgia State University, Atlanta.

Assays. OTCase activity was determined by measuring citrulline formation at 30°C as described by Prescott and Jones (30). The reaction mixture, unless otherwise stated, contained 100 mM triethanolamine buffer, pH 8.0, 2 mM ornithine, 1 mM lithium carbamylphosphate, and enzyme, in a final volume of 2 ml. The reaction was started by the addition of carbamylphosphate or enzyme. Samples (0.5 ml) were withdrawn at intervals and mixed with 0.5 ml of the color mixture (antipyrine-sulfuric acid-oxime) to stop the reaction. Control experiments lacking enzyme, ornithine, or carbamylphosphate were routinely included. All determinations were made in the range in which reaction rates were constant and proportional to enzyme concentration. One enzyme unit catalyzes the formation of 1 μmol of citrulline per min under these conditions. For kinetic experiments with purified enzyme, dilutions were made in 0.05 M triethanolamine buffer, pH 8.0, containing 0.5 mM ethylenediaminetetraacetic acid (EDTA) and 0.05% bovine serum albumin. For measurement of initial rates as a function of carbamylphosphate concentration, a 5-cm cell was used, and absorbance at 466 nm was read in a Cary 17-D spectrophotometer. This permitted the accurate determination of citrulline in the range of 1 to 10 nmol. In inhibition experiments, in which arginine or citrulline was present, [^{14}C]ornithine was used. The reaction mixture (final volume, 0.4 ml) contained enzyme, 100 mM triethanolamine buffer (pH 8.0), 0.2 mM ornithine (2×10^6 cpm/μmol), and 1 mM carbamylphosphate. The reaction was started by the addition of carbamylphosphate and terminated, after 4 min, by the addition of 100 μl of 1.0 N HCl. A sample (usually 0.4 ml) of each reaction mixture was applied to a column of Bio-Rex 70 (6 by 0.9 cm) equilibrated with 50 mM lithium acetate (pH 5.3), and the [^{14}C]citrulline was eluted with 4 ml of water. A 2-ml sample of the eluate was placed in a vial containing 10 ml of the counting mixture described by Patterson and Greene (26) and counted in a Packard Tri-Carb liquid scintillation spectrometer. Controls without enzyme contained approximately 0.1% of the counts added. In certain experiments, after termination of the reaction by the addition of HCl, 10-μl samples were spotted on silica gel thin-layer plates (Eastman Kodak Co.) and dried with hot air. Plates were developed in 1-butanol–acetone–diethylamine–water (40:40:8:20), dried, and exposed to X-ray films overnight. The spots corresponding to citrulline were cut out, placed in scintillation vials containing 10 ml of the counting solution, and counted.

Protein was determined by the method of Lowry et al. (20), with crystallized bovine serum albumin as standard.

Enzyme purification. The enzyme was purified from an *argR*$^-$ derivative of *S. typhimurium* (14), which produced an enzyme titer 20-fold higher than that of wild type. Cells were grown on glucose minimal medium (35) containing 100 μg of proline (the strain carried an incidental proline marker), harvested, washed once with water, suspended at 0.4 g (wet weight)/ml in 0.1 M potassium phosphate buffer, pH 7.6, containing 0.5 mM EDTA and 5 mM ornithine, and disrupted by sonication. The crude cell extract was centrifuged at 27,000 × *g* for 30 min.

Protamine sulfate (4 ml of a 5% solution adjusted to pH 7.0 per 10 ml of centrifuged extract) was added at 0°C with stirring for 10 min. After centrifugation at 18,000 × *g* for 30 min, the supernatant was fractionated with solid ammonium sulfate. The fraction precipitating between 50 and 60% saturation, which contained 95% of ornithine transcarbamylase activity, was dissolved in 0.02 M potassium phosphate buffer, pH 7.7, containing 0.5 mM EDTA and 5 mM ornithine; this solution was dialyzed against the same buffer.

The dialyzed enzyme solution was pumped at a rate of 60 ml/h onto a column (60 by 1.6 cm) of diethylaminoethyl-cellulose (Whatman DE-52) equilibrated with 0.02 M potassium phosphate buffer, pH 7.7. After the solution was washed with 100 ml of the equilibrating buffer, protein was eluted with a linear gradient of potassium phosphate buffer, pH 7.0, containing 0.5 mM EDTA. Ornithine transcarbamylase eluted between 0.04 and 0.13 M, with the peak of activity at 0.07 M. Fractions containing enzyme activity were combined, concentrated by precipitation with ammonium sulfate (70% saturation), and dissolved in 0.1 M potassium phosphate buffer, pH 7.0, containing 0.5 mM EDTA.

This solution was applied to a Sephadex G-150 column (110 by 1.6 cm; void volume, 79 ml), which was eluted at a rate of 7 ml/h with 0.05 M potassium

phosphate buffer, pH 7.0, containing 0.5 mM EDTA and 5 mM ornithine. The elution volume for ornithine transcarbamylase was 106 ml.

Finally, the Sephadex eluate was dialyzed against 5 mM potassium phosphate buffer, pH 7.6, and applied at a rate of 30 ml/h to a column (37 by 1.6 cm) of hydroxylapatite (Calbiochem) equilibrated with 0.1 M buffer. Development of the column with a linear gradient of potassium phosphate buffer, pH 7.6, eluted the enzyme between 0.03 and 0.07, with the peak of activity at 0.05 M, a treatment that separated ornithine transcarbamylase from a minor protein contaminant. Table 1 summarizes the various steps in the purification.

The final enzyme preparation appeared to be homogeneous; Fig. 1 shows a photograph of the single band and the densitometer tracing observed after electrophoresis on 7.5% polyacrylamide gel at pH 8.0 (9). Removal of ammonium persulfate by pre-electrophoresis of the separating gel was essential for avoidance of artifacts, resulting possibly from oxidation of sulfhydryl groups on the enzyme.

Sucrose gradient ultracentrifugation. Density gradient centrifugation was carried out as described by Martin and Ames (23), using the IEC 404 rotor in an IEC B60 preparative centrifuge. Samples (0.2 ml) of enzyme preparation were layered on 13-ml linear sucrose gradients (5 to 20%, wt/vol) in a polyallomer centrifuge tube.

Molecular weight of OTCase was calculated from the sedimentation coefficient and Stokes radius by the following formula (4): $M = 6 \pi \eta N a s (1 - \bar{v}p)$, where M = molecular weight, a = Stokes radius, s = sedimentation coefficient, $\bar{v}$ = partial specific volume (a volume of 0.725 cm^3/g was assumed as representative of most proteins), η = viscosity of the medium, p = density of the medium, and N = Avogadro's number.

Preparation of immunoglobulin fraction against OTCase. Antibodies to OTCase from *S. typhimurium* were obtained by injecting the homogeneous enzyme into male New Zealand white rabbits. Each animal received 6 mg of OTCase in a series of intramuscular injections over a period of 6 weeks. Prior to injection, the enzyme was thoroughly mixed with the complete form of Freund adjuvant (Difco) at a volume ratio of 1:1. Rabbits were bled 1 week after the last injection, and the blood was allowed to clot overnight at 4°C. After removal of erythrocytes by centrifugation at low speed, the immunoglobulin fraction was separated from the serum by two successive ammonium sulfate fractionations at 0°C. The precipitate (0 to 50% saturation) was dissolved in a minimal volume of 0.01 M potassium phosphate buffer, pH 6.8, dialyzed against the same buffer, and further purified as described by Levingston (18) by application to a column (40 by 1.6 cm) of diethylaminoethyl-cellulose (Whatman DE-52) previously equilibrated with 0.01 M potassium phosphate buffer, pH 6.8. Washing the column with the equilibration buffer removes immunoglobulins, which do not bind to the adsorbent under these conditions. Fractions containing antibodies were combined and treated with ammonium sulfate (60% saturation), yielding a precipitate, which was dissolved in 0.01 M potassium phosphate buffer, pH 6.8, and dialyzed against the same buffer. This purified antibody preparation was used in antigen titrations and immunodiffusion experiments.

TABLE 1. *Purification of carbamylphosphate synthetase from S. typhimurium*

Fraction	Vol (ml)	Protein (mg/ml)	Sp act (units/mg of protein)	Recovery (%)
Extract[a]	1,000	25.1	34	100
Protamine sulfate	1,340	16.3	37	96
Ammonium sulfate (50–60%)	80	44.1	170	70
Diethylaminoethyl-cellulose	59	7.8	850	46
Sephadex G-150	68	3.0	1,769	43
Hydroxylapatite	30	2.6	1,901	35

[a] An *argR* derivative of *S. typhimurium* was grown as described in the text. The starting material was 360 g (wet weight) of cells.

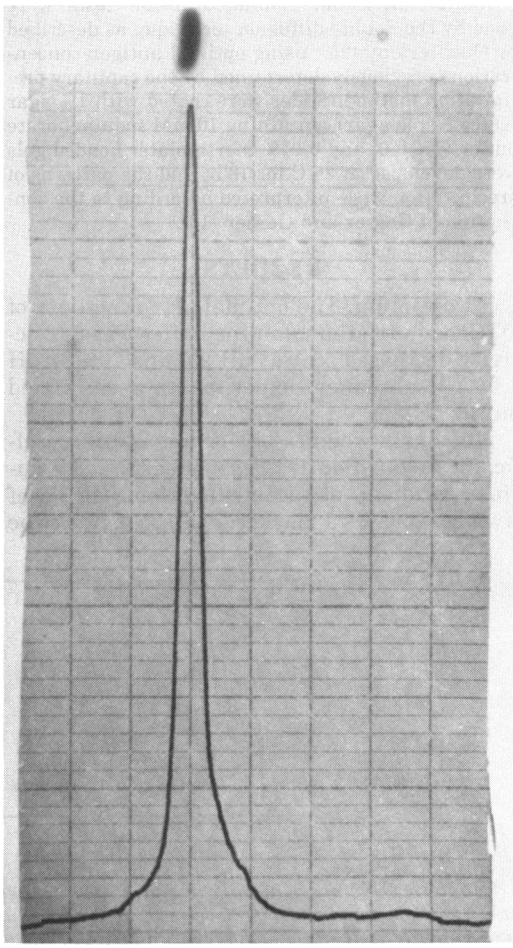

FIG. 1. *Densitometer tracing of polyacrylamide gel after electrophoresis at pH 8.0 of purified OTCase (130 μg of protein was applied).*

Titration of OTCase activity with antibodies. *S. typhimurium* LT2 and *E. coli* K-12, B, and W were grown in glucose minimal medium (35). *S. cerevisiae* was grown in a medium consisting of yeast nitrogen base (Difco) containing 3% glucose. *Bacillus subtilis* was grown in glucose minimal medium containing 100 μg of glutamate per ml to reduce lysis of cells (5). In all cases, cells were suspended in 0.1 M potassium phosphate buffer, pH 7.6, containing 0.5 mM EDTA and ruptured by passage though a French pressure cell. The crude cell extract was centrifuged at 30,000 $\times$ *g* for 30 min and then diluted in 0.05 M triethanolamine buffer, pH 8.0, containing 0.05% bovine serum albumin and 0.5 mM EDTA to 0.05 OTCase unit per ml for titration with antibodies. An appropriate dilution of the immunoglobulin fraction was added in progressive amounts to 100 μl of diluted enzyme, and the final volume was adjusted to 300 μl with the same dilution buffer. The mixture was incubated at 30°C for 15 min and assayed for OTCase activity by the colorimetric procedure described above.

Immunodiffusion. Immunodiffusion tests were done by the double-diffusion technique as described by Ouchterlony (25), using optimal antigen concentrations previously determined by the capillary precipitation method. Slides were coated with 1% agar (Difco Noble agar) containing 10 mM sodium borate buffer (pH 8.6) and 0.01% merthiolate. Loaded gels were developed at 25°C for 18 h, and the patterns of precipitation were interpreted according to the convention of Gasser and Gasser (10).

RESULTS

pH optimum. The optimal pH for activity of OTCase in triethanolamine buffer was 8.0; activity decreased 50% at pH 9.0 and 70% at pH 7.0. All subsequent experiments were carried out at pH 8.0.

Molecular weight. The sedimentation coefficient of purified OTCase, measured by sucrose gradient ultracentrifugation with beef liver catalase as a marker, was estimated to be 6.5*S* (Fig. 2a). The molecular Stokes radius of the enzyme, determined from a plot of elution volumes from Sephadex G-150 against the Stokes radii for chymotrypsinogen, bovine serum albumin, and yeast alcohol dehydrogenase (4), was 40 $\times$ 10^{-8} cm (Fig. 2b). The molecular weight of OTCase was calculated to be 116,000.

The sedimentation coefficient of OTCase in crude extracts of cells grown on glucose minimal medium containing either ammonium sulfate or arginine as a sole nitrogen source was also determined. Extracts were layered on sucrose gradients in the absence and presence of 1 mM arginine, 15 mM ornithine, and 0.2 M KCl, i.e., conditions shown to favor the binding of arginase to OTCase from *S. cerevisiae* (24). Gradients contained either 0.05 M potassium phosphate buffer, pH 7.6, or 0.05 M triethanolamine buffer, pH 8.0. Under all these conditions, the value obtained for the sedimentation coefficient of OTCase was similar to that of the purified enzyme.

Subunit composition. When homogeneous OTCase was subjected to disc electrophoresis in the presence of sodium dodecyl sulfate (37), a single band was obtained corresponding to a molecular weight of 39,000; when cross-linked in 0.05 M triethanolamine (pH 8.5) by dimethyl suberimidate (8) prior to treatment with sodium dodecyl sulfate and mercaptoethanol, three bands were observed, corresponding to molecular weights of 39,000, 78,000, and 117,000 (Fig. 3). In more concentrated enzyme solutions, an additional band corresponding to 234,000 (a hexamer) was observed. The molecular weight of the trimer (117,000) is in excellent agreement with that obtained from measurements of the sedimentation coefficient and the Stokes radius of the native enzyme. Thus,

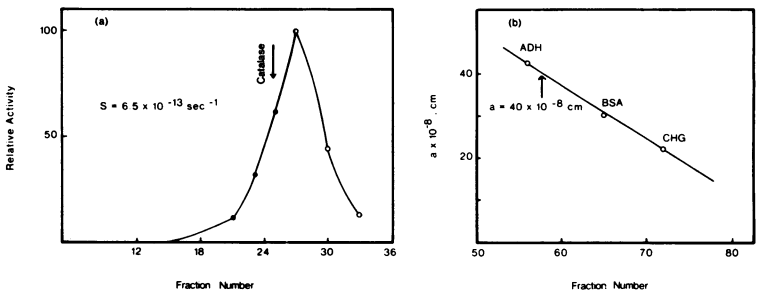

FIG. 2. *(a) Sucrose density gradient ultracentrifugation; 0.2 ml of purified OTCase was layered on a 13-ml linear sucrose gradient (5 to 20%) in 0.05 M potassium phosphate buffer, pH 7.6, containing 0.5 mM EDTA and centrifuged at 39,000 rpm for 15 h at 4°C. Fractions of 8 drops were collected. Peak OTCase activity was taken as 100%. Beef liver catalase (S = 11.3) was used as a marker. (b) Calibration curve for the determination of the Stokes radius by gel sieving on a Sephadex G-150 column (110 by 1.6 cm; void volume, 79 ml). Fractions of 50 drops (1.82 ml) were collected. The arrow indicates the elution volume of purified OTCase.*

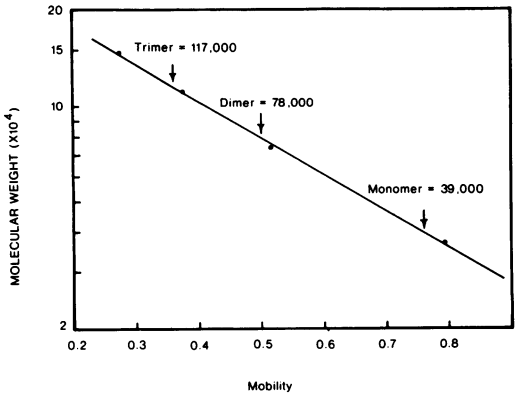

FIG. 3. *Estimation of the molecular weights of the subunit and the cross-linked enzyme. Yeast alcohol dehydrogenase was cross-linked by dimethyl suberimidate (8). The cross-linked enzyme (bands at 37,000, 74,000, 111,000, and 148,000) was used as a standard. The electrophoretic mobilities were determined as described by Weber and Osborn (37). The arrows indicate the mobilities for the monomer, dimer, and trimer of OTCase after cross-linking and electrophoresis in the presence of sodium dodecyl sulfate (37).*

the enzyme is composed of three subunits with identical molecular weights.

Kinetic analysis. Ornithine saturation curves were determined at several fixed concentrations of carbamylphosphate. Double reciprocal plots of these data gave a family of curves that intersect to the left of the vertical axis (Fig. 4a), suggesting a sequential kinetic mechanism (32). The secondary plots of intercepts and slopes against the reciprocal of carbamylphosphate concentration were linear (Fig. 4b and c). The limiting Michaelis constant for carbamylphosphate (K_{m_A}), determined from the intercept replot, is 0.057 mM. The dissociation constant for carbamylphosphate (K_{ia}), determined from the slope replot, is 0.026 mM. When carbamylphosphate was the variable substrate at different concentrations of the fixed substrate ornithine, an intersecting pattern (Fig. 5a) was again obtained. The limiting Michaelis constant for ornithine, determined from the intercept replot (Fig. 5b), is 0.2 mM. Values for K_{ia} and K_{m_A} are in reasonable agreement with those obtained from Fig. 4 (K_{m_A} = 0.053 mM and K_{ia} = 0.019 mM).

Experiments with phosphate as a product inhibitor showed that it acted as a linear competitive inhibitor with respect to carbamylphosphate (Fig. 6a), as expected if both ligands bind to the same enzyme form, E. Inhibition experiments by phosphate were also carried out with ornithine as a variable substrate (Fig. 6c). Here, the inhibition pattern was more difficult to establish in view of the inhibitory effect by ornithine, which appears in the presence of phosphate. However, at low phosphate concentrations, the inhibition pattern appeared to be of the mixed type with respect to ornithine, as expected in an ordered sequence. Equilibrium dialysis experiments (unpublished data) indicate that the dissociation constant for ornithine decreased by approximately threefold in the presence of 2 mM phosphate. The inhibitory effect by ornithine was examined with carbamylphosphate as a variable substrate in the presence of several fixed and high concentrations of ornithine. The inhibition pattern was uncompetitive with respect to carbamylphosphate (Fig. 7). Such a pattern is characteristic of ordered systems and indicates that ornithine (B) reacts in dead-end fashion with the enzyme-phosphate (EQ) complex (page 824, reference 32).

The effect of citrulline on reaction rate was examined by using [^{14}C]ornithine as a substrate, as described above. Citrulline at 10 mM had no effect on the reaction rate, possibly due to a high inhibition constant.

Dead-end inhibition analysis was performed using norvaline, which is a structural analogue of ornithine. The results show that norvaline gives rise to an uncompetitive inhibition pattern when carbamylphosphate is the variable substrate (Fig. 8a) and to a competitive inhibition pattern when ornithine is the variable substrate (Fig. 8c).

All the above results are consistent with an ordered Bi Bi mechanism (32), in which carbamylphosphate is the first substrate added and phosphate is the last product released.

Inhibition by arginine. Arginine at 5 mM inhibited the activity of purified OTCase by 42% (Table 2), as indicated by the standard radioactive assay (see Materials and Methods). These results were confirmed by separating the [^{14}C]citrulline product on thin-layer plates and counting its radioactivity. At saturating concentrations of carbamylphosphate and ornithine (1 and 2 mM, respectively), OTCase activity was inhibited 20%. The inhibition by arginine was examined at pH 7.0 in triethanola-

TABLE 2. *Inhibition of OTCase activity by arginine*

Addition to reaction mixture	cpm of [^{14}C]citrulline formed/4 min[a]
None	13,934 (100)
Arginine, 2 mM	10,450 (75)
Arginine 5 mM	8,200 (58)

[a] The enzyme was assayed by using [^{14}C]ornithine, as described in the text. Numbers in parentheses give percentages.

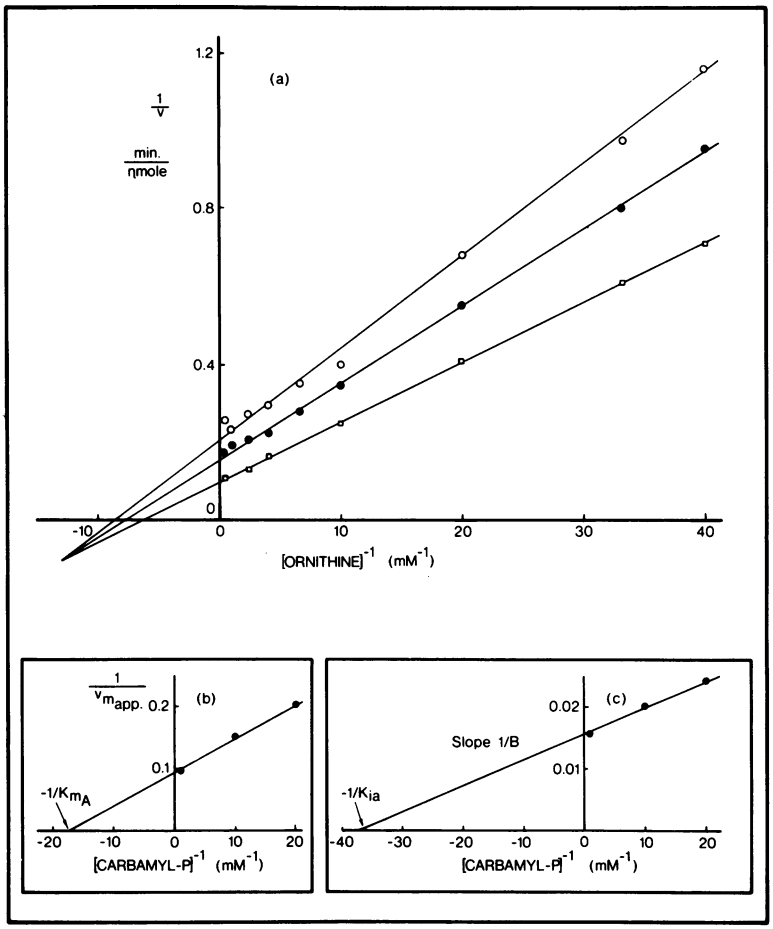

FIG. 4. *(a) Double reciprocal plots for ornithine. Carbamylphosphate concentrations were held constant at the following values:* ○, *0.05 mM;* ●, *0.1 mM;* □, *0.2 mM. (b) 1/v axis intercept replot,* K_{m_A} = *0.057 mM. (c) Slope replot,* K_{ia} = *0.026 mM.*

mine to determine whether the enzyme is more sensitive at this pH, but the extent of inhibition was similar to that at pH 8.0. Putrescine, which was shown to inhibit the catabolic OTCase from *Pseudomonas fluorescens* (33), had no effect on OTCase activity at a concentration of 5 mM.

Immunological cross-reactivity. The immunological relatedness of OTCase from several microbial sources was compared using purified immunoglobulin against OTCase from *S. typhimurium*. Antibodies prepared against homogeneous OTCase from *S. typhimurium* react as effectively with OTCase from *E. coli* B or W as they do with the homologous enzyme (Fig. 9). They fail to react with OTCase from *S. cerevisiae* and react partially with OTCases from *B. subtilis* and *E. coli* K-12 (C600). However, the intermediate cross-reactivity of the enzyme from *E. coli* K-12 (C600) appears to be characteristic only of that strain of *E. coli* K-12; experiments with other strains of *E. coli* K-12 (wild type and 3000 X 111) showed that the enzymes from these strains have cross-reactivity identical to that of the enzyme from *S. typhimurium*.

The results of a limited number of immunodiffusion experiments are in agreement with those obtained by immunotitration. Thus, the enzymes from *E. coli* B and W appear identical to that from *S. typhimurium*, whereas OTCase from *S. cerevisiae* gives no precipitation band.

DISCUSSION

The data presented here show that *S. typhimurium* produces a single form of OTCase, a result consistent with the presence of a single gene (*argI*) encoding OTCase in this organism (34). In this respect *S. typhimurium* is similar to *E. coli* B (13, 15) and W (16) but differs from *E. coli* K-12, which carries two OTCase genes,

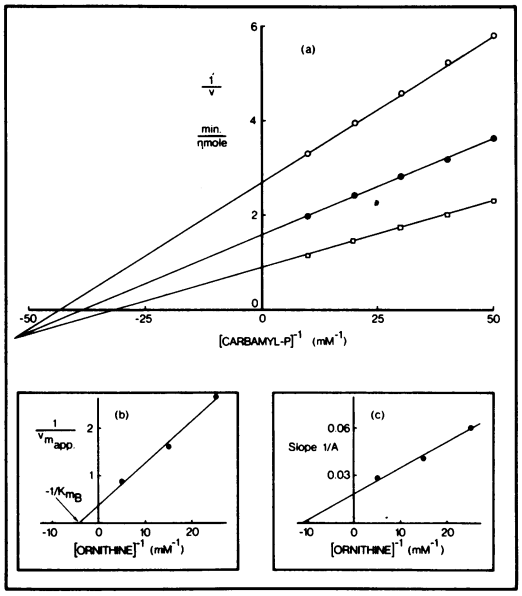

FIG. 5. *(a) Double reciprocal plots for carbamylphosphate. Ornithine concentrations were held constant at the following values: ○, 0.04 mM; ●, 0.0666 mM; □, 0.2 mM. (b) 1/v axis intercept replot, K_{m_B} = 0.2 mM. (c) Slope replot, K_{m_A} = 0.053 mM.*

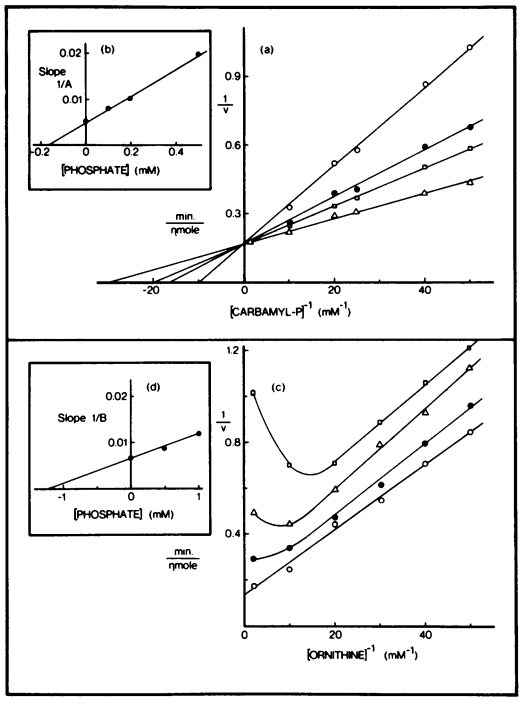

FIG. 6. *Product inhibition by phosphate. (a) Reciprocal velocity versus 1/[carbamylphosphate] with 0.2 mM ornithine and 0 (△), 0.1 (□), 0.2 (●), and 0.5 (○) mM phosphate. (b) Replot of slope versus phosphate concentration. (c) Reciprocal velocity versus 1/[ornithine] with 0.1 mM carbamylphosphate and 0 (○), 0.5 (●), 1 (△), and 3 (□) mM phosphate. (d) Replot of slope versus phosphate concentration.*

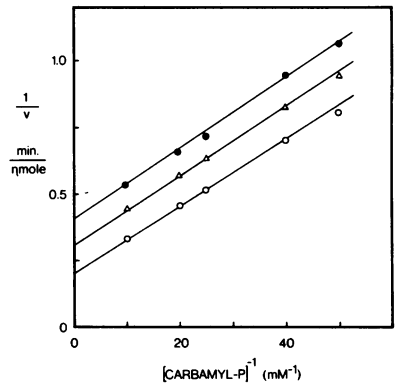

FIG. 7. *Substrate inhibition by ornithine. Reciprocal velocity versus 1/[carbamylphosphate] with 10 (○), 20 (△), and 40 (■) mM ornithine.*

argF and *argI*, the products of which interact to form four trimeric molecules which are separable on diethylaminoethyl-cellulose (15).

In the case of *E. coli* there is a discrepancy between the reported molecular weights of native OTCase determined by gel filtration (140,000) and by dodecyl sulfate-gel electrophoresis (105,000) of the cross-linked enzyme (15). Legrain et al. pointed out that this discrepancy may reflect the unusual shape of this enzyme on the estimation of its molecular weight by gel filtration (15). A similar discrepancy is observed for OTCase from *S. typhimurium*, however; when the values of both the Stokes radius and the sedimentation coefficient are taken into account in calculating the molecular weight, a value of 116,000 is obtained, which is in excellent agreement with that obtained by dodecyl sulfate-gel electrophoresis (117,000). This value, in turn, is similar to that obtained by dodecyl sulfate-gel electrophoresis for the enzymes from *E. coli* K-12 (15), *E. coli* W (16), *S. cerevisiae* (27), and the anabolic enzyme from *P. fluorescens* (33). All these anabolic enzymes share the unusual trimeric quaternary structure. In contrast, OTCases with catabolic functions are somewhat different: the enzymes from *P. fluorescens* (11) and *Streptococcus faecalis* (21) have molecular weights of 312,000 and 228,000 corresponding to eight and six identical subunits, respectively. A high molecular weight (360,000) was also reported for the catabolic OTCase from *Mycoplasma hominis* (31). An exceptional anabolic OTCase might be that from *B. subtilis*, for which a molecular weight of 280,000 was reported (12).

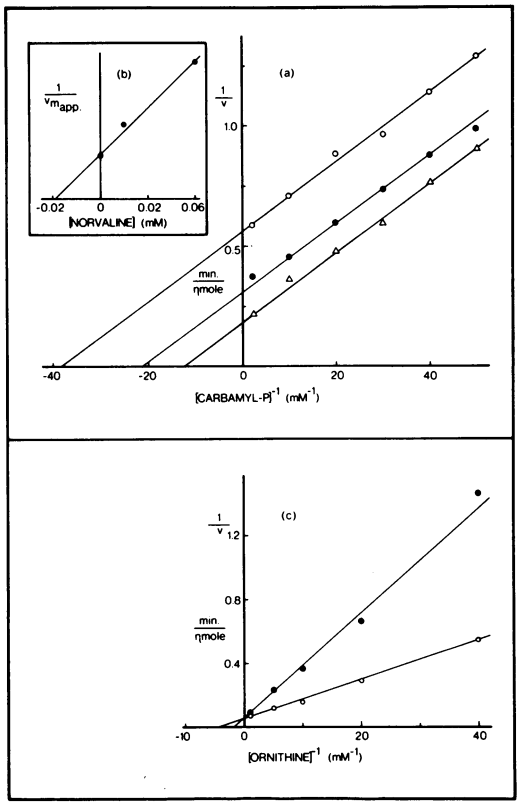

FIG. 8. *Dead-end inhibition by norvaline. (a) Reciprocal velocity versus 1/[carbamylphosphate] with 0.2 mM ornithine and 0 (△), 0.01 (●), and 0.04 (○) mM norvaline. (b) Replot of 1/v axis intercept versus norvaline concentration (K_i = 0.02 mM). (C) Reciprocal velocity versus 1/[ornithine] with 1 mM carbamylphosphate and 0 (○) and 0.05 (●) mM norvaline.*

Messenguy et al. (24) showed that in *S. cerevisiae* an arginine-inducible enzyme (arginase) binds to OTCase in the presence of arginine and ornithine, thereby inhibiting its activity but leaving arginase activity unaffected. This regulation, by reversible stoichiometric interaction between these two enzymes, one with a catabolic and the other with an anabolic function, prevents recycling of ornithine to arginine when the latter is used as a source of nitrogen; OTCase of *B. subtilis* was reported to be regulated in a similar manner (12). In both *S. cerevisiae* and *B. subtilis*, the association between the two enzymes is favored in the presence of arginine and ornithine. The results presented here show that the sedimentation coefficient for OTCase from *S. typhimurium* grown with arginine as a sole nitrogen source is similar to that from cells grown in its absence. Thus, the control mechanism found in *S. cerevisiae* and *B. subtilis*, for which the term "epi-enzymatic control" has been coined, appears to be absent from *S. typhimurium*. The interesting question as to the pathway by which *S. typhimurium* and *E. coli* metabolize arginine as a sole nitrogen source is yet to be answered.

The steady-state kinetic data for OTCase from *S. typhimurium* are consistent with an ordered Bi Bi mechanism, with carbamylphosphate adding first and ornithine second; then the product citrulline is released first, and finally phosphate is released. A sequential ordered mechanism has also been reported for the anabolic OTCase from *E. coli* W (16) and the catabolic enzyme from *S. faecalis* (22). It appears, therefore, that this is indeed a general mechanism for enzymatic transfer of carbamyl groups, because the related enzyme aspartate transcarbamylase also follows an ordered Bi Bi mechanism (28).

The finding that arginine inhibits OTCase from *S. typhimurium* was surprising because such sensitivity has not been reported for this enzyme from any other microbial source. Although only relatively high concentrations of arginine (5 mM) significantly inhibit the enzyme, these results contrast with those obtained with the enzyme from *E. coli* W, which is unaffected even by 100 mM arginine (16). It seems unlikely that the inhibition of OTCase from *S. typhimurium* by arginine is itself physiologically significant but, when interacting in vivo with CPSase, it might play a role in the regulation of carbamylphosphate utilization. In

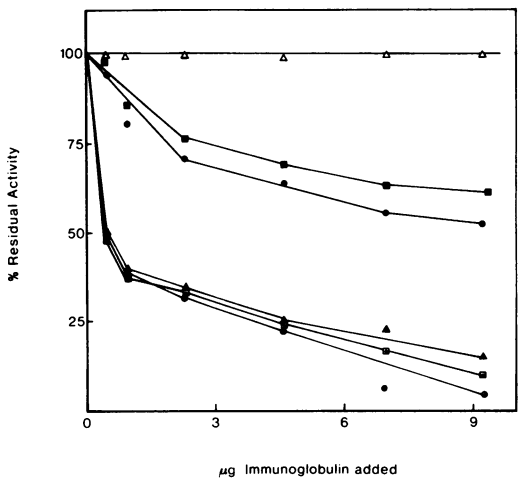

FIG. 9. *Inactivation of the OTCase activity from different microbial sources by the immunoglobulin fraction of serum raised against homogenous OTCase from S. typhimurium. For details, see Materials and Methods. Symbols: △, S. cervesiae; ■, B. subtilis; ●, E. coli K-12 (c600); ▲, E. coli W; □, E. coli B; ○, S. typhimurium LT2.*

this organism, a single CPSase is responsible for the synthesis of carbamylphosphate for both arginine and pyrimidines (2, 3), and biochemical and genetic evidence (1) indicate a close in vivo association between OTCase and CPSase. The inhibition by arginine might be intensified by CPSase and thereby serve to divert carbamylphosphate to the synthesis of pyrimidines when arginine is present at high concentrations. Examination of this possibility has to await studies on the interactions between purified OTCase and CPSase.

The conservation of the trimeric quaternary structure and molecular weight of the anabolic OTCases from various microbial sources prompted us to study the immunological relatedness of the enzyme from several microorganisms. The available evidence (29) suggests that among isofunctional proteins, immunological cross-reactivity is in fact an index of similarity of primary sructure. The results of immunological tests suggest that the enzymes from *S. typhimurium* and *E. coli* B and W are quite similar. OTCases from wild-type *E. coli* K-12 and strain 3000 X 111, which carry *argF* and *argI* (17), yield the same type of inactivation curves as that obtained with the enzyme from *S. typhimurium*. The *argI* and *argF* products exhibit identical kinetic parameters and can only be distinguished by different thermolability and chromatographic behavior on diethylaminoethyl-cellulose (17). In view of the apparent immunological similarity reported here, the physical differences between the *argI* and *argF* products might be the result of minor differences in the primary sequence. These results, therefore, support the conclusion (17) that *argF* might be the product of a relatively recent duplication of *argI*. We expect to elucidate the extent of sequence similarity among OTCases from *S. typhimurium* and the several *E. coli* strains with the more sensitive technique of micro-complement fixation (29).

In the case of *E. coli* K-12 (C600) the intermediate cross-reactivity indicated by immunotitration experiments shows that at least one of the two structural genes specifying OTCase in this strain encodes a product significantly different from OTCases in other strains of *E. coli*. This is possibly due to a mutation in *argI* or *argF* as a result of the several mutagenic treatments encountered in the pedigree of this strain (6).

Preliminary data (unpublished) show that the enzymes from *Citrobacter freundii* and *Shigella sonnei* are similar to OTCase from *S. typhimurium*, suggesting considerable conservation of the amino acid sequence for this enzyme among some members of the *Enterobacteriaceae*. In fact, the significant inhibition of OTCase from the gram-positive *B. subtilis* by antibodies against the enzyme from *S. typhimurium* (Fig. 9) indicates unusual conservation of enzyme structure among widely different taxonomic groups. This is contrasted, for example, with data reported for aldolases where antibodies against the enzyme from *S. faecalis* react only with aldolases from other lactic acid bacteria (19). Although the trimeric structure and molecular weight of OTCase from *S. cerevisiae* are similar to those of the enteric enzyme, no detectable cross-reactivity was observed with this eucaryotic enzyme. Such a complete loss of immunological reactivity for other isofunctional proteins was estimated to result from a 25 to 40% difference in primary sequence (29).

We feel that a detailed immunological analysis of OTCases from microbial sources might yield valuable information of the evolutionary development of this enzyme and, indeed, offers the possibility to study relatedness among procaryotic groups.

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LITERATURE CITED

1. **Abdelal, A., E. Griego, and J. L. Ingraham.** 1976. Arginine-sensitive phenotype of mutations in *pyrA* of *Salmonella typhimurium:* the role of ornithine carbamyltransferase in the assembly of mutant carbamylphosphate synthetase. J. Bacteriol. 128:105–113.
2. **Abdelal, A., and J. L. Ingraham.** 1969. Control of carbamylphosphate synthesis in *Salmonella typhimurium*. J. Biol. Chem. 244:4033–4038.
3. **Abdelal, A., and J. L. Ingraham.** 1975. Carbamylphosphate synthetase from *Salmonella typhimurium*. Regulation, subunit composition, and function of the subunits. J. Biol. Chem. 250:4410–4417.
4. **Andrews, P.** 1969. Estimation of molecular size and molecular weights of biochemical compounds by gel filtration, p. 1–53. *In* D. Glick (ed.), Methods of biochemical analysis, vol. 18. Interscience Publishers, New York.
5. **Armstrong, R. L., N. Harford, R. H. Kennett, M. L. St. Pierre, and N. Sueoka.** 1970. Experimental methods for *Bacillus subtilis*, p. 36–59. *In* H. Tabor and C. W. Tabor (ed.), Methods in enzymology, vol. 17A. Academic Press Inc., New York.
6. **Bachmann, B. J.** 1972. Pedigrees of some mutant strains of *Escherichia coli* K-12. Bacteriol. Rev. 36:525–557.
7. **Cunin, R., N. Kelker, A. Boyen, Huey-Lang Yang, G. Zubay, N. Glansdorff, and W. K. Maas.** 1976. Involvement of arginine in *in vitro* repression of transcription of arginine genes, C, B and H in *Escherichia coli* K12. Biochem. Biophys. Res. Commun. 69:377–382.
8. **Davies, G. E., and G. R. Stark.** 1970. Use of dimethyl

suberimidate, a cross-linking reagent, in studying the subunit structure of oligomeric proteins. Proc. Natl. Acad. Sci. U.S.A. 66:651-656.
9. **Gabriel, O.** 1971. Analytical disc gel electrophoresis, p. 565-578. *In* W. B. Jakoby (ed.), Methods in enzymology, vol. 22. Academic Press Inc., New York.
10. **Gasser, F., and C. Gasser.** 1971. Immunological relationships among lactic dehydrogenases in the genera *Lactobacillus* and *Leuconostoc*. J. Bacteriol. 106:113-125.
11. **Halleux, P., C. Legrain, V. Stalon, A. Pierard, and J. Wiame.** 1972. Regulation of the catabolic carbamyltransferase of *Pseudomonas fluorescens*. Eur. J. Biochem. 31:386-393.
12. **Issaly, I. M., and A. S. Issaly.** 1974. Control of ornithine carbamyltransferase activity by arginase in *Bacillus subtilis*. Eur. J. Biochem. 49:485-495.
13. **Jacoby, G. A.** 1971. Mapping the gene determining ornithine transcarbamylase and its operator in *Escherichia coli* B. J. Bacteriol. 108:645-651.
14. **Kelln, R. A., K. F. Foltermann, and G. A. O'Donovan.** 1975. Location of the *argR* gene on the chromosome of *Salmonella typhimurium*. Mol. Gen. Genet. 139:277-284.
15. **Legrain, C., P. Halleux, V. Stalon, and N. Glansdorff.** 1972. The dual genetic control of ornithine carbamoyltransferase in *Escherichia coli*. A case of bacterial hybrid enzymes. Eur. J. Biochem. 27:93-102.
16. **Legrain, C., and V. Stalon.** 1976. Ornithine carbamoyltransferase from *Escherichia coli* W. Purification, structure and steady-state kinetic analysis. Eur. J. Biochem. 63:289-301.
17. **Legrain, C., V. Stalon, and N. Glansdorff.** 1976. *Escherichia coli* ornithine carbamoyltransferase isoenzymes: evolutionary significance and the isolation of λ*argF* and λ*argI* transducing bacteriophages. J. Bacteriol. 128:35-38.
18. **Levingston, D. M.** 1974. Immunoaffinity chromatography of proteins, p. 723-731. *In* W. B. Jacoby and M. Wilchek (ed.), Methods in enzymology, vol. 34B. Academic Press Inc., New York.
19. **London, J., and K. Kline.** 1973. Aldolase of lactic acid bacteria: a case history in the use of an enzyme as an evolutionary marker. Bacteriol. Rev. 37:453-478.
20. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193:265-275.
21. **Marshall, M., and P. Cohen.** 1972. Ornithine transcarbamylase from *Streptococcus faecalis* and bovine liver. I. Isolation and subunit structure. J. Biol. Chem. 247:1641-1653.
22. **Marshall, M., and P. Cohen.** 1972. Ornithine transcarbamylase from *Streptococcus faecalis* and bovine liver. II. Multiple binding sites for carbamyl-P and L-norvaline, correlation with steady state kinetics. J. Biol. Chem. 247:1654-1668.
23. **Martin, R. G., and B. N. Ames.** 1961. A method for determining the sedimentation behavior of enzymes: application to protein mixtures. J. Biol. Chem. 235:1372-1379.
24. **Messenguy, F., M. Pennickx, and J. Wiame.** 1971. Interaction between arginase and ornithine carbamoyltransferase in *Saccharomyces cerevisiae*. Eur. J. Biochem. 22:277-286.
25. **Ouchterlony, Ö.** 1967. Immunodiffusion and immunoelectrophoresis, p. 655-706. *In* D. M. Weir (ed.), Handbook of experimental immunology. F. A. Davis Co., Philadelphia.
26. **Patterson, M. S., and R. C. Greene.** 1965. Measurement of low energy beta-emitters in aqueous solution by liquid scintillation counting of emulsions. Anal. Chem. 37:854-857.
27. **Pennickx, M., J. Simon, and J. Wiame.** 1974. Interaction between arginase and L-ornithine carbamoyltransferase in *Saccharomyces cerevisiae*. Eur. J. Biochem. 49:429-442.
28. **Porter, R. W., M. O. Modebe, and G. R. Stark.** 1969. Aspartate transcarbamylase. Kinetic studies of the catalytic subunit. J. Biol. Chem. 244:1846-1859.
29. **Prager, E. M., and A. C. Wilson.** 1971. The dependence of immunological cross-reactivity upon sequence resemblance among lysozymes. II. Comparison of precipitin and micro-complement fixation results. J. Biol. Chem. 246:7010-7017.
30. **Prescott, L. M., and M. E. Jones.** 1969. Modified methods for the determination of carbamylaspartate. Anal. Biochem. 32:408-419.
31. **Schimke, R. T., C. M. Berlin, E. W. Sweeney, and W. R. Caroll.** 1966. The generation of energy by the arginine dihydrolase pathway in *Mycoplasma hominis* O7. J. Biol. Chem. 244:2228-2236.
32. **Segel, I. H.** 1975. Enzyme kinetics: behavior and analysis of rapid equilibrium and steady-state enzyme systems. Wiley-Interscience, New York.
33. **Stalon, V., F. Ramos, A. Pierard, and J. Wiame.** 1972. Regulation of the catabolic ornithine carbamoyltransferase of *Pseudomonas fluorescens*. A comparison with the anabolic transferase and with a mutationally modified catabolic transferase. Eur. J. Biochem. 29:25-35.
34. **Syvanen, J. M., and J. R. Roth.** 1972. Structural genes for ornithine transcarbamylase in *Salmonella typhimurium* and *Escherichia coli* K-12. J. Bacteriol. 110:66-70.
35. **Vogel, H. J., and D. M. Bonner.** 1956. Acetylornithinase of *Escherichia coli*: partial purification and some properties. J. Biol. Chem. 218:97-106.
36. **Vogel, R. H., W. L. McLellan, A. P. Hirovonen, and H. J. Vogel.** 1971. The arginine biosynthetic system and its regulation, p. 464-485. *In* H. J. Vogel (ed.), Metabolic pathways, vol. 5. Academic Press Inc., New York.
37. **Weber, K., and M. Osborn.** 1969. The reliability of molecular weight determinations by dodecyl sulfate-polyacrylamide gel electrophoresis. J. Biol. Chem. 244:4406-4412.