Substrate Specificity and Protonation State of Ornithine Transcarbamoylase As Determined by pH Studies†

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ABSTRACT: The ornithine transcarbamoylase catalyzed reaction and its inhibition by L-norvaline have been investigated between pH 5.5 and 10.5. The steady-state turnover rate (k_{cat}) of the enzyme from *Escherichia coli* increases with pH and plateaus above pH 9. Its change with pH conforms to a single protonation process with an apparent pK_a of 7.3. The effect of pH on the apparent Michaelis constant (K_M^{app}) of L-ornithine suggests that this diamino acid in its cationic form is not the substrate. Treating only the zwitterions of ornithine as substrate, the pH profile of the pseudo-first-order rate constant (k_{cat}/K_M^z) of the reaction is a bell-shaped curve characterized by pK_a's of 6.2 and 9.1 and asymptotic slopes of ±1. Similar pK_a's (6.3 and 9.3) are obtained for the pK_i profile of zwitterionic L-norvaline, a competitive inhibitor. The pK_i profile further indicates that the α-amino group of the inhibitor must be charged for binding. Together, these pH profiles provide sufficient information to suggest that only the minor zwitterionic species of ornithine, $H_2N(CH_2)_3CH(NH_3^+)COO^-$, binds the enzyme productively. The selection of this substrate form by the enzyme leads to a Michaelis complex in which ornithine is poised for nucleophilic attack. Following such binding, the need for deprotonation of the δ-NH_3^+ group is avoided, and transcarbamoylation becomes energetically more feasible. Reaction schemes accounting for the effects of pH are proposed for the enzymic reaction.

An enzyme in the urea cycle, ornithine transcarbamoylase (EC 2.1.3.3), catalyzes the formation of L-citrulline and inorganic phosphate from L-ornithine and carbamoyl phosphate. The reaction also generates a proton. Little is known about the mechanistic details of the transcarbamoyl reaction catalyzed by this enzyme. The enzyme normally observes Michaelis-Menten kinetics, and binding of substrates and release of products are both sequential (Marshall & Cohen, 1972; Legrain et al., 1976). In the presence of Zn^{2+}, the enzyme from *Escherichia coli* also displays allosteric interactions (Kuo et al., 1982; Kuo, 1983).

Anabolic ornithine transcarbamoylase is a trimeric molecule of ~110 000 daltons and is composed of identical subunits (Legrain et al., 1977). The primary structures of the enzyme from *E. coli* and human liver are now known from nucleotide sequencing (Bencini et al., 1983; Horwich et al., 1984). Also, single crystals that diffract to a nominal Bragg spacing of 5 Å have been obtained for the *E. coli* enzyme (L. C. Kuo and W. N. Lipscomb, unpublished experiments). However, knowledge of the active-site environment of the enzyme remains limited to results of chemical modification studies; residues found to respond to substrate binding include at least a lysine, a cysteine, and an arginine (Marshall & Cohen, 1977, 1980a–d; Fortin et al., 1981). For the closely related enzyme aspartate transcarbamoylase, which catalyzes an analogous reaction in the pyrimidine biosynthesis pathway, crystallographic studies have located lysinyl, argininyl, and histidinyl residues in the immediate vicinity of its active site (Ke et al., 1984). The amino acid sequences of the two transcarbamoylases are highly homologous (Houghton et al., 1984).

The effect of pH on ornithine transcarbamoylase from liver has been investigated previously (Snodgrass, 1968; Marshall & Cohen, 1972). In both studies, the experiments are restricted to pH 6–8. An apparent pK_a of 6.5–6.8 is reported for V_{max}. There are insufficient data on the K_M of ornithine for a complete analysis.

In this paper, we report the pH dependence of ornithine saturation in the transcarbamoyl reaction catalyzed by the *Escherichia coli* enzyme. Substrate specificity and the enzyme protonation state in productive binding are elucidated. The results illustrate kinetic features of the enzyme that are important in mechanistic delineation.

Experimental Procedures

Materials

The following reagent-grade chemicals were purchased from Sigma Chemical Co. and used without further purification: L-citrulline, L-ornithine, 2,3-butanedione monoxime, ethanolamine, 3-(cyclohexylamino)propanesulfonic acid (CAPS), diethanolamine, 2-(*N*-morpholino)ethanesulfonic acid (MES), *N*-ethylmorpholine, and tris(hydroxymethyl)aminomethane (Tris).

The dilithium salt of carbamoyl phosphate, also purchased from Sigma, was recrystallized in the manner prescribed by Gerhart & Pardee (1962). Stock solutions were prepared in H_2O at 4 °C and frozen in 5-mL aliquots for storage until use. Particular attention was directed toward minimizing carbamoyl phosphate decomposition. During enzymic assays, carbamoyl phosphate was kept at 0 °C. Less than an estimated 2% of carbamoyl phosphate decomposed in the course of a 5-min reaction. Marshall & Cohen (1972) reported a rate constant of 0.0029 min^{-1} for the decomposition of carbamoyl phosphate at 25 °C and pH 7.9. The actual concentration of carbamoyl phosphate in the reaction mixtures was calibrated by assay

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in the presence of excess ornithine and enzyme. Stock 0.1 M L-ornithine was also kept frozen in aliquots until use. Distilled, deionized water of resistivity greater than 12 MΩ-cm at 25 °C was used throughout this investigation.

Ornithine transcarbamoylase from the *argI* gene of *Escherichia coli* K12 was purified as described (Kuo et al., 1982). The AH–Sepharose 4B column employed previously was substituted with a Matrex Gel Blue A column (Amicon) equilibrated in 20 mM Tris–acetate buffer, pH 7.5. Proteins that did not chelate to the triazinyl dye ligands were first eluted with Tris buffer (50 mL) at room temperature. Ornithine transcarbamoylase was subsequently eluted by using a 100-mL linear gradient of 0–0.5 M potassium chloride in 20 mM Tris–acetate at pH 7.5. Homogeneous ornithine transcarbamoylase was indicated by a single band on a sodium dodecyl sulfate–polyacrylamide gel. The specific activity of the enzyme was identical with that of several other preparations obtained by the same procedure. Normally two genes (*argI* and *argF*) code for the ornithine transcarbamoylase trimer (Legrain et al., 1972). A deletion of the pro-*lac* region of the *E. coli* DNA assured that only the trimer from the *argI* gene product was obtained (Fortin et al., 1981; Kuo et al., 1982).

Methods

Enzyme Assays. Enzyme reactions were initiated with the addition of carbamoyl phosphate. The ionic strength of the buffer system was either 0.1 or 0.2, and the assay temperature was maintained within ±1 °C with a circulating water bath. We monitored the initial velocity of the enzymic reaction in the production of L-citrulline by use of the method of Pastra-Landis (1981); the optical density at 466 nm was measured with a Cary 210 spectrophotometer at ambient temperature. The sensitivity of this assay allowed the detection of as little as 2 μM citrulline. We varied the length of the reaction time between 2 and 5 min according to the velocity of the reaction and the amount of optical density produced for accurate data analysis. The quantity of enzyme used was kept low in all assays so that a sufficiently high ratio of [S]/[E] was maintained to ensure steady-state turnover. Under all circumstances, the rates collected were the initial velocities of the enzymic reaction. The details are described in legends of the figures.

pH Measurements and Titrations. An Orion Research Digital Ionalyzer (Model 601A) equipped with a GK 2123c Type VW electrode and accurate to ±0.005 pH unit was used for all pH measurements. Calibration was performed with buffer standards at pH 4.00, 7.00, and 10.00. Buffers were titrated to the desired pH with either acetic acid or potassium hydroxide. During pH adjustment, the temperature of the reaction mixtures was maintained constant to within ±1 °C inside a water bath.

The acid dissociation constants of L-ornithine were determined by titration with 0.05 M NaOH at ambient temperature. In this procedure, a small aliquot (15 μL) of 0.1 M HCl was added to an aqueous solution (2.985 mL) of 0.02 M ornithine, bringing the total concentration of H_3O^+ to 0.045 M. The resultant pH of this solution was 3.6. Titration was performed from pH 3.6 to pH 12 with a Radiometer TTT80 titrator that was coupled to an ABU80 autoburet, an M82 standard pH meter, and a REC80 servograph.

Data Analysis. Saturation kinetic data were fitted to the Michaelis–Menten equation iteratively on a VAX PDP11/780 computer using a nonlinear least-squares program, DEAP11, for the calculation of kinetic parameters. Because ornithine transcarbamoylase was found to be allosteric in the presence of Zn^{2+} at pH 8.3, we have also fitted the saturation data in the entire pH range investigated to the Hill equation in order to affirm that the enzyme without the metal is not cooperative at any pH. For the saturation curves of both ornithine and carbamoyl phosphate, the Hill coefficient was found to be unity at all pH values. In order to calculate the apparent pK_a values, we employed the BELL and HABELL algorithms of Cleland (1979) on the VAX PDP11/780 computer. The dissociation constants, K_i, of the substrate analogue L-norvaline were calculated from secondary plots by using the parameters k_{cat} and K_M obtained from ornithine saturation curves at three concentrations of the inhibitor.

RESULTS

Buffer System. The first step in a pH study is to find a suitable buffer system. A buffer system of constant ionic strength in which each component of the mixture buffers a different portion of a pH profile is preferable to a series of single-component buffers. The tribuffer diethanolamine/MES/*N*-ethylmorpholine (0.051 M/0.1 M/0.051 M) prescribed by Ellis & Morrison (1982) is suitable for our purpose. Ornithine transcarbamoylase is stable in the tribuffer. After incubation of the enzyme in this buffer system at 25 °C for 15 min between pH 6.0 and 10.5, the enzyme activity deviates less than 2% from the control when assayed at pH 8.5.

Enzyme activity at 0.5 pH unit above and below the pK_a of individual buffer components has also been monitored as a function of buffer concentration (0.01–0.2 M). Dixon plots of the initial velocity data obtained for each of the three buffers with both substrates held at subsaturating levels yield horizontal lines. The results show that components of the tribuffer neither inhibit nor activate ornithine transcarbamoylase. In the course of our search for a buffer system, we have found that ethanolamine and CAPS are both strong inhibitors of the enzyme.

The tribuffer system is designed to maintain constant ionic strength throughout its useful buffering pH range. By use of the program of Ellis & Morrison (1982), an ionic strength of 0.1 or 0.2 is found to deviate less than 3% in the pH 5–11 range, except for pH 7.0–7.3 where deviation approaches 6%. In the pH 8–9 range, the values of V_{max} and K_M are statistically identical for the enzymic reaction carried out in 0.1 and 0.2 ionic strength tribuffers (see legend of Figure 1).[1]

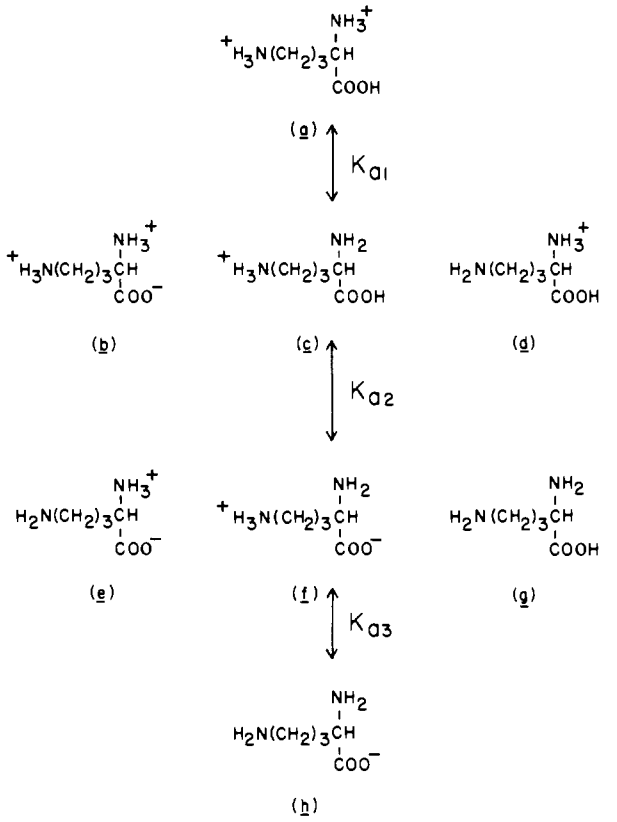
The combined results indicate that the activity of ornithine transcarbamoylase is unaffected by the buffer system and is a function only of pH, temperature, and inhibitor concentration.

Ionic Species of L-*Ornithine.* The dibasic L-ornithine exists in multiple forms in the pH 5–11 range, and their concentrations are pH dependent. Because these species may not all be substrates of the enzyme, it is necessary to consider them as separate entities in interpreting our kinetic results.

The *molecular* dissociation constants of L-ornithine, determined in H_2O at ambient temperature, are 8.68 and 10.78 with standard errors between 0.03 and 0.06 pH unit. These values are in complete agreement with those reported by Batchelder & Schmidt (1940). We have not determined the low pK_a value which is primarily associated with the ionization of the carboxyl group of the amino acid, inasmuch as this value

[1] We note here two typographical errors in our earlier paper (Kuo et al., 1982). The amount of enzyme used previously in kinetic assays is 5.75 ng, not 5.75 pg, and the V_{max} observed in 0.05 M Tris–acetate at pH 8.3 and 25 °C is 8.4 ± 10^2 mmol min^{-1} g^{-1}, not 8.4 × 10^8 mmol min^{-1} g^{-1}. With a molecular weight of 114 465 for the *E. coli* enzyme (Bencini et al., 1983), the correct specific activity is equivalent to 0.96 × 10^5 min^{-1}.

Scheme I



can be disregarded in estimating the relative concentration of the ionic species of ornithine above pH 5.

The successive dissociation steps of ornithine are shown in Scheme I. Because of the ease of ionization of the α-carboxyl group, the forms c, d, and g are present in negligible amounts at any pH. The total concentration of ornithine is then [a + b + e + f + h]. In the pH 5–11 range, ornithine exists predominantly in forms b, e, f, and h.

The fraction of zwitterionic ornithine, [e + f], can be calculated by using the following relationships:

$$\frac{K_{a1}}{[H]} = \frac{[b]}{[a]} \qquad \frac{K_{a2}}{[H]} = \frac{[e+f]}{[b]} \qquad \frac{K_{a3}}{[H]} = \frac{[h]}{[e+f]} \quad (1)$$

where K_{a1}, K_{a2}, and K_{a3} are the *molecular* dissociation constants shown in Scheme I. The ratio [e + f]/[a + b + e + f + h] is equal to

$$10^{pH-pK_{a2}}/(1 + 10^{pK_{a1}-pH} + 10^{pH-pK_{a2}} + 10^{2pH-pK_{a2}-pK_{a3}}) \quad (2)$$

For $pK_1 \ll$ pH, eq 2 is simplified to

$$10^{pH-pK_{a2}}/(1 + 10^{pH-pK_{a2}} + 10^{2pH-pK_{a2}-pK_{a3}}) \quad (3)$$

The populations of form e and form f cannot be determined from the relationships in eq 1, but their ratio can be estimated by using Greenstein's equation (1932) in which the *group* pK_a of the NH_3^+ group of an amino acid is given by

$$pK_{NH_3^+} = 10.72 - 0.9/D \quad (4)$$

In eq 4, D is the number of carbon atoms separating the dissociating amino group from the carboxyl group. For a dibasic amino acid such as ornithine or lysine, the effect of the second NH_2 group on the dissociating NH_3^+ group is small and can be neglected (Edsall, 1943). Hence

$$pK_e = 10.72 - 0.9/1 = 9.8$$

and

$$pK_f = 10.72 - 0.9/4 = 10.5$$

Table I: Kinetic Parameters of the Steady-State Reaction of *E. coli* Ornithine Transcarbamoylase in the Presence of L-Norvaline[a]

[norvaline] (mM)	k_{cat} ($\times 10^{-5}$ min^{-1})	K_M^{app} (mM)	K_M^{app}/k_{cat} ($\times 10^6$ mM/min)
	(A) Ornithine Saturation[b]		
0	1.07 ± 0.01	0.151 ± 0.006	1.41
0.2	1.04 ± 0.01	0.435 ± 0.015	4.18
0.4	1.01 ± 0.02	0.663 ± 0.024	6.56
0.8	0.98 ± 0.04	0.861 ± 0.021	8.79
	(B) Carbamoyl Phosphate Saturation[c]		
0	0.85 ± 0.09	0.121 ± 0.022	1.41
0.2	0.70 ± 0.07	0.097 ± 0.018	1.38
0.4	0.58 ± 0.05	0.079 ± 0.014	1.37
0.8	0.41 ± 0.02	0.057 ± 0.008	1.38

[a] Assays were carried out in duplicate as described (Kuo et al., 1982) at 25 °C in 0.05 M Tris–acetate, pH 8.3. [b] Carbamoyl phosphate was fixed at 2.4 mM; ornithine was varied between 0.1 and 6.0 mM. [c] Ornithine was fixed at 0.8 mM, and carbamoyl phosphate was varied between 15 and 180 μM.

The *group* dissociation constants K_e and K_f are related to each other by

$$K_e = [h][H]/[e] \quad (5a)$$

and

$$K_f = [h][H]/[f] \quad (5b)$$

It follows then that

$$[e]/[f] = 0.17/0.83 \quad (6)$$

This ratio is pH independent.

Using the relationships $K_g = [h][H]/[g]$ and $pK_g = 4.3$, we estimated the concentration of form g to be 1 part per 2×10^6 of the total ornithine population with zero net charge. The value of pK_g represents the ionization of a carboxyl group under the influence of an uncharged neighboring amino group in α-amino acids; the effect of the uncharged distal δ-NH_2 group is negligible (Edsall, 1943).

The effect of a carboxyl substituent in decreasing the basicity of an amino group has been documented by Edsall (1943). A primary amine is roughly 10^3 times stronger as a base than is an amino acid ester. As the distance between the amino group and the carboxyl group increases, the effect of the carboxylate diminishes. For a dibasic acid, the α-amino group is always a weaker base than is the distal amino group, but it is erroneous to treat the *molecular* pK_a values of 8.68 and 10.78 as *group* pK_a's by assigning them to the α- and δ-amino groups of ornithine.

pH Profiles of Ornithine Saturation. The steady-state kinetics of ornithine transcarbamoylase are sequential and ordered for the enzyme from bovine liver between pH 6.6 and 8.8 (Marshall & Cohen, 1972) and for the enzyme from *E. coli* W at pH 8.0 (Legrain & Stalon, 1976). The data in Table I indicate that this mechanism also holds for the enzyme from *E. coli* K12 (*arg F*$^-$). When ornithine is the variable substrate, k_{cat} remains unchanged, but K_M^{app}/k_{cat} increases as the concentration of norvaline increases. When carbamoyl phosphate is the variable substrate, k_{cat} decreases as the concentration of norvaline is raised while K_M^{app}/k_{cat} is a constant. Thus, novaline is competitive toward ornithine but uncompetitive toward carbamoyl phosphate. This inhibition pattern arises when ornithine is the second substrate bound in a sequential mechanism. However, the ordered mechanism has been questioned for the *E. coli* enzyme at low pH. Wargnies et al. (1978) have proposed that substrates at low concentration appear to bind to the enzyme from *E. coli* W randomly at pH 6.8, even though the reaction proceeds via an enzyme–car-

Table II: Apparent K_M of Carbamoyl Phosphate as a Function of pH in the *E. coli* Ornithine Transcarbamoylase Reaction[a]

pH	K_M^{app} (mM)	[ornithine] (mM) total[b]	[ornithine] (mM) zwitterions[c]
6.8	0.032 ± 0.004	147.50	1.56
7.0	0.028 ± 0.003	74.70	1.56
7.3	0.028 ± 0.002	38.20	1.56
7.7	0.040 ± 0.006	16.20	1.57
7.9	0.026 ± 0.004	8.90	1.57
8.2	0.035 ± 0.003	5.00	1.49
8.6	0.042 ± 0.002	3.05	1.57
9.5	0.136 ± 0.008	1.99	1.57
9.8	0.192 ± 0.015	1.86	1.58
10.0	0.334 ± 0.044	1.92	1.57

[a] Assays were carried out at 25 °C in the tribuffer system diethanolamine/MES/*N*-ethylmorpholine (0.051 M/0.1 M/0.051 M) as described in Figure 1. The concentration of carbamoyl phosphate was varied from 5.9 to 700 μM. [b] Total ornithine concentration added to reaction buffer. [c] Concentration of ornithine in zwitterionic forms (from eq 3). The ratio of the two zwitterions is insensitive to pH (see eq 6).

bamoyl phosphate binary complex when substrates reach their K_M levels. To ensure that only the sequential Bi-Bi mechanism is followed by the *E. coli* K12 enzyme at all pHs, carbamoyl phosphate must be present at a saturating level. This condition can be met easily by raising the concentration of carbamoyl phosphate in the reaction mixture. This substrate, unlike ornithine, does not introduce substrate inhibition.

We have demonstrated that 5 mM carbamoyl phosphate (CP) is sufficient to saturate the enzyme completely at all pHs. The results are shown in Table II. As the pH is raised above 9, K_M^{CP} also increases but remains less than 0.3 mM. The amount of ornithine used varies with pH so that its zwitterion concentration is kept constant at ~1.6 mM. The reason for this procedure will become clear later. We now present the k_{cat}, k_{cat}/K_M, and pK_i profiles of ornithine saturation as a function of pH.

(A) log k_{cat} Profile. The effect of pH on k_{cat} at 25 °C is shown in Figure 1. Since the concentration of carbamoyl phosphate used is 15–1500-fold in excess of its K_M value (Table II), the values of k_{cat} in Figure 1 can be considered as the maximal turnover rates of the enzyme when both substrates are at total saturation. The data are fitted iteratively to

$$k^{obsd} = k^{lim}/(1 + 10^{pK_a - pH}) \quad (7)$$

where $k = k_{cat}$. The apparent pK_a obtained is 7.3 ± 0.05. The limiting k_{cat} in the pH-independent region (pH > 9) is (0.91 ± 0.09) × 10^5 min^{-1}. We have repeated the kinetic experiments over the entire pH range 3 times at 25 °C. The apparent pK_a's obtained for individual data sets are within ±0.1 pH unit of each other.

(B) log (k_{cat}/K_M) Profile. The effect of pH on the apparent K_M values of ornithine is shown in Table III. As the pH is lowered, K_M^{app} drops gradually to a minimum around pH 9, and then it increases rapidly at pH < 7.5. The concentration of ornithine above which substrate inhibition becomes noticeable also increases as the pH is lowered (Table III).

From eq 3 and the *molecular* pK_a's of ornithine, it can be shown that over 93% of the amino acid is in the cationic b form at pH < 7.5. Thus, the abrupt increase in K_M^{app} at low pH suggests that ornithine with both its α-amino and its δ-amino groups charged may not be the substrate. For this reason, we have calculated[2] the K_M values for the zwitterions of ornithine,

[2] This analysis assumes that the pK_a's of L-ornithine do not change on binding.

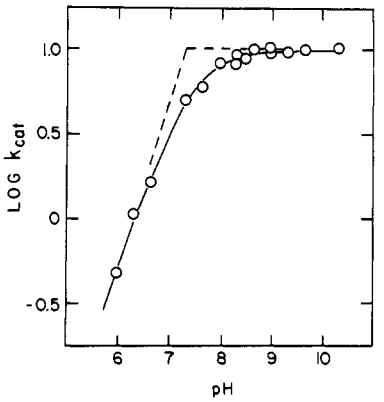


FIGURE 1: Effect of pH on the specific activity of *E. coli* ornithine transcarbamoylase. Each data point in the diagram represents the value derived from a complete ornithine saturation curve and has been divided by the computer-determined maximal (pH-independent) value of k_{cat}. The solid line is the theoretical line calculated from eq 7. The dashed lines are the asymptotes with slopes of 0 and +1. The best-fit value of pK_a is 7.3 ± 0.05, and the limiting k_{cat} is (0.91 ± 0.09) × 10^5 min^{-1}. Kinetic assays were conducted at 25 °C and an ionic strength of 0.1 in 1.0 mL of diethanolamine/MES/*N*-ethylmorpholine (0.051 M/0.1 M/0.051 M) containing 5.0 mM carbamoyl phosphate, 0.1–12 mM ornithine, and 5.22 ng of enzyme.[1] To compensate for loss of activity and increase in K_M^{app} at pH <7, the concentrations of both enzyme and ornithine in the reaction mixture were increased 10-fold. The pH of the reaction buffer was measured at 25 °C in the absence of carbamoyl phosphate shortly before assays. Preliminary runs indicated that the strength of the buffer was sufficient to maintain constant pH over the course of the experiment. The enzymic reaction was initiated by addition of 50 μL of 100 mM carbamoyl phosphate and quenched after 2–5 min with 1.0 mL of color mix as prescribed by Pastra-Landis et al. (1981). Under these conditions, steady-state turnover of substrates to products was maintained at all pHs, and the velocities monitored were initial rates. All data shown were collected in a single experiment.

Table III: Values of K_M^{app} and K_M^z for Ornithine Saturation in the *E. coli* Ornithine Transcarbamoylase Reaction[a]

pH	K_M^{app} (mM)	K_M^z (mM)	[ORN] (mM)[b]
5.7	29.60 ± 8.76	0.031	120
6.1	5.40 ± 0.47	0.013	100
6.7	2.71 ± 0.07	0.030	80
7.0	2.09 ± 0.13	0.048	30
7.4	0.50 ± 0.02	0.025	15
7.8	0.59 ± 0.01	0.064	9
8.1	0.47 ± 0.01	0.095	6
8.5	0.32 ± 0.01	0.120	6
8.9	0.23 ± 0.01	0.142	6
9.1	0.44 ± 0.02	0.307	6
9.7	0.56 ± 0.01	0.474	6
9.9	0.66 ± 0.02	0.551	6
10.1	1.49 ± 0.06	1.20	6
10.2	1.30 ± 0.05	1.01	6

[a] Assay conditions are described in the legend of Figure 1. K_M^z is the Michaelis constant of the forward reaction if only the zwitterions of L-ornithine are substrates of the enzyme. The values of K_M^z are calculated from K_M^{app} by using eq 3. [b] Total ornithine concentration above which mild inhibition due to this substrate is detectable in direct saturation plots.

K_M^z. The large increase in K_M^{app} at low pH is abolished. The values of K_M^z are also listed in Table III.

Figure 2 illustrates the effect of pH on k_{cat}/K_M^z at 25 °C. The data are fitted iteratively to

$$k^{obsd} = k^{lim}/(1 + 10^{pK_{a1} - pH} + 10^{pH - pK_{a2}}) \quad (8)$$

where $k = k_{cat}/K_M^z$. The pK_a's obtained are 6.2 ± 0.11 and 9.1 ± 0.06, reflecting, respectively, an enzyme deprotonation step and an enzyme protonation step. (Since K_M^z's are employed, ionizations uncovered in the profile cannot be due to ornithine.)

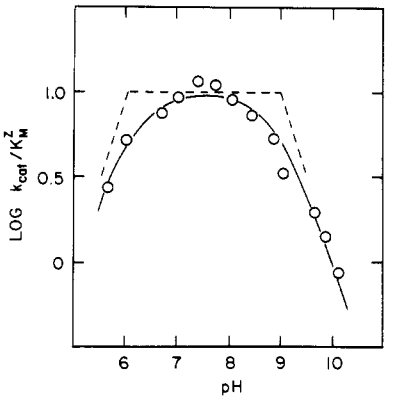


FIGURE 2: pH dependence of k_{cat}/K_M^z for the reaction catalyzed by *E. coli* ornithine transcarbamoylase at 25 °C. Each data point has been divided by the computer-determined limiting value of k_{cat}/K_M^z. The solid line is the theoretical line calculated from eq 8 by using the best-fit pK_a values of 6.2 ● 0.11 and 9.1 ± 0.06. The dashed lines show the asymptotic slopes of +1, 0, and −1. The limiting k_{cat}/K_M^z is (8.94 ± 0.65) × 10^8 M^{-1} min^{-1} at pH 7.55. Assay conditions are given in Figure 1.

If only one of the zwitterionic species is used in the calculation, the profile is displaced vertically along the ordinate but its shape remains unaltered, because the ratio of the two zwitterions, [e]/[f], is pH independent (eq 6). For the same reason, the data in Figure 2 alone cannot discriminate whether one or both zwitterions of ornithine bind to the enzyme.

We have also plotted log (k_{cat}/K_M^{app}) against pH (figure not shown). The resultant profile is also a bell-shaped curve. When theoretical lines generated for two or more ionizations are superimposed onto the data, both the ascending and the descending limbs of the curve can only be described by two consecutive ionizations. The result reveals that four ionizations are involved in the log (k_{cat}/K_M^{app}) profile when all ionic species of ornithine are treated as substrate. The pK_a's obtained by visual inspection using theoretical lines computed for consecutive ionizations are approximately 6.1 and 8.5 for the ascending limb and 9.5 and 10.6 for the descending limb. This finding supports the interpretation of Table III and Figure 2 that two of the ionizations belong to groups on ornithine and the other two on the enzyme. A more accurate estimate of the pK_a's in this profile cannot be obtained since extensive data are needed to distinguish regions with asymptotic slopes of ±1 and ±2.

(C) pK_i Profile. The *molecular* pK_a's of L-norvaline are 2.4 and 9.7. Among the four ionic species of the amino acid, cationic norvaline is present in negligible amounts at high pH (<0.025% at pH ≥6). Using pK_a's of 4.3 and 9.8 for the α-carboxyl (Edsall, 1943) and α-amino groups, respectively, we can estimate the ratio of the uncharged species to the zwitterion in the same manner as shown for ornithine (see Ionic Species of L-Ornithine) and found it to be approximately 1 to 316 000 at any pH. Hence, both the cationic and the uncharged species of norvaline can be neglected in the pH 6–10 range, and the inhibitor exists as zwitterion and anion.

The K_i values of norvaline at various pHs are presented in Table IV. In calculating the apparent inhibition constant, we have explored two possibilities: (1) only the zwitterion binds to ornithine transcarbamoylase, and (2) both the zwitterion and anion bind to the enzyme. Under the first assumption, the inhibition constants correspond to the K_i values corrected for the fraction of norvaline that exists as zwitterion. Within pH 6–8, these data are equivalent to the experimental observed K_i with respect to the total norvaline concentration since the concentration of anion is negligible in this region.

Table IV: Inhibition Constant of L-Norvaline as a Function of pH in the *E. coli* Ornithine Transcarbamoylase Reaction[a]

pH	K_i^z (μM)[b]	K_i^{obsd} (μM)[c]
6.0	17.6	17.6
6.5	13.4	13.4
7.0	8.1	8.1
7.7	9.1	9.1
7.9	5.8	5.8
8.6	6.5	8.9
8.9	10.0	11.2
9.1	11.0	14.1
9.5	12.9	20.9
9.7	26.7	51.9

[a] Assay conditions are described in the legends of Figures 1 and 3. [b] Inhibition constants corrected for the fraction of norvaline that exists as zwitterion. [c] Experimentally determined inhibition constants with respect to the total norvaline concentration added.

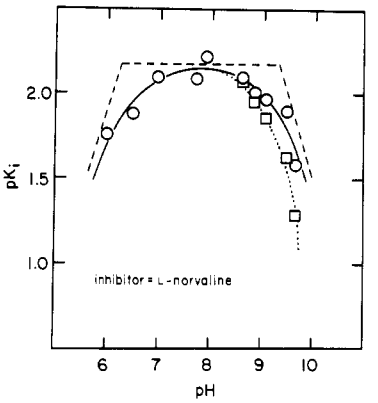


FIGURE 3: Effect of pH on the inhibition of *E. coli* ornithine transcarbamoylase by L-norvaline at 25 °C. (○) Dissociation inhibition constants of norvaline if only the zwitterionic form of the inhibitor binds the enzyme; (□) dissociation inhibition constants of norvaline when both the zwitterion and anion bind the enzyme (see Table IV and text for details). The solid line is drawn according to eq 9 by using the best-fit pK_{a1} and pK_{a2} values of 6.3 ± 0.16 and 9.3 ± 0.14, respectively. The dotted line is the theoretical line computed from eq 10 by using pK_{a2} = 9.3 (the high pK_a of the solid line in the diagram) and pK_{a3} = 9.72 (the high pK_a of norvaline in H_2O). The dashed lines are the asymptotes with slopes of −1, 0, and +1. The limiting K_i is 6.7 ± 0.82 μM at pH 7.8. At each pH shown, ornithine saturation curves were collected at three fixed levels of norvaline; the concentrations of norvaline used were 0–0.06 mM at pH 6–7, 0–0.1 mM at pH 7–8, and 0–0.2 mM at pH >8. To maintain a constant pH in the presence of both ornithine and norvaline, the ionic strength of the buffer was increased to 0.2 for assays above pH 9. Norvaline inhibition did not vary with ionic strength. Assays were otherwise conducted as described in Figure 1. The data shown were collected in a single experiment. Statistically unchanged values of pK_a's were obtained when this experiment was repeated twice.

Under the second assumption, the inhibition constants are the experimental observed K_i's with respect to the total norvaline concentration.

The pK_i profile of norvaline is shown in Figure 3. Two single ionizations are observed under assumption 1 (open circles). The data can be described by

$$K_i^z = K_i^{lim}(1 + 10^{pK_{a1}-pH} + 10^{pH-pK_{a2}}) \quad (9)$$

The values of pK_{a1} and pK_{a2} obtained from iterative fits are 6.3 ± 0.16 and 9.3 ± 0.14, statistically identical with those found in k_{cat}/K_M^z. These pK_a's are less accurately defined since every data point represents assays of three saturation curves each at a different norvaline level. Because only the zwitterion is included in this analysis, the ionizations belong to groups on the enzyme and not the analogue.

Under assumption 2, the values of pK_i^{obsd} at pH <8 are unchanged from those computed for the zwitterion. Above pH 8, the pK_i^{obsd} values (open squares) drop relative to those

Table V: Temperature Dependence of the Maximal Turnover Rate and Apparent pK_a's in the Forward Reaction of *E. coli* Ornithine Transcarbamoylase

	k_{cat} profile		k_{cat}/K_M^z profile	
temp (°C)	k_{cat} ($\times 10^{-5}$ min^{-1})	pK_a	pK_{a1}	pK_{a2}
19	0.56 ± 0.04	7.37 ± 0.06	6.72 ± 0.17	9.01 ± 0.16
25	0.91 ± 0.09	7.26 ± 0.05	6.17 ± 0.11	9.05 ± 0.04
32	1.12 ± 0.07	7.38 ± 0.05	5.95 ± 0.20	9.02 ± 0.09
41	1.72 ± 0.19	7.31 ± 0.09	5.73 ± 0.32	9.03 ± 0.21

obtained for the zwitterion. The high limb of the profile can no longer be described by a single ionization process, but the data in this limb agree nicely with a theoretical line calculated for two consecutive deprotonation steps with pK_a's of 9.3 and 9.7. The latter value is the high pK_a of norvaline (Edsall, 1943). The equation used to generate the theoretical line for the portion of the data above pH 8 is

$$K_i^{obsd} = K_i^{lim}(1 + 10^{pH-pK_{a'}} + 10^{2pH-pK_{a2}-pK_{a3}}) \quad (10)$$

where $K_{a'} = K_{a2} + K_{a3}$ and pK_{a2} and pK_{a3} refer to ionizations of the enzyme and norvaline, respectively. This finding indicates that if the anion is also included as an inhibitor, an additional ionization at pK_{a3} = 9.7 is necessary for binding.

Temperature Effects. The effect of temperature on the maximal (pH-independent) turnover number of ornithine transcarbamoylase, k_{cat}^{lim}, has been determined from the pH profiles of k_{cat} at 19, 25, 32, and 41 °C. Within this temperature range, the data appear to observe the Arrhenius rate law. The values of k_{cat}^{lim} are listed in Table V. The energy of action of the enzymic reaction is 8.79 kcal/mol from linear regression analysis.

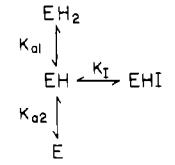
The dependence of the pK_a's obtained from pH profiles of k_{cat} and k_{cat}/K_M^z at four different temperatures is also analyzed. The results are shown in Table V. The enthalpy of ionization determined from a plot of pK_a vs. $1/T$ is 0.33 kcal/mol for the ionization observed in the k_{cat} profile (figure not shown). Enthalpy changes related to pK_{a1} and pK_{a2} in the k_{cat}/K_M^z profile are 17.69 and 0.31 kcal/mol, respectively.

Discussion

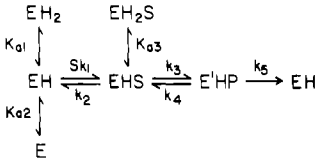
For transcarbamoylation, a lone pair of electrons is required on the δ-amino group of L-ornithine for nucleophilic attack. The reactive substrate is the one which has its δ-amino group uncharged. However, the species which binds the enzyme–carbamoyl phosphate binary complex need not initially be in the correct protonation state for reaction.

The rapid increase in the K_M^{app} of ornithine at low pH (Table III), as first pointed out by Snodgrass (1968), suggests that the cationic species is not a substrate. The remaining candidates are the zwitterions and the anion. Four ionizations are involved in the optimal binding of ornithine as revealed in the pH profile of k_{cat}/K_M^{app}, but only two are observed in the k_{cat}/K_M^z plot in which only the zwitterions are considered as substrate. The pK_a's obtained from the k_{cat}/K_M^z profile are 6.2 ± 0.11 and 9.1 ± 0.06, and they belong to ionization of groups on the enzyme. The simplest explanation for the two additional pK_a's seen in the k_{cat}/K_M^{app} profile is that they correspond to the pK_a's of ornithine and that they govern the substrate's ionization prior to its combination with the enzyme to form a productive reaction complex. In support of this interpretation is the observation that the same pK_a's (6.3 ± 0.16 and 9.3 ± 0.14) are seen in the pK_i profile of zwitterionic norvaline. Because this competitive inhibitor is structurally similar to ornithine, it binds tightly to the enzyme (K_i^{opt} = 7 μM); this finding indicates that the same enzymic groups must be involved in the binding of both amino acids in their zwitterionic forms. Furthermore, when the anionic form of norvaline is also included in the pK_i profile, an additional protonation process with a pK_a = 9.7 is necessary for optimal binding of the inhibitor (Figure 3). This pK_a corresponds to the high pK_a of norvaline. In other words, the anion must first be protonated, and thus, a positively charged α-amino group is a prerequisite for binding (norvaline has no δ-amino group). By analogy, it may be concluded that ornithine also binds with a protonated α-NH_3^+ group and that the δ-amino group of the substrate is, therefore, uncharged.

Scheme II



Scheme III



The zwitterion $H_2N(CH_2)_3CH(NH_3^+)COO^-$ constitutes only 17% of the ornithine population which has zero net charge. At pH ~7–8 where the enzyme is most efficient and the substrate exists in three ionic forms, of which the cation is the overwhelming majority (83–98%), our data are consistent with the interpretation that only the species of the lowest concentration binds productively. However, the stringent selection of this substrate form by the enzyme generates a Michaelis complex in which ornithine is poised for nucleophilic attack. Subsequent to such binding, deprotonation of the δ-NH_3^+ group is avoided and transcarbamoylation becomes energetically more feasible. The price paid is a much higher *apparent* K_M for the substrate.

The simplest model which describes the pH dependence of K_i of norvaline is depicted in Scheme II. The enzyme–carbamoyl phosphate complex is denoted by E and the zwitterion by I. Because EHI is at equilibrium in the steady state and the only enzyme forms are E, EH, and EH_2 under the conditions used to determine the apparent K_i, the inhibition constant is a simple thermodynamic dissociation constant. Thus, the pK_a's observed in Figure 3 correspond to ionization of enzymic groups *prior* to inhibitor binding. Neither EH_2I nor EI is detected between pH 6 and pH 10; their formation would have led to leveling off in the drop of pK_i at low and high pH (Cleland, 1977), and this effect is not seen in Figure 3.

We show in Scheme III the minimum scheme which accounts for the pH dependence of ornithine transcarbamoylase in the forward direction. The enzyme–CP and enzyme–P_i complexes are denoted by E and E′, respectively, and P represents citrulline. The model describes only steps from the binding of ornithine because carbamoyl phosphate is saturating at all pHs. Product release is treated in one step. The constants K_{a1} and K_{a2} correspond to the ionizations seen in k_{cat}/K_M^z, while K_{a3} corresponds to that observed in k_{cat}. The neutral substrate, a zwitterion which has an α-NH_3^+, combines productively with a single protonic form of the enzyme, EH. Because the same values are obtained for K_{a1} and K_{a2} from the pK_i profile of norvaline, the relationship of rate constants is $k_2 \gg k_3$, and thus, the substrate is *not* sticky. Nonproductive

binding of S to the unprotonated E is ruled out because only k_{cat}/K_M^z, but not k_{cat}, is sensitive to [H] at pH >8.5. A k_{cat} profile will fail to show the pK_a of a group that allows binding only when correctly protonated.

The assignment of pK_a's to free enzyme from the pH profile of k_{cat}/K_M is now established on two assumptions (Peller & Alberty, 1959; Knowles, 1976): (1) proton transfer to and from the free enzyme is much faster than the formation and breakdown of the Michaelis complex, and (2) productive binding occurs upon binding to a single protonation state of the enzyme. The proton equilibrium assumption is probably a reasonable one for most enzymes (Cornish-Bowden, 1976), but its validity has been questioned (Ottolenghi, 1971), and exceptions may exist. For Scheme III, this assumption is necessary *only* if EH_2 binds S. In such an event, the protonations represented by K_{a1} and K_{a2} are presumed to be fast and at equilibrium, but the rate of other steps, including those related to K_{a3}, need not be specified. We note that EH_2, however, does not bind I. Assumption 2, on the other hand, is unnecessary for nonsticky substrates (i.e., when $k_2 \gg k_3$), and the Alberty treatment is a good approximation. For several enzymes, however, this assumption is not strictly correct, and justification for its application is probably required in general [e.g., chymotrypsin (Renard & Fersht, 1973)]. Cleland (1977) has discussed the case in which both EHS and EH_2S can be converted to products. When this situation applies, the shape of the curves for both log V and log (V/K) against pH is a wave which has asymptotes of zero slope at low and high pH. This shape is not seen for ornithine transcarbamoylase.

The possibility of reverse protonation of the enzymic groups associated with pK_{a1} and pK_{a2} is ruled out. If the residue which has pK_a ~6 had to be protonated and that which has pK_a ~9 had to be deprotonated, only 0.1% of the total enzyme would be in the correct protonation state at pH ~7.5 to bind ornithine. Hence, the pseudo-first-order rate constant k_{cat}/K_M^z cannot be greater than 10^{-3} times the diffusion-limiting rate constant. At pH 7.55, k_{cat}/K_M^z is 1.5×10^7 M^{-1} s^{-1}. Therefore, a reverse-protonation scheme is not feasible. We may conclude that the protonation state of the free enzyme at pH 6–11 (strictly, the enzyme–CP complex) is adequately described by Scheme III.

Interpretation of the pK_a value from the pH dependence of k_{cat} is less straightforward. The constant K_{a3} may differ from K_{a1} because substrate binding changes the dissociation constant of the relevant group in the free enzyme or because the constants refer to different ionizations. Most often, the k_{cat} profile shows the effect of substrate binding on the pK_a of the enzyme. Since no covalent enzyme–substrate intermediate is formed and transcarbamoylation requires only concerted nucleophilic attack of ornithine, direct participation of a specific enzymic group in carbamoyl transfer is unlikely. However, an enzymic residue could be involved in the deprotonation of the secondary amine of the newly formed ureido group of citrulline. This step should not affect the overall rate. Finally, product release steps are often rate determining when the substrate is nonsticky and protonation of EHP to EH_2P may occur. The pK_a observed in the k_{cat} profile would then be a mean value weighted in the ratio of the steady-state concentration of EHS and EHP. Additional information by means of chemical modification or site-specific mutagenesis is necessary for a more definitive assignment.

The effect of temperature on the maximal catalytic rate shows that within the 19–41 °C range, k_{cat} obeys approximately the Arrhenius law (figure not shown). Since this parameter is a composite of individual rate constants of the entire reaction, the result suggests that the steady-state velocity is apparently dominated by one rate constant. The observed apparent energy of activation (E_{act}) is ~9 kcal/mol. Within the limited experimental temperature range, the entropy of activation ($\Delta S^{\ddagger}$) is −17 eu. We report these values of $E_{act.}$ and $\Delta S^{\ddagger}$ without interpretation because the rate-determining step(s) has (have) not been identified and because of the complication introduced by expected changes in solvent structure and possible conformational changes in the protein folding.

We have also calculated the enthalpy of ionization (ΔH_{ion}) associated with the pK_a's seen in k_{cat} and k_{cat}/K_M^z (Table V). Only the group associated with the acidic pK_a in the k_{cat}/K_M^z profile has a ΔH_{ion} significantly different from zero. The value of ΔH_{ion} is influenced not only by the chemical nature of the ionizing group but also by its environment. For an ionization process in aqueous solution, this parameter could be dominated by solvation effects that involve compensating changes in enthalpy and entropy. Assignment of specific amino acid residues to observed pK_a's with use of ΔH_{ion} is therefore unwarranted in the absence of additional information.

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Registry No. Ornithine transcarbamoylase, 9001-69-8; carbamoyl phosphate, 590-55-6; L-ornithine, 70-26-8; L-norvaline, 6600-40-4.

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Theoretical Studies on the Activation of the Pterin Cofactor in the Catalytic Mechanism of Dihydrofolate Reductase†

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ABSTRACT: Two mechanisms for facilitating hydride ion transfer from NADPH involving preprotonation of the pteridine rings of the dihydrofolate reductase substrates folate and dihydrofolate have been investigated by ab initio quantum mechanical methods. Protonation energies and effective solution pK_as have been calculated for four protonated forms, three of which are nonpreferred in aqueous solution and therefore not directly accessible to experimental study. The pattern and degree of redistribution of the positive charge over the component rings of the N-heterobicyclic π-system in these protonated forms have been analyzed in terms of changes in the electron populations of the ring atoms and total ring charges. The effects of such changes in promoting hydride ion transfer to C7 in folate and C6 in dihydrofolate have been evaluated by considering the extent of development of partial carbonium ion character at these carbon atoms and also the degree of electron deficiency in the pyrazine ring as a whole. The results illustrate that perturbations due, for instance, to protonation may be propagated by π-electron coupling effects over medium-range distances of 4–6 Å across the pteridine ring. The two mechanisms have been assessed in terms of the calculated absolute and relative pK_as of the protonated species taking into account experimental information regarding possible stabilization of these forms in the enzyme active site and also the effectiveness of the various protonations in assisting the hydride ion transfer step. Judged against these criteria, the theoretical results favor the generally proposed mechanism involving preprotonation of N8 in folate and N5 in dihydrofolate. However, some support was also found for the alternative novel mechanism involving O4-protonation of both folate and dihydrofolate.

The enzyme dihydrofolate reductase (DHFR) has been the subject of intensive investigation for over 25 years, the continuing interest being prompted mostly by its importance as the biological target for a large class of drugs—the "antifolates" (Blakley, 1969, 1981; Hitchings & Smith, 1979; Gready, 1980; Freisheim & Matthews, 1984). DHFR[1] (tetrahydrofolate:NADP$^+$ oxidoreductase, EC 1.5.1.3) catalyzes two reactions:

$$\text{folate} + 2\text{NADPH} + 2\text{H}^+ \rightarrow 5,6,7,8\text{-tetrahydrofolate} + 2\text{NADP}^+ \quad (1)$$

$$7,8\text{-dihydrofolate} + \text{NADPH} + \text{H}^+ \rightarrow \text{H}_4\text{folate} + \text{NADP}^+ \quad (2)$$

However, in mammalian cells at physiological pH the maximal rate of reaction 1 is, at most, a few percent that of reaction 2 while for some bacterial DHFRs reaction 1 is undetectable (Blakley, 1969). Most of the vertebrate enzymes have two pH optima for H_2folate reduction at ca. pH 4–5 and pH 7–8, while most bacterial DHFRs have a single acidic pH optimum (Blakley, 1969; Freisheim & Matthews, 1984). For folate reduction, a single pH optimum between ca. pH 4 and pH 5 has been reported for DHFRs from bacterial (Nixon & Blakley, 1968; Baccanari et al., 1975) and vertebrate (Mathews & Huennekens, 1963; Gupta et al., 1977; Smith et al., 1979) sources: at these low pH values folate-reducing activity may be 10–20% that for H_2folate. K_m values for H_2folate and NADPH are typically in the micromolar range while the enzyme turnover numbers are typically low (4–30 s^{-1}) (Blakley, 1969; Freisheim & Matthews, 1984), thus suggesting slow catalysis rather than poor substrate-binding affinity; although the K_m values for folate reduction are typically higher than

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[1] Abbreviations: pterin, 2-aminopteridin-4(3*H*)-one; DHFR, dihydrofolate reductase; folate, 6-[[[*p*-(L-glutamocarbonyl)phenyl]-amino]methyl]pterin ≡ pteroylglutamate; H_2folate, 7,8-dihydrofolate; H_4folate, 5,6,7,8-tetrahydrofolate.

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