Substrate Specificity and pH Dependence of Dipeptidases Purified from *Escherichia coli* B and from Mouse Ascites Tumor Cells†

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ABSTRACT: Differences in substrate specificity and pH dependence have been used to define properties of the active sites of soluble dipeptidases purified from ELD mouse ascites tumor cells and from *Escherichia coli* B. The enzymes hydrolyze only unsubstituted L-α-dipeptides; however, D-Leu-Gly and Gly-D-Leu are linear competitive inhibitors of L-Ala-Gly hydrolysis. Maximum velocities and Michaelis constants were determined at pH 8.3 for the hydrolysis of 50 dipeptides. The tumor enzyme has the highest maximum velocities with substrates containing uncharged bulky C-terminal and small, uncharged N-terminal side chains, while the bacterial dipeptidase hydrolyzes most rapidly substrates with long or flat N-terminal R groups and small or positively charged C-terminal groups. With the best substrates (Ala-Ile, tumor enzyme; Met-Ala, bacterial), the molecular activities of both enzymes are 2×10^4 sec^{-1}. The molecular weights are 85,000 ± 5000 and 100,000 ± 5000, respectively, and the bacterial enzyme contains two identical subunits. The enzymes differ in the charged groups related to activity; the pK values of the free enzymes being 8.0 and 8.2 (bacterial) and 7.3 and 8.8 (tumor). The pK's of the enzyme–Ala-Gly complexes are 6.7 and 9.1, and 7.2 and 9.4, respectively. It is clear that the active sites of the two dipeptidases must differ in the shape and hydrophobicity of the "pockets" by which side chains are bound, and in the nature or in the environment of proton donors and acceptors that take part in the reaction.

By a combination of crystallographic and classical enzymatic techniques, it is now possible to describe in considerable detail the active sites of several proteolytic enzymes (Hartsuck and Lipscomb, 1971; Blow, 1971; Hess, 1971; Robertus *et al.*, 1972) but little progress has been made on the dipeptidases, possibly because of difficulties in purification. Campbell *et al.* (1966) and René and Campbell (1969) have purified a particulate dipeptidase from log kidney and have described its substrate specificity, pH dependence, and Zn content. This enzyme clearly differs from the soluble dipeptidase that we (Hayman and Patterson, 1971) have purified from ELD mouse ascites tumor cells. In the present paper, the substrate specificity and pH dependence of another soluble dipeptidase purified from late log phase *E. coli* B, a prokaryotic source, are compared with the properties of the enzyme from ascites tumor cells, a eukaryotic source. These materials were chosen because rapidly growing cells have been shown to be high in Ala-Gly dipeptidase activity (Linderstrøm-Lang, 1952). Although both enzymes have similar high maximal molecular activities and molecular weights, their substrate specificities and pH dependencies differ in ways which permit preliminary conclusions to be drawn about features of their active sites. Future papers will deal with the ways the activities of these enzymes depend on intrinsic and extrinsic metal ions.

Materials and Methods

Materials. The peptides were purchased from Schwarz/Mann, Sigma, and Cyclo Co.; crystallized bovine serum albumin from Pentex; ammonium sulfate and sucrose, special enzyme grade, from Schwarz/Mann; DEAE-cellulose (microgranular DE-32) from Whatman; Sephadex G-150 from Pharmacia; and Bio-Gel HTP from Bio-Rad. Analytical grade chemicals and glass-distilled water (greater than 10^6 ohms resistence) were used for all solutions.

General Conditions. Precautions to reduce metal ion contamination were as outlined previously (Hayman and Patterson, 1971). In addition, a clean air hood was run continuously in the room where most of the operations were carried out.

Methods. The dipeptidases were routinely assayed during purification by measuring the amount of hydrolysis for 5 min at 40° of the substrate L-Ala-Gly (50 mM). The substrate solution was adjusted to pH 8.3 by addition of NaOH. The enzyme was diluted in 0.02 M phosphate buffer (pH 8.2), containing bovine serum albumin (2 mg/ml) to ensure stability during the incubation period. Seven microliters of 0.1 M Ala-Gly solution was added to 7 μl of enzyme solution and the reactions were run in the absence of extrinsic metal ions. The extent of hydrolysis was measured by a modification (Hayman and Patterson, 1971) of the ninhydrin method of Matheson and Tattrie (1964). A unit of enzyme activity is defined as 1 μmol of substrate hydrolyzed/min at 40°, and specific activity is defined as enzyme units/mg of protein. Protein was assayed by a modified (Hayman and Patterson, 1971) method of Nayyar and Glick (1954).

Michaelis constants and maximum velocities (30°) were determined (Hayman and Patterson, 1971) by means of an extrapolation of the linear portion of Lineweaver–Burk plots. The hydrolysis of L-α-dipeptides over a five–tenfold concentration range was followed by monitoring the loss of ultraviolet absorbance of the peptide bond (230 or 235 nm) with a modification of the method of Schmitt and Siebert (1961). The substrate solution (150 μl) was warmed to 30° in a microcuvette and 5 μl of enzyme solution rapidly stirred in. No metal ions were added. Absorbancy changes were recorded

† From The Institute for Cancer Research, Fox Chase, Philadelphia, Pennsylvania 19111. *Received March 9, 1973.* This research has been supported by U. S. Public Health Service Grants CA-03102, CA-06927, and RR-05539, and by an appropriation from the Commonwealth of Pennsylvania.

TABLE I: Purification of a Dipeptidase from *E. coli* B.

Fraction	Protein (mg)	Units	Sp Act. (Units/mg)	% yield
Streptomycin supernatant	5180	15,400	3.0	
30–50% $(NH_4)_2SO_4$	540	11,300	21	73
G-150 filtrate	225	9,090	40	59
DEAE-cellulose	31	5,700	184	37
DEAE-cellulose (fraction 51)	2.0	350	175	
Hydroxylapatite	0.24	183	760	

with a Gilford spectrophotometer using an expanded scale of 0–0.3. The substrate solutions were adjusted to pH 8.3 with Kolthoff phosphate–borate–NaOH buffers (Britton, 1942). Changes in absorbancy/min were converted to rates of hydrolysis by use of factors calculated for each substrate from the measured differences in extinction between the dipeptide and its constituent amino acids under the given experimental conditions. To allow for differences in enzymatic activity in separate experiments with various substrates, the rate of hydrolysis of a 12.5 mM Ala-Gly standard was measured routinely. Maximum velocities were calculated relative to that with Ala-Gly and converted to molecular activities [V_M/[E], mol of substrate hydrolyzed at 40°/sec per mol of enzyme] by use of previously determined molecular weights and specific activities. For these calculations, it was assumed that the highest specific activities (2600, tumor enzyme; 750, bacterial enzyme; Ala-Gly, 40°) obtained represented pure enzyme.

For the pH studies, the phosphate–borate–NaOH buffers were used to obtain the desired pH of Ala-Gly solutions which were measured at 25°. Spot checks of the reaction mixtures after hydrolysis confirmed that there was no significant change in pH. The extrapolated maximum velocities obtained from Lineweaver–Burk plots were expressed as the percentage ($V_{M'}$) of the maximum velocity at pH 8.3 as calculated from rates obtained with 12.5 mM Ala-Gly solutions.

Inhibition constants (K_I) and type of inhibition were determined by a two-step procedure. Ala-Gly at pH 8.3 was used as substrate. A preliminary estimate of the class of inhibition and K_I was made from Lineweaver–Burk plots in the presence and absence of an inhibitor. The linearity of the inhibition was studied by means of an experiment in which the inhibitor concentration [I] was varied from 0 to 4 times the estimated K_I, and velocities (v) were measured at two noninhibitory substrate concentrations (3.2 and 6.25 mM). From plots of $1/v$ *vs.* [I], the degree of linearity was assessed and K_I determined, if the plots were indeed linear (Cleland, 1970; Dixon and Webb, 1964), from the point of intersection of the two lines.

Purification of Dipeptidases. Procedures for the growth of ELD ascites tumor cells in HaIcr:ALB mice and purification of the dipeptidase from the soluble cell fraction have been reported previously (Hayman and Patterson, 1971). Starting material for the *E. coli* B purification consisted of an ammonium sulfate precipitated supernatant solution derived from streptomycin precipitation of an extract of late-log-phase *E. coli* B cells (Richardson *et al.*, 1964). This material was kindly contributed by Dr. Lawrence Loeb of this Institute. Since Simmonds (1970) showed that the activity of intracellular dipeptidases depended on the pH and metal ion content of the growth media, it should be noted that the bacteria were grown in a medium containing 1.1% K_2HPO_4, 0.85% KH_2PO_4, 0.6% Difco yeast extract, and 1% glucose. The purification steps (ammonium sulfate precipitation, Sephadex G-150 gel filtration, and DEAE-cellulose chromatography) were similar to those used for the tumor material with the following exceptions: the bacterial enzyme was precipitated between 30 and 50% ammonium sulfate, and a final step of hydroxylapatite chromatography was added. Table I gives the results of a purification in which in the final step a single fraction from the peak of enzyme activity eluted from DEAE-cellulose was concentrated against 0.01 M sodium phosphate (pH 7.5) and applied to a column of Bio-Gel HTP hydroxylapatite equilibrated with the above buffer solution. Elution was accomplished with a phosphate gradient. These steps resulted in a 250-fold purification from the streptomycin supernatant and 350-fold from the cell extract (specific activity 2.3). It was later observed that the hydrolysis of 50 mM Ala-Gly was stimulated by Mn^{2+} (S. Hayman, J. S. Gatmaitan, and E. K. Patterson, in preparation); in the presence of this metal, the highest specific activity was 4400, compared with the best value of 2600 for the tumor enzyme.

The bacterial dipeptidase having a specific activity of 4400 was judged to be 88% pure by planimetry of densitometric (650 nm) traces of Amido Black stained bands resulting from acrylamide gel electrophoresis at pH 3 in 2.5 M urea according to Panyim and Chalkley (1969). The sample was loaded in 8 M urea and 5% β-mercaptoethanol. There was one very heavy band and four very weak bands. When samples from all fractions in the peak of enzyme activity after hydroxylapatite chromatography were subjected to this procedure, the density of the principal band was roughly proportional to the enzyme activity in each fraction; therefore, the heavy band was assumed to be the dipeptidase. Subunit molecular weight determination was carried out by the method of Weber and Osborn (1969) with the modification that the sample was heated at 100° for 5 min in the presence of 8 M urea, 5% mercaptoethanol, and 1% sodium dodecyl sulfate. Six markers spanning the range of 17,500–130,000 in molecular weight were used on gels of 13.2 cm.

Results

Molecular Weight. By means of Sephadex G-150 gel filtration of samples of the *E. coli* B dipeptidase along with standard proteins, a molecular weight of 100,000 ± 5000 was obtained in two separate experiments. The method was identical with that used for the tumor enzyme for which 85,000 ± 5000 was determined (Hayman and Patterson, 1971). The bacterial enzyme moved as a single heavy band of 53,000 on sodium dodecyl sulfate acrylamide gel electrophoresis and therefore may be composed of two identical subunits. Unfortunately, the application of this technique to the purified (specific activity 2300) tumor dipeptidase resulted in multiple bands and left unsettled the question of whether or not subunits were present.

Stability. Both dipeptidases are stable to freezing at −30° in the presence of 0.02 M K_2PO_4 buffer, 0.25 M sucrose (pH 8.3), and stable to dialysis (18 hr) at 5° against 0.02 M phosphate buffer at pH 7.5 or 8.3. Solutions of both enzymes diluted with 2 mg/ml of bovine serum albumin, 0.02 M K_2PO_4 (pH 8.3), retained their full activity at 0° for 24 hr. However, when diluted without bovine serum albumin in phosphate–borate buffers, the two enzymes diverged in their stability at various pH's at 0°. The bacterial dipeptidase (0.4 mg/ml) retained 100% activity for 6 hr at pH's from 6.2 to 9.0, whereas

TABLE II: Comparison of Kinetic Constants[a] of the Tumor and *E. coli* B Dipeptidases for Hydrolysis of Neutral Dipeptides.

	Tumor Dipeptidase			*E. coli* Dipeptidase		
Dipeptide	V_M/[E] (sec^{-1}) ($\times 10^{-3}$)	K_m (mM)	V_M/K_m[E] (sec^{-1} mM^{-1}) ($\times 10^{-3}$)	V_M/[E] (sec^{-1}) ($\times 10^{-3}$)	K_m (mM)	V_M/K_m[E] (sec^{-1} mM^{-1}) ($\times 10^{-3}$)
Gly-Ile	6.5	5.7	1.1	0		0
-Nle	12	2.1	5.7	5.2	1.5	3.5
-Leu	7.1	2.5	2.8	0.91	2.4	0.38
-Val	8.3	5.1	1.6	0		0
-Abu	9.4	20	0.47	1.0	0.82	1.2
-Ala	3.5	22	0.16	3.2	0.88	3.6
-Ser	0.94	12	0.078	1.0	1.6	0.62
-Gly	0.30	24	0.012	4.1	15	0.27
Gly-Phe	3.6	1.7	2.1	0.84	0.50	1.7
-Tyr	4.7	4.3	1.1	0.60	0.43	1.4
Ala-Ile	23	1.6	14	0		0
-Nle	8.8	0.75	12	7.1	0.78	9.1
-Leu	3.0	0.28	11	2.2	0.86	2.6
-Nva	4.9	0.50	9.8	3.6	0.32	11
-Val	5.6	1.1	5.1	0		0
-Abu	8.3	1.3	6.4	3.1	0.35	8.8
-Ala	8.8	3.5	2.5	5.7	1.0	5.7
-Gly	5.9	3.4	1.7	11	3.3	3.3
Ala-Phe	3.0	0.59	5.1	3.0	0.45	6.7
Abu-Ile	9.4	0.88	11	0		0
-Ala	3.8	0.44	8.6	12	0.72	17
Val-Ile	7.7	1.0	7.7	0		0
-Leu	4.7	0.68	6.9	0		0
-Val	6.5	0.99	6.6	0		0
-Ala	3.0	0.45	6.7	3.3	0.30	11
-Gly	2.3	1.4	1.6	3.6	3.6	1.0
Leu-Phe	2.9	0.29	10	2.0	0.25	8.0
-Leu	3.1	0.25	12	0.1	0.3	0.3
-Val	2.8	0.44	6.4	0		0
-Ala	5.0	0.37	14	3.8	0.55	6.9
-Gly	1.8	0.33	5.4	4.3	2.0	2.2
Nle-Ala	2.4	0.41	5.8	3.1	0.21	15
-Gly	0.4	0.36	1.1	3.2	0.36	8.9
Ile-Ile	1.8	0.28	6.4	0		0
-Leu	2.9	0.57	5.1	0		0
-Val	3.5	0.45	7.8	0		0
-Ala	2.8	0.50	5.6	5.4	0.51	11
Phe-Ala	0.77	0.60	1.3	12	0.57	21
-Gly	0.22	4.4	0.050	11	1.6	6.9
Met-Leu	2.5	0.28	8.9	1.6	0.29	4.6
-Ala	3.0	0.61	4.9	16	0.88	18
-Gly	1.6	0.60	2.7	16	1.4	11

[a] The procedure for determining the kinetic constants is described under Methods. Values less than 1% of the V_M for Ala-Gly are listed as zero.

the tumor enzyme (0.07 mg/ml) lost 80% of its activity in 6 hr at pH 6.0 and 6.7 and 25–30% at pH 7.0 and 8.9, but was completely stable at pH 8.1 to 8.3. For the periods of reaction at 40° (5 min) and 30° (2–30 min), the enzymes at pH 8.3 remained stable. With longer periods at 30°, the bacterial (2.3 mg of protein/ml) and tumor (0.7 mg of protein/ml) dipeptidases in 0.02 M K_2PO_4 and 0.25 M sucrose (pH 8.3) slowly lost activity at equal rates, decreasing to 60% of the initial activity in 24 hr.

Effects of Thiols and SH Reagents. Neither dipeptidase appears to be a sulfhydryl enzyme. Mercaptoethanol, dithiothreitol, and cysteine (1.0 mM) inhibit both enzymes and the degree of inhibition is roughly parallel to the metal chelating ability of these reducing substances (Hayman and Patterson, 1971). Iodoacetate (1.0 mM) has no inhibitory effect on either dipeptidase even after 60-min prior incubation (25°) of the enzymes with this alkylator of SH groups. Iodosobenzoate (1.0 mM) gives a time-dependent inhibition that is similar for the tumor and bacterial dipeptidases. Prior incubation (25°) of the enzymes with this compound results in 20% inhibition

TABLE III: Comparison of Kinetic Constants[a] of the Tumor and *E. coli* B Dipeptidases for Hydrolysis of Charged Dipeptides.

	Tumor Dipeptidase			*E. coli* B Dipeptidase		
Dipeptide	V_M/[E] (sec^{-1}) ($\times 10^{-3}$)	K_m (mM)	V_M/K_m[E] (sec^{-1} mM^{-1}) ($\times 10^{-3}$)	V_M/[E] (sec^{-1}) ($\times 10^{-3}$)	K_m (mM)	V_M/K_m[E] (sec^{-1} mM^{-1}) ($\times 10^{-3}$)
Ala-Lys	1.6	1.4	1.1	15	1.2	12
Gly-Lys	0.48	5.6	0.086	11	2.0	5.5
Ala-Arg	0.5	0.7	0.7	2.0	1.2	1.7
Lys-Gly	0		0	0.1	2	0.05
Gly-Asn	2.6	29	0.090	1.6	1.8	0.89
Gly-Asp	0		0	1.3	2.4	0.54
Asp-Gly	0		0	0		0

[a] See footnote *a* of Table II.

of Ala-Gly (50 mM) hydrolysis after 30 min, 40% after 60 min, and 60% after 120 min, and the inhibition is not reversed by cysteine which itself is an inhibitor. Therefore, the slow iodosobenzoate inhibition may be caused by oxidation of a group in the enzyme other than cysteine.

Substrate Specificities of the Two Enzymes. Both enzymes are true dipeptidases in that they hydrolyze only L-α-dipeptides with both a free amino and a free carboxyl group. MeGly-Ala and Pro-Gly are hydrolyzed at less than 1% of the rate of hydrolysis of Ala-Gly. Although neither enzyme hydrolyzes Gly-D-Leu, D-Ala-Gly, D-Leu-Gly, β-Ala-Gly, L-leucinamide, Z-Gly-L-Leu, L-Leu-Gly-Gly, or L-Met-L-Leu-Gly, some of these compounds are competitive inhibitors of the dipeptidases (*vide infra*). The kinetic constants for the hydrolysis of a variety of neutral dipeptides by the two enzymes are listed in Table II. The substrates are grouped according to increasing size of the N-terminal residues (R_N)[1] with the C-terminal residues (R_C) in descending order of size or bulk within each group. From the V_M/[E] (molecular activity) data, it can be seen that the substrate preferences of the two enzymes differ markedly; the best substrate for the tumor enzyme is Ala-Ile, whereas Met-Ala and Met-Gly are hydrolyzed most rapidly by the bacterial enzyme. In general, good substrates for the tumor enzyme have small R_N groups and long hydrophobic R_C groups although this enzyme also hydrolyzes a variety of neutral peptides. On the other hand, the bacterial enzyme splits most rapidly dipeptides with long straight-chain or flat (Phe) R_N groups and small or straight-chain R_C groups, and barely hydrolyzes dipeptides having R_C -Val or -Ile. Many dipeptides are split by both enzymes at comparable rates.

Table III lists kinetic data for hydrolysis of dipeptides with acidic and basic residues by the tumor and bacterial dipeptidases. While the *E. coli* enzyme does not distinguish between C-terminal -Asp and -Asn, the tumor dipeptidase does not hydrolyze Gly-Asp and splits Gly-Asn at a moderate rate. Asp-Gly is not hydrolyzed by either enzyme, and Lys-Gly is not affected by the tumor dipeptidase and split very slowly by the bacterial enzyme, which rapidly hydrolyzes the reverse dipeptide, Gly-Lys. Ala-Arg is a much poorer substrate than Ala-Lys for both enzymes.

A few patterns may be discerned in the K_m data shown in Tables II and III. The K_m values for the *E. coli* B dipeptidase show less variation (15–0.2 mM) than those (29–0.3 mM) obtained for the tumor enzyme. The highest K_m values for the tumor enzyme are for N-terminal glycyl-dipeptides, with a range from 29 mM for Gly-Asn to 1.7 mM for Gly-Phe. The corresponding spread for the bacterial dipeptidase is 15 mM for Gly-Gly to 0.43 mM for Gly-Tyr. In general, the K_m values for most dipeptides are in the 1–2 mM range.

The ratio V_M/[E]K_m (in units of sec^{-1} mM^{-1}) is also included in Tables II and III to provide an indication of the rates at which the dipeptides would be hydrolyzed under the expected low physiological concentrations. Although the highest ratios are obtained for the best substrates, 14×10^3 for Ala-Ile with the tumor enzyme and 21×10^3 for Phe-Ala or 18×10^3 for Met-Ala with the *E. coli* enzyme, the low K_m values for some R_N Leu-, Nle-, or Ile- peptides are reflected in high ratios for both enzymes. Exceptionally low ratios are observed for the action of the tumor enzyme on some R_N Gly-dipeptides because of the high K_m values for these substrates.

Evidence That the Bacterial Dipeptidase Preparations Contain a Single Dipeptidase Activity. In the past, investigators have assumed (Smith, 1948, 1960) that different enzymes catalyzed the hydrolysis of Ala-Gly, Gly-Gly (Co^{2+} activated), and Pro-Gly (Mn^{2+} activated), but several investigators (Capobianco and Vescia, 1967; Cordonnier, 1966) have more recently believed that a single enzyme might be responsible for all these reactions. As is the case with the tumor dipeptidase (Hayman and Patterson, 1971), the hydrolysis by the bacterial enzyme of Gly-Gly is activated by Co^{2+} and that of Pro-Gly by Mn^{2+}. These activities, however, were co-purified at a constant ratio to the activity toward Ala-Gly, and, moreover, all three activities, as well as those toward Met-Gly and Gly-Lys, decayed at the same rate at both 30 and 40°. Studies of metal activation of the hydrolysis of dipeptides by the dipeptidases will be discussed in a future paper (also see Hayman and Patterson, 1973).

Linear Competitive Inhibitors of L-Ala-Gly Hydrolysis. Since dipeptides containing D residues are not hydrolyzed by either dipeptidase, selected compounds were tested as inhibitors (Table IV). Both D-Leu-Gly and Gly-D-Leu are linear competitive inhibitors of the hydrolysis of L-Ala-Gly by both enzymes; the K_I of D-Leu-Gly is lower with the bacterial enzyme than with the tumor dipeptidase, whereas Gly-D-Leu has the same K_I with the two enzymes. The tumor enzyme is quite insensitive to inhibition by the product, L-alanine, which is an effective inhibitor of the bacterial enzyme.

[1] Abbreviations used are: R_C represents the C-terminal side chain and R_N represents the N-terminal side chain; Nle, norleucine; MeGly, *N*-methylglycine; Z, benzoyloxycarbonyl; Abu, 2-aminobutyric acid.

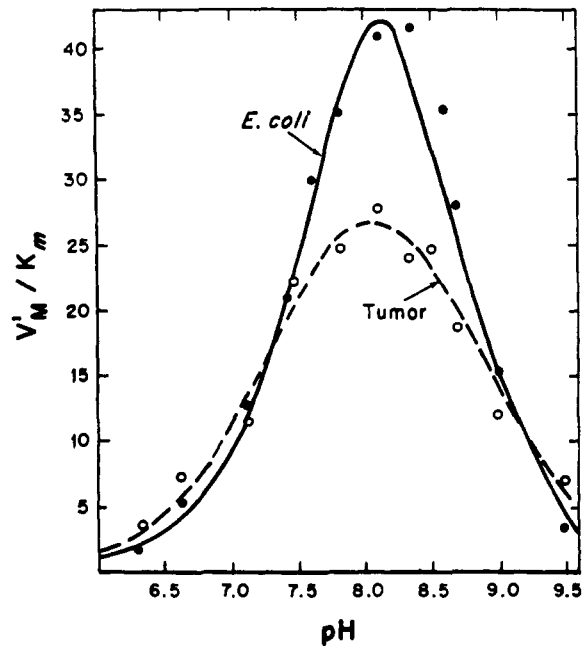

FIGURE 1: Plots of V_M'/K_m *vs.* pH for the hydrolysis of Ala-Gly by the ascites tumor (○) and *E. coli* (●) enzymes. The lines were calculated according to eq 1. The K_m values are millimolar. V_M' is expressed as the percentage of the maximum velocity at pH 8.3. V_M' and K_m were determined from Lineweaver–Burk plots at each pH, and V_M' was normalized to pH 8.3 by use of a standard assay.

Relationship of V_M' and K_m to pH. The fact that the tumor enzyme hydrolyzes Gly-Asn but not Gly-Asp whereas the bacterial dipeptidase makes no distinction between these two peptides implied that there might be differences in the ionizing groups in the active sites of the enzymes. In order to investigate this possibility, the V_M' and K_m values for the hydrolysis of Ala-Gly by the two dipeptidases were determined as a function of pH. Ala-Gly was chosen for these studies since this substrate is hydrolyzed at a relatively rapid rate by both enzymes. Plots of V_M'/K_m *vs.* pH for the tumor and bacterial enzymes are shown in Figure 1. The points were experimentally determined, and the lines were calculated by use of the following equation for a bell-shaped curve (Alberty, 1956).

$$\frac{V_M'}{K_m} = \frac{V_{M,lim}'}{K_{m,lim}} \frac{1}{1 + [H^+]/K_{aE} + K_{bE}/[H^+]} \quad (1)$$

where $V_{M,lim}'$ is the limiting value for the maximal velocity, $K_{m,lim}$ is a limiting value for the Michaelis constant, and K_{aE} and K_{bE} are dissociation constants of acid and basic groups in the enzyme. The assumption that the ionizations of only two groups control the activity is implicit in this oversimplified treatment. The curves for the two enzymes are nearly superimposed at their bases, but the peak of the bacterial dipeptidase curve is much sharper. The calculated dissociation constants are listed in Table V.

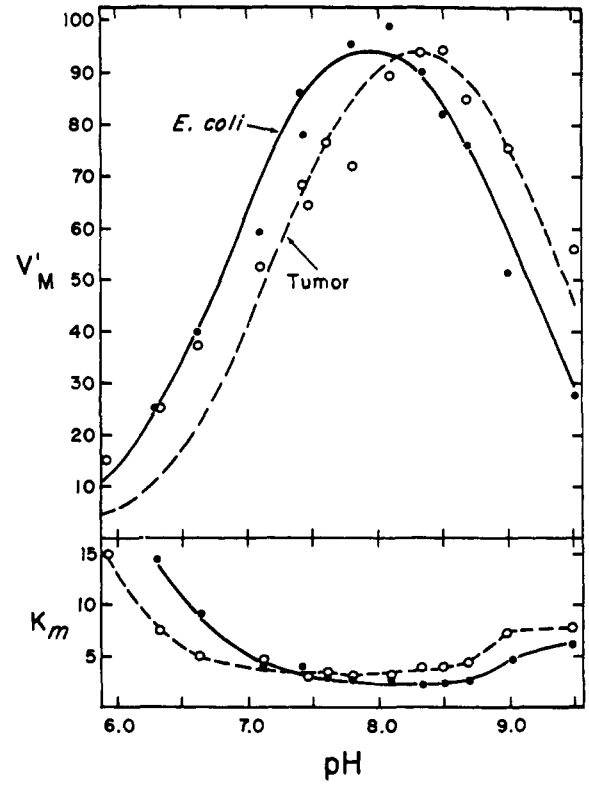

FIGURE 2: (upper portion) Plots of V_M' *vs.* pH for the tumor (○) and *E. coli* (●) enzymes. The lines were calculated according to eq 2. (lower portion) Plots of K_m *vs.* pH for the tumor (○) and *E. coli* (●) enzymes. For clarity the lines are drawn connecting the points rather than being calculated. The theoretical line for the *E. coli* enzyme fits the data reasonably well, but the line for the tumor enzyme is flat below pH 8 while fitting the higher pH points.

From plots of V_M' *vs.* pH (Figure 2), it is apparent that the optimum pH of the ascites enzyme is 8.3 and that of the bacterial enzyme, 8.0. The lines were drawn according to eq 2 (Alberty, 1956).

$$V_M' = V_{M,lim}' \frac{1}{1 + [H^+]/K_{aES} + K_{bES}/[H^+]} \quad (2)$$

where K_{aES} and K_{bES} are acid and basic dissociation constants of the enzyme–substrate complexes. Although the theoretical line fits the data for the bacterial enzyme reasonably well, the velocities of the tumor enzyme at low pH are higher than predicted by the equation. The calculated pK values are given in Table V.

TABLE IV: Linear Competitive Inhibitors of L-Ala-Gly Hydrolysis.

Inhibitor	K_I[a] Tumor (mM)	K_I[a] *E. coli* (mM)
D-Leu-Gly	2.5	0.35
Gly-D-Leu	9.0	9.0
L-Alanine	71	5.9

[a] The procedure for determination of K_I is described in Methods.

TABLE V: pK Values[a] of the Tumor and *E. coli* Dipeptidases.

Enzyme	pK_{aE}	pK_{bE}	pK_{aES}	pK_{bES}
Tumor	7.3	8.8	7.2	9.4
E. coli	8.0	8.2	6.7	9.1

[a] The substrate used was Ala-Gly. The listed pK values were used in the calculation of the curves shown in Figures 1 and 2.

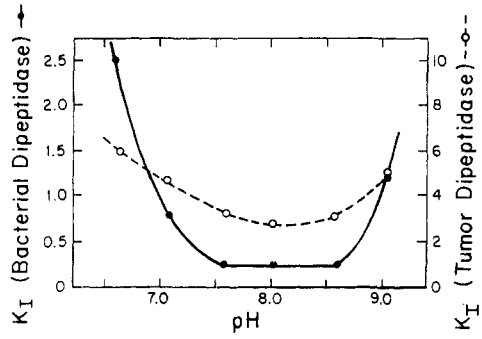

FIGURE 3: The variation of K_I (mM) of the competitive inhibitor D-Leu-Gly with pH for the hydrolysis of Ala-Gly by the bacterial (●) and tumor (○) dipeptidases. The K_I values were determined from Dixon plots in which the concentration of the competitive inhibitor, D-Leu-Gly, was varied over a fivefold range and the substrate, Ala-Gly, was used at two suitable concentrations. The lines are not theoretical.

Table V. The two enzymes have very similar values for the enzyme–substrate dissociation constants, those for the ascites tumor enzyme being 0.3–0.5 pH unit higher than those of the bacterial enzyme. The pK_{aE} of the tumor enzyme is unchanged when substrate is bound, whereas the pK_{bE} is raised 0.6 pH unit. In contrast, both pK's of the free bacterial enzyme are shifted an entire pH unit in the enzyme–substrate complex, the pK_{aE} to a lower pH and the pK_{bE} to a higher value. The Michaelis constants (Figure 2) for Ala-Gly hydrolysis by both enzymes have broad, flat minima, that for the tumor dipeptidase occurring between pH 6.7 and 8.7, and that for the bacterial enzyme between pH 7.1 and 8.7. When the K_I for D-Leu-Gly for Ala-Gly hydrolysis by the dipeptidases was studied as a function of pH (Figure 3), the minimum was also flat and lay between pH 7.6 and 8.6. The curve for the bacterial enzyme rose more sharply on the acid and basic sides of this pH range than that of K_m *vs.* pH, whereas the curve for the tumor enzyme was more similar to that of the K_m.

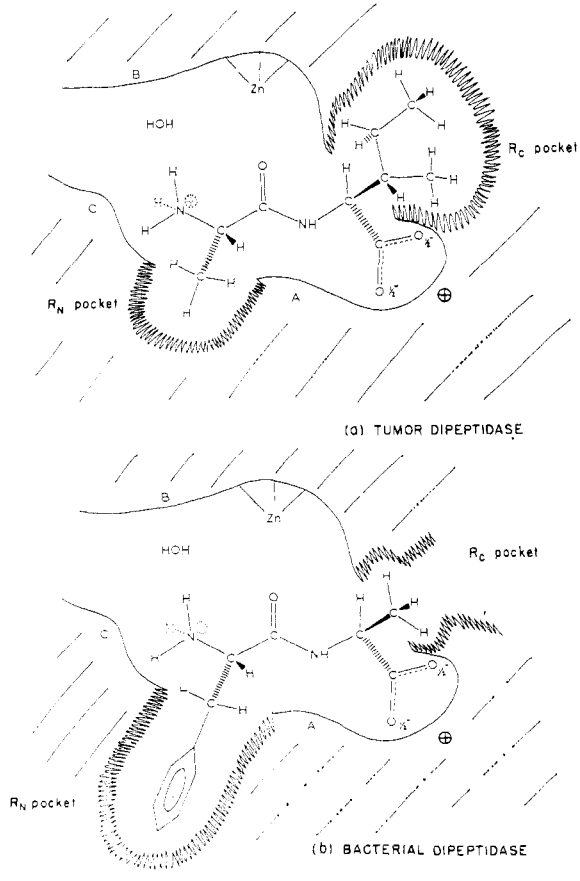

FIGURE 4: Proposed models for the structure of the active sites of the dipeptidases. (a) Tumor dipeptidase with Ala-Ile as substrate; (b) bacterial dipeptidase with Phe-Ala as substrate. The dotted H in the amino group indicates this hydrogen would be present 50% of the time at pH 8.3, the pH at which the reactions were carried out, and the approximate pK of the amino group. The designations of A, B, and C on the enzymes indicate residues with groups that may react with the amide, carbonyl, and amino groups, respectively, of the substrates.

TABLE VI: Comparison of Dipeptidase Active Sites.

Substrate

Enzyme	R Groups Which Fit R_N Pocket	pK_E of Ionizing Groups	R Groups Which Fit R_C Pocket
Tumor	Uncharged small	7.3, 8.8	Uncharged > ⊕ bulky, branched
E. coli	Uncharged long straight chain or planar	8.0, 8.2	⊕ charged ≥ neutral > ⊖ small or straight chain not β branched

Discussion

The active sites of dipeptidases represent a special case in the class of proteolytic enzymes because the substrates to be bound consist of L-α-dipeptides rather than a chain of residues requiring binding at subsites in addition to sites around the scissile peptide bond (Blow, 1971; Hartsuck and Lipscomb, 1971). Interpretation of the dipeptidase data, however, has been facilitated by consideration of the detailed kinetic (Vallee, 1964; Kaiser and Kaiser, 1972) and X-ray crystallographic (Lipscomb *et al.*, 1968; Hartsuck and Lipscomb, 1971) studies of carboxypeptidase A, which like the dipeptidases (Hayman and Patterson, 1971, 1973; S. Hayman, J. S. Gatmaitan and E. K. Patterson, in preparation) is an exopeptidase and a Zn-metalloenzyme.

Three types of attachment of substrates to the dipeptidase active sites can be proposed (Table VI and Figure 4): (1) hydrophobic interactions between pockets in the enzymes and the R_N and R_C groups of the substrate; (2) an electrostatic bond between a positively charged enzyme residue and the negatively charged carboxyl group of the substrate; and (3) possibly a hydrogen bond between a group on the enzyme and the amino nitrogen of the substrate. Once the substrate is bound, interactions between active site groups and the peptide bond of the substrate would promote catalysis. These suggestions will now be discussed in order.

The R_C and R_N groups of the substrates clearly have a marked effect on maximum velocities of hydrolysis by the dipeptidases (Tables II and III) but it is hard to discern a relationship between substrate structure and K_m, except in the case of the high K_m's for the hydrolysis of glycyl peptides by the tumor enzyme. Therefore, discussion of the effect of R

groups shall be confined primarily to V_M data. The substrate specificities of the two dipeptidases suggest that the R_C and R_N groups are held in the active site by two hydrophobic pockets (Table VI and Figure 4), similar to the one in carboxypeptidase A (Lipscomb *et al.*, 1968) which binds the C-terminal R group of the substrate. That the pockets differ in shape and hydrophobicity in the tumor and bacterial dipeptidases is apparent from the data. In general, the R_N pocket seems to be a positive determinant of reactivity for the bacterial enzyme and the R_C pocket for the tumor enzyme. The R_N pockets of both enzymes must be strongly hydrophobic because neither enzyme will hydrolyze dipeptides with polar R_N groups (Table III), but the shapes of these pockets (Figure 4) are dissimilar. As depicted in Figure 4, the R_N pocket of the bacterial enzyme is long and shallow, well-adapted to accommodating phenyl or methionyl residues, whereas the R_N pocket of the tumor enzyme is better suited to smaller groups, as is apparent from the following ranking of V_M values (Table II): Ala-Ile > Abu-Ile > Val-Ile $\gg$ Ile-Ile. However, since glycyl peptides have higher K_m's than do alanyl peptides with the tumor enzyme, the interaction of an R_N group with the R_N pocket is essential for good catalysis.

The substrate specificity data (Tables II and III) also indicate that the R_C pockets differ in the two enzymes. Since Ala-Ile is by far the best substrate for the tumor enzyme, its hydrophobic R_C pocket may be of such a shape (Figure 4) as to allow a precise fit for the isoleucyl R group, while also accommodating the straight chain norleucyl group. In contrast, the failure of the bacterial dipeptidase to hydrolyze dipeptides containing β-branched R_C groups implies that the entrance to its R_C pocket (Figure 4) may tend to be constricted; however, smaller groups such as the alanyl residue are well bound. The R_C pocket of the bacterial enzyme has been depicted in the figure as open-ended because of its ability to accommodate charged and long residues such as lysyl R-groups. The rate of hydrolysis of C-terminal -Lys compounds is enhanced for the bacterial enzyme and diminished for the tumor dipeptidase as compared to corresponding neutral R_C -Nle dipeptides. The inferiority of Ala-Arg to Ala-Lys as a substrate for both enzymes may be due either to the high pK of the guanidinium group or less probably to steric hindrance by this bulky group. Negative charge of the R_C group appears to have no effect on its affinity to the corresponding pocket of the bacterial enzyme (*cf.* Table III, Gly-Asn, Gly-Asp; Table II, Gly-Abu) but does prevent binding to the R_C pocket of the tumor enzyme.

The competitive inhibition (Table IV) by dipeptides with D residues is in accord with the hypothesis of pockets to bind R groups and steric specificity of the position of the amino and carboxyl groups in relation to the R groups. The lower K_I value (0.35 mM) for D-Leu-Gly found for the bacterial enzyme in comparison with that (2.5 mM) for the tumor enzyme may reflect the greater affinity of the R_N pocket of the former dipeptidase for large groups. The identical K_I values (9 mM) for inhibition by Gly-D-Leu may be related to the similar K_m values (2.5 and 2.4 mM) for hydrolysis of Gly-L-Leu by the tumor and bacterial enzymes. The high K_I for alanine for the tumor dipeptidase compared to the bacterial enzyme may mean that product is released more rapidly by the former enzyme.

It must be concluded from the requirement of these dipeptidases for substrates having unsubstituted amino and carboxyl groups in defined steric relationship, as evidenced by failure to hydrolyze β-Ala-Gly, tripeptides, and blocked dipeptides, that there exist in the active sites of the enzymes two groups in the proper geometric configuration to bind the amino and carboxyl groups of the substrates. The dipeptide carboxyl group, which has a pK of 3 (Cohn and Edsall, 1943), would be deprotonated above pH 5. Therefore, it is reasonable to propose that there are positively charged groups (designated $\oplus$ in Figure 4) in the enzymes' active sites which bind the negatively charged carboxyls of the substrates. The mechanism by which the amino group is bound to the enzyme is more difficult to define from the available data which are primarily the dependence of K_m and K_I on pH. Although K_I is the dissociation constant of the enzyme–inhibitor complex, K_m cannot be assumed to be a dissociation constant; the present studies do not permit any conclusions to be drawn about the values of the rate constants comprising K_m. The equation for the pH dependence of K_m is the quotient of eq 2 divided by eq 1, and the pH dependence of K_I is formally similar. Both equations contain the ionization constants of the free enzyme and those of either the enzyme–substrate or the enzyme–inhibitor complex, and the shapes of plots of K_m (Figure 2) or K_I (Figure 3) should be governed by these active site ionizations. These equations make no provision for possible ionizations of the substrate or inhibitor; in the cases illustrated in Figures 2 (lower) and 3, the only ionization that would occur is that of the amino group of the dipeptide (either substrate or inhibitor) with a pK of 8.2 (Cohn and Edsall, 1943). If the deprotonation of the amino group impeded binding, we might have expected to see a tenfold increase in K_m or K_I between 7.7 and 8.7. That the plots are flat in this region implies either that protonation of the amino group is not obligatory for interaction or less probably that there has occurred a fortuitous cancellation of effects because of the number of ionizations involved. The accuracy of the data for Figure 3 did not permit fitting a theoretical curve to the points, and therefore the ionization constants of the enzyme–inhibitor complexes could not be calculated. When pK_I was plotted *vs.* pH, the shapes of the curves (Webb, 1963) indicated two ionizations with pK's too close for accurate determination, but clearly different from 8.2, the pK of the dipeptides, and therefore these ionizations are attributable to groups on the enzyme. Consequently, we conclude that binding of the substrate or inhibitor is independent of protonation of their amino groups. That steric restriction in the active site may be the reason for the requirement for a free amino group is supported by the extremely slow hydrolysis of MeGly-Ala as compared to Gly-Ala, dipeptides of relatively similar amino group pK's. Since ionic bonding of the amino group to the enzyme has been shown to be unlikely, the nitrogen may be bound by hydrogen bonding to a group (labeled C in Figure 4), the ionization of which might be represented by one of the pK values measured (Table VI).

Although it is impossible to assign any definite role to the ionizing groups whose pK's have been measured, plausible interpretations of the pK_E values are that they represent: (1) catalytic groups interacting with the amide nitrogen and carbonyl carbon of the substrate (denoted A and B, respectively, in Figure 4); (2) groups responsible for maintaining the enzyme in active conformation; and/or (3) residues hydrogen bonding to the dipeptide amino group. The group on the enzyme that binds the carboxyl group would probably have a pK higher than 9; *e.g.*, in carboxypeptidase A (Lipscomb *et al.*, 1968), the free carboxyl of the substrate is bound to the guanidinium group (pK = 12.5) of Arg-145. Kinetic studies (Auld and Vallee, 1970) of the pH dependence of hydrolysis of peptide substrates by this enzyme have yielded pK values in the region from 6 to 9 which have been attributed to groups

interacting with the amide and carbonyl of the peptide bond. On the other hand, in chymotrypsin (Hess *et al.*, 1970) a basic pK is due to the ionization of a group responsible for maintaining the proper conformation of the enzyme. Thus, the dipeptidase ionizations which we measured could be involved in controlling catalysis, conformation or binding.

It is also impossible at this time to attribute the pK_E values to specific residues in the enzymatic active sites. Groups ionizing in the pH region between 7 and 9 include the α-amino and imidazole (Cohn and Edsall, 1943). Another candidate could be the dissociation of a water molecule bound to zinc (Kaiser and Kaiser, 1972). Moreover, Hass and Neurath (1971) have attributed a pK of 7.0 to Glu-270 in carboxypeptidase A_γ^{Leu}. The variation ($\pm$0.7) in pK values in the two enzymes may be due either to diversity in ionizing groups in their active sites or to pK shifts in identical groups caused by shielding by the protein chains in the rest of the enzyme molecule. The latter possibility is supported by the similarities in pK_{ES} values (Table V) for the two enzymes.

Comparison with dipeptidases purified by others emphasizes the diversity of these enzymes. Recently, Das and Radhakrishnan (1972) reported purification of a dipeptidase from monkey small intestine with a specific activity (substrate Gly-Leu, 12 mM, 37°, pH 7.7) of 4100 and a molecular weight of 107,000, values which are comparable to those found for the *E. coli* B dipeptidase. The intestinal enzyme has a somewhat lower pH optimum (7.75) than we found for the tumor (8.3) and bacterial (8.0) dipeptidases. Gly-Leu is hydrolyzed faster than Gly-Val or Gly-Ile and much faster than Ala-Leu or Leu-Gly. Gly-D-Leu and Gly-Gly are not split. This mammalian enzyme, therefore, has some resemblance to the tumor dipeptidase in substrate specificity. The major differences are the activation of the intestinal enzyme by 0.1 mM Zn and the necessity for stabilization by dithiothreitol. The Zn-metallodipeptidase highly purified by Campbell *et al.* (1966) from hog kidney cortex particles has a molecular weight of 47,200, a pH optimum of 7.6, and a pK_E of 7.4 (René and Campbell, 1969) similar to the tumor dipeptidase pK_{aE} value of 7.3. The substrate specificity of the kidney dipeptidase differs radically from that of the *E. coli* B and tumor dipeptidase in that peptides containing C-terminal D-amino acids are hydrolyzed. Dipeptidases purified from *E. coli* K-12 by Simmonds (1972) and from *E. coli* B by Brown (1973) differ from our *E. coli* B dipeptidase in their requirement for -SH stabilization by β-mercaptoethanol. All three bacterial enzymes rapidly hydrolyze N-terminal methionyl-dipeptides but it is difficult further to assess similarities among the substrate specificities of these enzymes since both Simmonds and Brown conducted their assays in the presence of Mn^{2+} ions which we find have differing effects, dependent on substrate and substrate concentration, on rates of hydrolysis by our bacterial dipeptidase (S. Hayman *et al.*, in preparation). The *E. coli* B dipeptidase M of Brown resembles our *E. coli* B enzyme in that the molecular weight is 94,000 and two subunits are present.

It must be noted that despite the prominent differences between our tumor and bacterial dipeptidases, these enzymes have many features in common. They have similar molecular weights and high molecular activities. Although the best substrates for the two enzymes are strikingly dissimilar, both enzymes hydrolyze Ala-Gly readily and also handle a great many other dipeptides with similar kinetic constants. Also, the contrasting effects of charged R_C groups on the activities of the two enzymes can be compared with the failure of both dipeptidases to split substrates with polar R_N groups. The mammalian and bacterial enzymes are affected in parallel ways by sulfhydryl reagents. Moreover, as will be shown in a future paper, both enzymes are zinc metalloenzymes.

It is thus evident, both from the work of others on dipeptidases and from the present study of the hydrolyses of 50 dipeptides by two dipeptidases of different evolutionary origins, that the steric configuration of the active sites of dipeptidases can differ while the rates of hydrolysis of given dipeptides are similar. The high molecular activities (2×10^4/sec) relative to other proteolytic enzymes (trypsin and chymotrypsin $1-2 \times 10^2$, Neurath and Schwert, 1950; carboxypeptidase $1-2 \times 10^2$, Auld and Vallee, 1970) may be attributed to rapid positioning of the small substrates for hydrolysis and faster dissociation of the products. The effect of intrinsic and extrinsic metal ions on dipeptide hydrolysis by the ascites and bacterial dipeptidases will be discussed in a forthcoming paper (S. Hayman, J. S. Gatmaitan and E. K. Patterson, in preparation).

Acknowledgment

We wish to thank Dr. Alfred Zweidler and Henry Miziorko of this Institute for carrying out the acrylamide gel electrophoreses.

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Choline Acetyltransferase. Evidence for an Acetyl-Enzyme Reaction Intermediate†

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ABSTRACT: Choline acetyltransferase (EC 2.3.1.6) catalyzes the following reversible reaction: acetyl-coenzyme A + choline ⇌ acetylcholine + coenzyme A. The partially purified bovine brain enzyme catalyzed the [^{14}C]choline–acetylcholine exchange reaction in the absence of detectable coenzyme A thereby providing indirect evidence for a covalent acetyl-enzyme intermediate. When the enzyme was incubated with [^{14}C]acetyl-coenzyme A or [^{14}C]acetyl[^{3}H]choline, and the reaction mixture was passed through a Sephadex G-50 column, ^{14}C label was associated with the protein in the eluent. This [^{14}C]acetyl-enzyme intermediate was chemically competent in that it reacted with choline or coenzyme A to give the corresponding acetyl derivative. The bond between the acetyl group and enzyme was not disrupted by hot trichloroacetic acid nor by 6 M guanidium-Cl treatment. The acetyl-enzyme bond was a thio ester by the following criteria: acid stability, cleavage by dilute alkali (pH 10), cleavage by 3 M salt-free hydroxylamine (pH 5.7) to give acetylhydroxamate and performic acid oxidizability. The choline acetyltransferase active-site sulfhydryl is most likely a cysteine SH since the enzyme did not contain detectable 4′-phosphopantetheine.

Acetylcholine is a neurotransmitter at the vertebrate neuromuscular junction and is a putative, but not proven, neurotransmitter in the mammalian central nervous system (*cf.* Iverson, 1970). Choline *O*-acetyltransferase (EC 2.3.1.6) catalyzes the bioformation of acetylcholine with the stoichiometry given in the following chemical equation: acetyl-coenzyme A + choline ⇌ acetylcholine + coenzyme A. The enzyme is adsorbed to membrane at low ionic strength and is readily desorbed at high ionic strength (Fonnum, 1968). The soluble bovine brain enzyme has an estimated molecular weight of 65,000 daltons determined by Sephadex gel filtration (Glover and Potter, 1971). Kinetic studies suggest a sequential mechanism in which acetyl-coenzyme A is probably the leading substrate (Potter *et al.*, 1968). White and Cavallito (1970) reported that [^{14}C]acetyl-coenzyme A formed a complex with crude bovine brain enzyme isolable by Sephadex G-100 gel filtration. They reported that this complex might represent a covalent [^{14}C]acetyl-enzyme or a noncovalent [^{14}C]acetyl-coenzyme A · enzyme complex.

More extensive mechanistic studies have been carried out with the hepatic arylamine *N*-acetyltransferase (EC 2.3.1.5). The results are consistent with the notion that acetyl-coenzyme A reacts with the enzyme to form an intermediate acetyl-enzyme and coenzyme A. Then the acetyl-enzyme reacts with the arylamine to form the *N*-acetylarylamine and the regenerated enzyme (Weber and Cohen, 1967; Steinberg *et al.*, 1971; Riddle and Jencks, 1971). Moreover, Jencks and coworkers (1972) suggest that the acetyl group forms a covalent thio ester intermediate with the enzyme.

The studies in the present paper support the hypothesis of an acetyl-enzyme intermediate in the choline acetyltransferase reaction. The occurrence of a [^{14}C]choline–acetylcholine exchange reaction provides indirect evidence for this notion. The isolation of an acetyl-enzyme intermediate by Sephadex gel filtration using labeled acetyl-coenzyme A or acetylcholine, and the demonstration of the chemical competence of this intermediate provides more direct evidence in support of the hypothesis. Finally, the acetyl-enzyme link exhibits the properties of a thio ester.

Experimental Section

Materials. [^{14}C]Acetyl-coenzyme A (50–60 Ci/mol), [^{14}C]-acetylcholine (4–5 Ci/mol), [^{3}H]acetylcholine (50 Ci/mol), [^{14}C]choline (6.2 Ci/mol), [^{3}H]choline (500 Ci/mol), and Liquifluor were obtained from New England Nuclear Corp.

† From the Department of Biochemistry, The University of Iowa, Iowa City, Iowa 52242. *Received April 11, 1973.* This work was supported by Grant GU-2591 of The National Science Foundation. A preliminary report of these experiments has appeared (Roskoski, 1973).