Amino Acid Esters of Phenols as Prodrugs: Synthesis and Stability of Glycine, β-Aspartic Acid, and α-Aspartic Acid Esters of p-Acetamidophenol

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Abstract □ pH–rate profiles were calculated for the hydrolysis of the glycine (II), β-aspartic acid (III), and α-aspartic acid (IV) esters of p-acetamidophenol (I) at 25° and μ = 1.0 *M*. The hydrolysis of esters II and III occurred predominantly *via* intermolecular reactions involving water, hydroxide ion, and the various ionic forms of the substrates. The hydrolysis of ester IV occurred predominantly *via* intramolecular reactions. The catalytic effects of formate, acetate, and phosphate ions as well as of tromethamine on the degradation on ester II were similar to their effects on the hydrolysis of ethyl dichloroacetate. The efficient catalysis of the hydrolysis of ester II in bicarbonate buffers was consistent with a mechanism that involved carbon dioxide and the formation and decomposition of a carbamate intermediate.

Keyphrases □ Prodrugs—of p-acetamidophenol, amino acid esters □ p-Acetamidophenol—amino acid esters as prodrugs, synthesis and analysis □ Phenols—amino acid esters as prodrugs, synthesis and analysis □ Amino acid esters—as prodrugs of p-acetamidophenol, synthesis and analysis

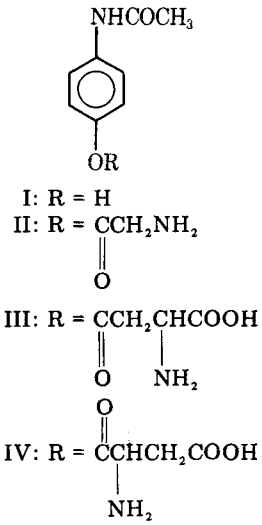
Amino acid esters of p-acetamidophenol (I) were proposed (1) as potentially useful prodrugs. Appropriate selection of the amino acid can yield derivatives having greater or less water solubility than p-acetamidophenol and a range of chemical stabilities with respect to transformation to the parent drug. When the amino acid is a normal dietary constituent, the transformation of the prodrug to I within the body will not yield a toxic byproduct.

Similar arguments can be used to support the investigation of amino acid esters of other phenolic drugs as prodrugs. This paper describes the synthesis and stability against degradation of the glycine (II), β-aspartic (III), and α-aspartic acid (IV) esters of I.

RESULTS

Characterization of Reactants—The elemental analysis and NMR spectrum of the glycine (II) derivative of p-acetamidophenol (I) were completely consistent with the conclusion that it was the hydrobromide salt of II. The I esters of L-aspartic acid had elemental analyses and NMR spectra consistent with the conclusion that they were the hydrochloride salts of the isomers III and IV. Only one isomer could be obtained pure. This isomer was concluded to be ester III rather than ester IV because its pKa value (2.04) was closer to that of β-ethyl aspartate (2.00) (2) than it was to that of α-ethyl aspartate (3.04) (2). Its pKa value also was very close to the first pKa values of aspartic acid (1.88) (3) and histidine (1.78) (3), two molecules in which the carboxyl group adjacent to an ammonium group is the first to ionize. The greater reactivity of IV made it difficult to isolate and characterize.

Decomposition of Esters II and III—Esters II and III decomposed by hydrolysis in formate, acetate, and phosphate buffers. Plots of the observed first-order rate constants at each pH value, k_{obs} values, *versus* the total concentration of buffer species, $[buffer]_T$, were linear over at least four buffer concentrations. Values of a buffer-independent first-order rate constant, k_{hyd} values, were calculated from the *y* intercepts, when $[buffer]_T = 0$, of these plots. Values of k_{hyd} are plotted *versus* pH in Fig. 1. Values of catalytic rate constants, k_{cat}, for the total buffer species were calculated in the case of hydrolysis of ester II from the slopes of the linear plots of k_{obs} *versus* $[buffer]_T$.



The relationship among k_{obs}, k_{hyd}, and k_{cat} values is given by:

$$k_{obs} = k_{hyd} + k_{cat}[buffer]_T \quad \text{(Eq. 1)}$$

Values of k_{cat} for decomposition of ester II are listed in Table I.

For phosphate catalysis, a plot of k_{cat}/f_{EH^+} *versus* f_b [where f_{EH^+} and f_b are the fraction of II that exists as its conjugate acid and the fraction of the buffer acid ($H_2PO_4^-$) that exists as its conjugate base (HPO_4^{2-}), respectively, for data at the various pH values] was essentially linear with a zero intercept when $f_b = 0$. Hence, the major reactions involved HPO_4^{2-} ions and the conjugate acid of II. On the assumption that the other re-

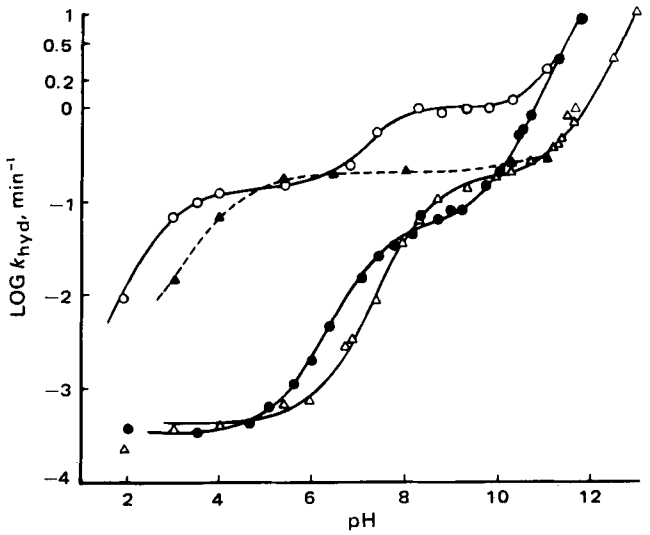


Figure 1—*Plots against pH of the logarithms of the buffer-independent pseudo-first-order rate constants,* k_{hyd}, *for the hydrolysis of esters II (●), III (△), and IV (○) and for the acid succinate of I (▲) in water at 25°,* μ *= 1.0 M. The lines were calculated as described in the text. The data for hydrolysis of the acid succinate of I were taken from Ref. 8.*

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Table I—Catalytic Rate Constants, k_{cat} (pH Dependent) and k_b (pH Independent), for Oxyanion- and Tromethamine-Catalyzed Hydrolysis of *p*-Acetamidophenyl Glycinate at 25°, μ = 1.0 *M*

Oxyanion	pH	f_b[a]	f_{EH^+}[b]	$10^3\,k_{cat}$, M^{-1} min^{-1}	$10^3\,k_b$, M^{-1} min^{-1}
Formate	3.53	0.50	1.000	1.02	2.04
Acetate	5.08	0.80	1.000	4.14	5.18
Phosphate	5.60	0.15	1.000	19.50	—
Phosphate	6.00	0.30	1.000	38.00	—
Phosphate	6.38	0.50	0.927	49.60	—
Phosphate	7.08	0.83	0.710	85.20	120.00
Tromethamine	7.44	0.16	0.52	333.6	—
Tromethamine	8.18	0.50	0.16	354.0	4102

[a] Fraction of the buffer that exists as HCO_3^-, CH_3COO^-, HPO_4^{2-}, or tromethamine. The pKa values used in the calculations were the pH values of half-neutralized solutions of the acids or conjugate acids and were: pKa^{HCO_3H}, 3.53; pKa^{CH_3COOH}, 4.68; $pKa^{H_2PO_4^-}$, 6.38; and $pKa^{tromethamine}$, 8.18. [b] Fraction of ester II that exists as the conjugate acid (pKa = 7.44).

actions were similar, values of a pH-independent base catalysis constant, k_b (which is equal to the value of the slope in the described type of plot), were calculated by dividing k_{cat} values by the term $f_{EH^+}f_b$ (Table I).

The decomposition of ester II in tromethamine and carbonate buffers also was studied. In tromethamine buffers (pH 7.44 and 8.18), plots of k_{obs} *versus* [buffer]$_T$ were linear, and k_{cat} values were calculated in the manner described for oxyanion catalysis. At pH 9.22, a plot of k_{obs} *versus* [buffer]$_T$ was paraboloid. This finding suggests that a reaction that was second order in tromethamine concentrations became important at higher pH. The reactions at higher pH were not studied further because they are unlikely to be important in biological systems.

The pH dependence of k_{cat} values also was such that a plot of k_{cat}/f_{EH^+} *versus* f_b was linear with a zero intercept when $f_b = 0$. The value of the pH-independent rate constant, k_b, calculated from the slope of this line is given in Table I. This value is only approximate since k_{cat} values were measured at two pH values only.

The hydrolysis of ester II in pH 6.00–8.85 buffers that contained various amounts of sodium bicarbonate was first order in sodium bicarbonate concentration. Phosphate buffers were used between pH 6 and 7, and tromethamine buffers were used at higher pH values. Plots of k_{obs} for these reactions *versus* sodium bicarbonate concentration (up to 10^{-2} *M*) were linear, and the k_{cat} values calculated from them are shown in Table II. At pH 6.0, this type of plot was linear up to 4×10^{-3} *M* $NaHCO_3$. At higher concentrations, k_{obs} values were independent of the sodium bicarbonate concentration. This behavior probably was caused by the fact that solutions at the higher concentrations were saturated with carbon dioxide.

Decomposition of Ester IV—The hydrolysis rate of ester IV was independent of the nature or composition of the buffer in all of the experiments. Hence, k_{obs} values were concluded to be k_{hyd} values (Fig. 1).

DISCUSSION

The results of this study provide information on the stability *versus* degradation of three potentially useful prodrugs of *p*-acetamidophenol (I). However, analysis of the results also provides information about the effect of substituents in the acyl portion of phenolic esters on their hydrolysis rates. Such information can be used to design esters of other phenolic drugs as prodrugs.

Mechanism of Water- and Hydroxide-Ion-Catalyzed Hydrolysis of Esters II–IV—The line for ester II in Fig. 1 was calculated on the basis that the major reactions involved in its hydrolysis at pH 2–13 are:

$$EH^+ + H_2O \xrightarrow{k_A^{II}}$$
$$EH^+ + OH^- \xrightarrow{k_B^{II}}$$
$$E + OH^- \xrightarrow{k_C^{II}}$$

where E represents the ester neutral molecule and EH^+ is its conjugate acid in which the amino group is protonated. The rate law for these reactions is:

$$\frac{-d[E]_T}{dt} = k_{hyd}[E]_T = k_A^{II}[EH^+][H_2O] + k_B^{II}[EH^+][OH^-] + k_C^{II}[E][OH^-] \quad \text{(Eq. 2)}$$

where $[E]_T$ is the total concentration of ester at time t. The acid dissociation constant of the conjugate acid of ester II was determined to be 3.63×10^{-8} at 25° and μ = 1.0 *M*. The values of k_A^{II} (the water concentration was assumed to be 55.5 *M* in the computation of this second-order rate constant), k_B^{II}, and k_C^{II} used to generate the line in Fig. 1 are included in Table III.

Table II—Effect of pH on the Carbon Dioxide-Facilitated Decomposition of Ester II, μ = 1.0 *M*, 25°

pH	k_{cat}, M^{-1} min^{-1}	f_E[a]	f_{CO_2}[b]	$k_{cat}/f_E f_{CO_2}$, M^{-1} min^{-1}
8.85	10.20	0.960	0.003	2951
8.60	9.60	0.940	0.006	1421
7.44	23.07	0.570	0.084	549.3
7.00	20.07	0.270	0.200	371.7
6.75	9.58	0.170	0.310	189.8
6.00	0.96	0.035	0.715	38.4

[a] Fraction of II that exists as a neutral molecule; pKa = 7.44. [b] Fraction of added sodium bicarbonate that exists as carbon dioxide or carbonic acid = 6.4.

The reaction pathway for the hydrolysis of II that is depicted as $EH^+ + OH^-$ is believed to predominate over the isokinetic pathway $E + H_2O$. This conclusion is based on the previously reported observation (4) that the second-order rate constant for the reaction between hydroxide ion and the methylbetaine methyl ester is virtually identical to that calculated for the reaction between OH^- and the protonated glycine methyl ester. Only the latter compound could alternatively or concurrently be hydrolyzing by the isokinetic reaction; water catalyzed reaction of the neutral molecule. The similarity in the values of the second-order rate constants for the hydroxide-ion reaction strongly suggests that both compounds hydrolyze by the same mechanism.

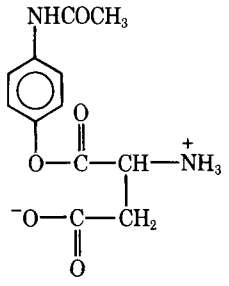
Further support for this conclusion comes from the fact that the value of the ratio for ester II, k_B^{II}/k_A^{II} (3×10^{10}) is of the same order of magnitude as the ratio of the rate constants (5) for the specific base- and water-catalyzed hydrolysis of phenyl acetate (10^{10}). The leaving group tendencies of phenol (pKa 9.86) and I (pKa 9.49) are expected to be similar; therefore, the hydrolytic behavior of phenyl acetate appears to be a good model for the hydrolyses studied here. Since the ratio of k_B^{II}/k_A^{II} for ester II is three times larger than the equivalent ratio for phenyl acetate, the presence of the positive charge in the cation of II probably favors hydroxide-ion attack more than it does water attack when these reactions are compared to the same reactions involving neutral phenyl acetate. Previous investigators (6, 7) observed a similar deviation when comparing the ratio of hydroxide-ion- and water-catalyzed hydrolysis rate constants for a series of α-substituted *o*-nitrophenyl esters. They concluded that the enhancement for a reaction between hydroxide ion and a cationic ester could be accounted for better by postulating favorable electrostatic interactions in the transition state rather than by invoking electrostatic effects on collision frequency.

Comparison of the rate constants for the reaction between the neutral molecule of II and hydroxide ion (k_C^{II}) and between hydroxide ion and the acetate of I (8) indicates that the introduction of an amino group into the acetate (*i.e.*, an α-amino group in the acyl moiety) increased the rate of this reaction 4.6 times. This figure is close to the fivefold increase in rate that follows the introduction of an amino group in the α-position of ethyl acetate (4). Protonation of the amino group in ester II produced a further 100-fold increase in rate. Hence, the introduction of the $^+NH_3$ group into the acyl moiety of the acetate of I produced a 500-fold increase in the magnitude of the hydroxide-ion catalysis constant. This figure is considerably larger than that (150) found (9) for the introduction of $^+NH_3$ into the acyl portion of ethyl acetate. The difference in magnitude of these two figures is consistent with the generally accepted postulate that the rate of formation of a tetrahedral intermediate is the rate-determining step in the hydroxide-ion-catalyzed hydrolysis of phenolic esters (10), whereas the rate of breakdown of a tetrahedral intermediate also is in-

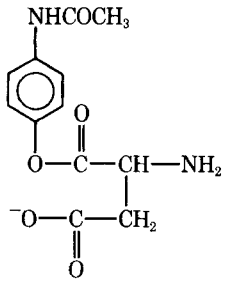
Table III—Values of Microscopic Rate Constants [a] for Hydrolysis of Esters II and III in Aqueous Solutions at 25°, μ = 1.0 *M*

Compound	$10^6\,k_A$[b], M^{-1} min^{-1}	$10^{-4}\,k_B$[c], M^{-1} min^{-1}	$10^{-2}\,k_C$[d], M^{-1} min^{-1}
II	6.10 (±0.52)	18.70 (±1.10)	14.40 (±0.50)
III	7.75 (±0.65)	3.97 (±0.33)	1.00 (±0.08)

[a] Calculated by a curve-fitting technique from the data in Fig. 1. [b] For the reactions $EH^+ + H_2O \rightarrow$ in the case of ester II and $EH^\pm + H_2O \rightarrow$ in the case of ester III. [c] For the reactions $EH^+ + OH^- \rightarrow$ in the case of ester II and $EH^\pm + OH^- \rightarrow$ in the case of ester III. [d] For the reactions $E + OH^- \rightarrow$ in the case of ester II and $E^- + OH^- \rightarrow$ in the case of ester III.



IV*a*



IV*b*

volved in determining the hydrolysis rate of esters of more basic alcohols (11). Hence, a substituent such as $^+NH_3$, which acts primarily by stabilizing the transition state for tetrahedral intermediate formation (6, 7), should have a greater effect on the hydrolysis rate of phenolic esters than on esters of aliphatic alcohols.

The line for ester IV in Fig. 1 was calculated on the basis that the major reactions of the α-aspartic acid ester (IV) between pH 1.5 and 11.0 were:

$$EH^{\pm} \xrightarrow{k_A^{IV}}$$
$$E^- \xrightarrow{k_B^{IV}}$$

where E^- and $EH^{\pm}$ are the zwitterion and anion of the ester, respectively. The rate law for these reactions is:

$$\frac{-d[E]_T}{dt} = k_{hyd}[E]_T = k_A^{IV}[EH^{\pm}] + k_B^{IV}[E^-] \qquad \text{(Eq. 3)}$$

The acid dissociation constants of ester IV could not be measured because of its extreme instability in water. However, it was expected that the values would be close to those of α-ethyl aspartic acid (2), *i.e.*, 9.12×10^{-4} (for the cation ionizing to yield $EH^{\pm}$) and 3.16×10^{-8} (for $EH^{\pm} \rightleftharpoons E^- + H^+$). The values of k_A^{IV} and k_B^{IV} that were used to calculate the curve in Fig. 1 are listed in Table IV.

The pH–rate profile for the hydrolysis of ester IV is markedly different from that of the esters II and III. At pH values slightly above the pKa of the carboxyl group of IV (~3.0), the decomposition rate of IV surpassed that of its isomer, III, almost 900 times. Also, the decomposition rate of IV was not catalyzed by any buffer species used in this study, and it was pH independent when the concentration of the zwitterion or the anion of IV was essentially constant. These phenomena can be accounted for best by postulating that the predominant reactions were intramolecular carboxylate-ion-catalyzed reactions involving the zwitterion IV*a* between pH 2 and 6 and the anion IV*b* between pH 6 and 11. The first-order rate constants for the first two reactions, k_A^{IV} and k_B^{IV}, are given in Table IV.

Similar mechanisms previously were proposed (8, 12) to account for the hydrolysis of the acid succinate ester of phenols including *p*-acetamidophenol. The pH–rate profile for the latter reaction (9) is shown as the dashed line in Fig. 1.

The hydrolysis of ester IV is subject to intramolecular nucleophilic catalysis, and its hydrolysis rate is much faster than that of the ester II between pH 2 and 10. It was shown previously (13) that both α- and β-aspartic acid esters of ethanol hydrolyze more slowly than the glycinate ester and that the pH–rate profiles for all three compounds have similar shapes. These results suggest that the predominant hydrolysis reactions of the aliphatic alcohol esters are intermolecular reactions and that intramolecular nucleophilic catalysis is not significant in aspartic acid esters with such strongly basic leaving groups at $C_2H_5O^-$.

The line for ester III in Fig. 1 was calculated on the basis that the major reactions that occurred during its hydrolysis were:

$$EH^{\pm} + H_2O \xrightarrow{k_A^{III}}$$
$$EH^{\pm} + OH^- \xrightarrow{k_B^{III}}$$
$$E^- + OH^- \xrightarrow{k_C^{III}}$$

where E^- represents the anion of III and $E^{\pm}$ is its zwitterionic form. The acid dissociation constant of III was calculated to be 5.01×10^{-9} at 25° and $\mu = 1.0\ M$. Values of the microscopic rate constants were calculated in the same way as the corresponding values for ester II (Table III).

The pH–rate profile for the hydrolysis of ester III is similar in shape to that for ester II and differs significantly from that for ester IV. Hence, it can be concluded that the predominant hydrolysis reactions of ester III involve inter- rather than intramolecular reactions. Apparently, the $^+NH_3$ or NH_2 substituent adjacent to the carboxylate group in the zwitterion or anion of III makes cyclization so unfavorable relative to the

Table IV—Values of Microscopic Rate Constants [a] for Hydrolysis of Ester IV in Aqueous Solution at 25°, $\mu = 1.0\ M$

Reaction	Rate Constant	Value, min^{-1}
$EH^{\pm} \rightarrow$ products	k_A^{IV}	0.135
$E^- \rightarrow$ products	k_B^{IV}	1.06

[a] Calculated by a curve-fitting technique from the data in Fig. 1.

succinate or α-aspartic acid esters that the intramolecular catalyzed reaction is unfavorable relative to the intermolecular reactions. The k_C^{III} and k_B^{III} values are smaller than the k_C^{II} and k_B^{II} values to the extent that could be expected (14, 15) to result from moving the NH_2 or $^+NH_3$ group one carbon atom farther away from the ester carbonyl group.

Buffer Catalysis of Ester II Hydrolysis—The catalytic effects of oxy anions (and water) on the hydrolysis of II were similar to what was found previously (16) for the same species on the hydrolysis of esters activated in the acyl portion such as ethyl dichloroacetate. This result is demonstrated by the linearity of the plot (Fig. 2) of log k_b values for the reactions of II *versus* the log k_b values for the reactions of ethyl dichloroacetate (log k_0). Although the leaving group in the hydrolysis of II is a phenolate ion (*i.e.*, a relatively weak base), it is expected (10) that the rate of breakdown of a tetrahedral intermediate rather than the formation rate would be the rate determining step for nucleophilic catalysis in the reactions of II and ethyl dichloroacetate that are catalyzed by water and the oxy anions except hydroxide ion. Furthermore, on the basis of previous arguments (10, 16), water and formate ion probably are acting as general base catalysts, acetate ion probably is acting as both general base and nucleophilic catalysts, and phosphate ion and hydroxide ion probably are nucleophilic catalysts.

The second-order rate constant for aminolysis of the II cation by a molecule of neutral tromethamine fell close to the line in Fig. 2. Thus, the characteristics of this aminolysis reaction apparently are also similar to those (16) of the aminolysis of other aromatic esters or of aliphatic esters activated in the acyl portion.

The catalysis of the hydrolysis of II in sodium bicarbonate solutions can be accounted for on the basis of the sequence of reactions (Scheme I) proposed by Hay and Main (17) to account for the carbon dioxide-catalyzed hydrolysis of *p*-nitrophenyl esters of glycine and other α-amino acids. Hay and Main observed that values of $k_{cat}/f_E f_{CO_2}$ for reactions of *p*-nitrophenyl esters of α-amino acids were pH independent, and they concluded that a rate-determining reaction between the α-amino acid ester and carbon dioxide was followed by the rapid formation of a Leuchs anhydride and *p*-nitrophenol.

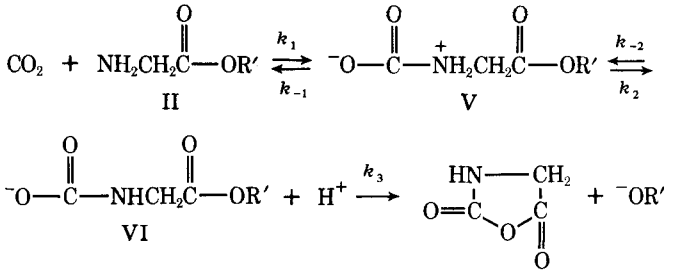
In contrast to this situation, values of $k_{cat}/f_E f_{CO_2}$ for reactions of ester II (Table II) in the presence of sodium bicarbonate were pH dependent throughout the 6.0–8.85 range. A plot of $f_E f_{CO_2}/k_{cat}$ *versus* the activity of hydrogen ion was linear ($r^2 = 0.999$) with an intercept of 5.43×10^{-4} and a slope of 2.55×10^4 when $a_{H^+} = 0$.

This behavior can be accounted for by assuming that, under the experimental conditions, the concentration of carbamate remained essentially constant. This assumption leads to:

$$\frac{f_E f_{CO_2}}{k_{cat}} = \frac{k_3(k_{-1} + k_2) + k_{-1}k_{-2}a_{H^+}}{k_1 k_2 k_3} \qquad \text{(Eq. 4)}$$

where f_{CO_2} is the fraction of bicarbonate buffer that exists as carbon dioxide and f_E is the fraction of ester that exists as a neutral molecule.

This assumption requires that the decomposition rate of carbamate to yield the Leuchs anhydride and phenol is not much greater than the carbamate decomposition rate to yield reactants. Conversely, the conclusion that the formation rate of the carbamate from reactants is rate determining in the reactions of *p*-nitrophenyl esters infers that the decomposition of the carbamates to Leuchs anhydride and *p*-nitrophenol



Leuchs anhydride

Scheme I

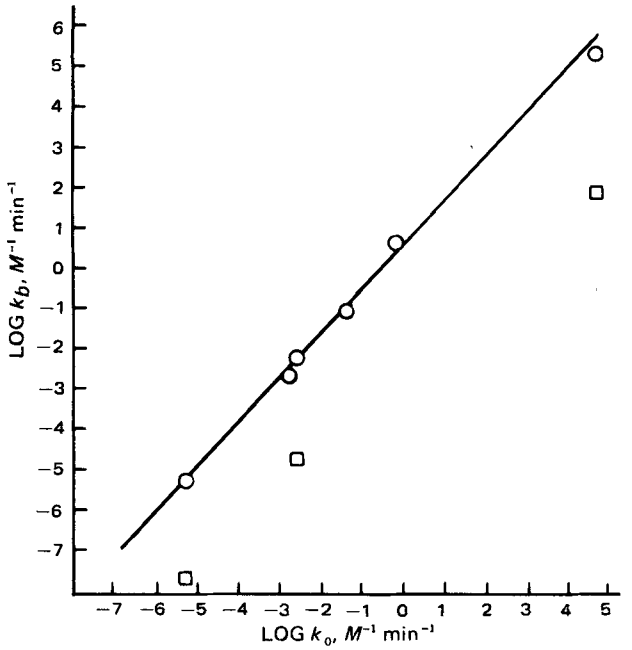


Figure 2—*Comparison of the second-order rate constants for the reactions of some general bases and nucleophiles with ester II* (k_b) (○) *to those for the reactions of the same reagents with ethyl dichloroacetate* (k_o). *The corresponding rate constants for the reactions of water, acetate ion, and hydroxide ion with phenyl acetate also are shown* (□).

in these reactions is substantially faster than its decomposition to yield reactants.

These conclusions are consistent with the expectation that *p*-nitrophenol is a better leaving group than *p*-acetamidophenol.

This efficient type of catalysis of amino acid ester hydrolysis by carbon dioxide is particularly interesting when considering the *in vivo* hydrolysis of amino acid esters of drugs since most biological fluids contain $\sim 3 \times 10^{-3}$ *M* CO_2.

EXPERIMENTAL

Materials—All chemicals were reagent grade unless otherwise specified. Morpholine was dried over anhydrous calcium sulfate and distilled. Freshly boiled double-distilled water was used in all kinetic experiments.

***p*-Acetamidophenyl-*N*-carbobenzoxyglycinate (VII)**—A suspension of *N,N'*-dicyclohexylcarbodiimide (0.26 *M*) in dry methylene chloride was added to an equal volume in dry tetrahydrofuran of a solution of *p*-acetamidophenol (I), and the mixture was stirred at 0 ± 2° for 40 hr. Then *N,N'*-dicyclohexylurea, mp 22–29° [lit. (18) mp 29–30°], was filtered off (70% yield). Gradual reduction in volume of the filtrate under reduced pressure initially resulted in precipitation of I, which was filtered off, and then resulted in an ~1:1 mixture of *p*-acetamidophenol and VII. The filtrate finally yielded a syrup, which, after treatment with acetic acid to decompose excess *N,N'*-dicyclohexylcarbodiimide, filtration, and cooling, yielded more product. The crude ester, together with some I, was recrystallized from acetone, mp 140–142°.

Hydrobromide of II—A solution of hydrobromic acid (2–3 *M*) in acetic acid was prepared by bubbling the gas through the acid. The ester VII was dissolved in this solution (10 g/100 ml) and then stirred for 2 hr. Precipitation of the product was completed by the addition of ether–acetic anhydride (9:1). The crude product was washed with warm ether and acetone and recrystallized from methanol–acetone. The resulting product (80% yield) had a melting point of 228–230°; NMR (D_2O): δ 1.95 (s, 3H, CH_3), 4.05 (d, 2H, CH_2), 7.00 (d, 2H, aromatic H *ortho* to oxygen), and 7.24 (d, 2H, aromatic H *ortho* to amide).

Anal.—Calc. for $C_{10}H_{13}BrN_2O_3$: C, 41.5; H, 4.5; N, 9.7. Found: C, 41.3; H, 4.6; N, 9.5.

***N*-Carbobenzoxy-L-aspartic Acid Anhydride (VIII)**—One part of *N*-carbobenzoxy-L-aspartic acid, which had been dried and powdered, was mixed with three parts of acetic anhydride. The mixture was agitated for 1 hr and then allowed to react at room temperature for 15 hr. The product that precipitated when the solution was poured over ice was washed with cold water and ether. The waxy platelets had a melting point of 108–109°.

α- and β-*p*-Acetamidophenyl-*N*-carbobenzoxy-L-aspartates (IX*a* and IX*b*)—A solution of I in aqueous sodium hydroxide (equimolar) was cooled to 1°. A suspension of VIII in methylene chloride (1 mole of VIII to 2 moles of I) gradually was added to the cooled solution of *p*-acetamidophenoxide with constant stirring. The final ratios of water and methylene chloride in the mixture were 1:2. The mixture was stirred at 1° for 50 min, after which time the layers were separated. The aqueous layer was acidified with sufficient 5 *N* HCl to cause the separation of an oily layer and then was refrigerated. A pasty product, obtained following decantation of the supernatant liquid, was dried over phosphorus pentoxide *in vacuo*.

TLC of the product [on plates[1] impregnated with a 0.4 *M* formate buffer at pH 4; developed with ethyl acetate–butanol (4:1)] indicated that it contained I and two other compounds, which were subsequently identified as IX*a* and IX*b*. A 2% solution of the crude product in ethyl acetate was extracted in succession with pH 5, 0.4 *M* acetate–pH 6.3, 0.2 *M* sodium phosphate (5:1). TLC indicated that the more acidic buffer contained an almost pure sample of one of the isomeric esters. It was expected, and later confirmed, that this ester was the isomer with a pKa of 2.04 at 25°, and it was concluded to be IX*b*. Acidification of this solution yielded a white solid, mp 154–157°.

Anal.—Calc. for $C_{20}H_{20}N_2O_7$: C, 59.6; H, 5.0; N, 7.0. Found: C, 58.2; H, 4.9; N, 7.5.

The other buffer, upon acidification, yielded solids with lower melting points than IX*b*. TLC suggested that these solids were mixtures of IX*a* and IX*b*, but no successful method of separating them was found.

Hydrochloride of III—A catalyst consisting of 10% palladium-on-charcoal (18) was added to a 10% solution of IX*b* in dry methanol. Hydrogen was washed in dioxane and passed *via* a calcium chloride tube through the solution under moderate pressure for 5 hr. Precipitation of the product was completed by the addition of dry ether. The precipitated product was freed from the catalyst by elution with methanolic hydrochloric acid. Addition of ether to the eluent resulted in the precipitation of the hydrochloride of III which, after recrystallization from methanolic hydrochloride, had the following characteristics: mp 189–190°; NMR (D_2O): δ 1.95 (s, 3H, CH_3), 3.10 (d, 1H, CH_2), 3.25 (s, 1H, CH_2), 4.35 (q, 1H, CH), 7.00 (d, 2H, aromatic H *ortho* to oxygen), and 7.24 (d, 2H, aromatic H *ortho* to amide); pKa, 2.04 ± 0.06; pKa_2, 8.22 ± 0.06.

Anal.—Calc. for $C_{12}H_{15}ClN_2O_5$: C, 47.6; H, 5.0; N, 9.3. Found: C, 47.4; H, 5.0; N, 9.3.

Mixture (50:50) of Hydrochlorides of III and IV—When a mixture of the esters IX*a* and IX*b* was treated with hydrogen and catalyst in the manner previously described and the reaction mixture was filtered to remove excess catalyst and then reduced in volume, an ~50:50 mixture of the hydrochlorides of III and IV was recovered. This mixture had the same elemental analysis as the hydrochloride of III, but its NMR spectrum also contained peaks at δ 3.05 (d, 1H, CH_2), 3.19 (s, 1H, CH_2), and 4.67 (q, 1H, CH). The conclusion that the mixture was 50:50 was based on comparisons of the areas under the proton peaks.

Acid Dissociation Constants—Dissociation constants were measured at 25.0 ± 0.1° by using the potentiometric methods described previously (19).

Rate Constants—Rate constants were calculated from changes in UV absorbance at 282 or 242 nm that followed the mixing of solutions of the esters (usually 1–3 μl of aqueous solutions of II or III or a methanolic solution of III and IV) with aqueous buffers (2.5 or 5.0 ml). Reactions that had a half-life of <20 sec were followed on a stopped-flow spectrophotometer after equal volumes of aqueous solutions were mixed. The ionic strength of all reaction solutions was 1.0 *M* (adjusted with potassium chloride), and the temperature was maintained at 25.0 ± 0.1°. The buffers and pH ranges used were: formate, 1.9–4.1; acetate, 4.7–5.1; phosphate, 5.4–7.1; glycine, 9.8–10.5; tromethamine, 7.4–9.2; pyrophosphate, 7.7–9.0; morpholine, 8.3–9.3; carbonate, 9.85–10.7; and sodium hydroxide, >11.5. The initial substrate concentration was always $<10^{-3}$ *M*.

Pseudo-first-order rate constants for the hydrolyses of II and III were calculated from linear plots of log ($A - A_\infty$) *versus* time (A and A_∞ being the absorbance at a particular time and infinite time, respectively). Plots of log ($A - A_\infty$) *versus* time were biexponential for the hydrolysis of the mixtures of III and IV. Values of the rate constants for hydrolysis of III were calculated from the terminal slopes of such plots, and they agreed well with values that were determined using pure III. Values of the rate constants for hydrolysis of IV were calculated from the initial portions of the curves by using a feathering technique (20).

Rate constants were reproducible in at least three runs to within ±5%.

[1] Polygram Sil N-HR.

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Absorption and Bioavailability of Captopril in Mice and Rats after Administration by Gavage and in the Diet

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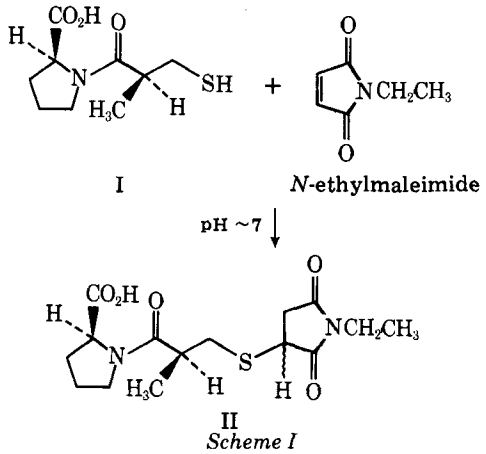
Abstract □ The absorption of captopril (I), a new antihypertensive agent, was studied in mice and rats at doses (50 and 1350 mg/kg) administered in the diet in chronic toxicological studies. ^{3}H- or ^{35}S-Labeled I was administered by gavage and in the diet to male and female animals in a two-way crossover study. Animals received daily doses of nonradiolabeled I in the diet for 25 days, except on Days 15 and 22 when radiolabeled I was administered either by gavage or in the diet. Absorption of the total radioactivity in 2-month-old mice averaged 49 and 48%, respectively, of the 50- and 1350-mg/kg doses given in the diet and 57 and 65%, respectively, of the doses given by gavage. The bioavailability of I in 2-month-old mice averaged 48 and 39% (diet) and 44 and 59% (gavage) of the 50- and 1350-mg/kg doses, respectively. In 2-month-old rats, absorption of the total radioactivity averaged 41% of the 50-mg/kg dose given in the diet. In 2- and 15-month-old rats, minimum absorption of the 1350-mg/kg dose averaged 36 and 45% (diet) and 51 and 71% (gavage), respectively; the minimum bioavailability averaged 29 and 29% (diet) and 39 and 44% (gavage), respectively. These studies demonstrate adequate absorption and bioavailability of I over a wide range of doses from the drug–diet mixtures and by young and old animals and also illustrate a useful experimental design for the estimation of relative oral absorption of a drug administered continuously in the diet over several days.

Keyphrases □ Captopril—absorption and bioavailability, gavage and dietary administration, mice and rats □ *Metabolism*—absorption and bioavailability of captopril, gavage and dietary administration, mice and rats □ Antihypertensives—absorption and bioavailability of captopril, gavage and dietary administration, mice and rats

Captopril (I), 1-[(2*S*)-3-mercapto-2-methyl-1-oxopropyl]-L-proline, is a potent and specific inhibitor of the enzyme that catalyzes the conversion of angiotensin I to angiotensin II (1) and was an orally effective antihypertensive agent in extensive clinical trials (2–4). The disposition of I in normal subjects was reported recently (5). Specific assays for determination of I as its *N*-ethylmaleimide derivative (II) also were reported (6, 7).

In oral toxicological and pathological studies over a 2-year period, I was administered daily in the diet to mice and rats at 50, 150, and 1350 mg/kg. The present study evaluated the effects of food and of repeated daily administration of 50- and 1350-mg/kg doses of I on its absorption and bioavailability in 2-month-old mice and rats and 15-month-old rats.

Investigations in animals and *in vitro* indicate that I is chemically unstable in biological fluids and undergoes rapid autoxidation to form III, the disulfide dimer of I, and other products. To prevent or minimize such processes, a procedure for immediate conversion of I to II in biological samples was established (5–7) (Scheme I). This procedure was utilized in the determination of nonlabeled I using GLC–mass spectrometry (7) and of radiolabeled I using thin-layer radiochromatography (6). Since radiolabeled I was necessary to determine absorption in the present study, thin-layer radiochromatography was used.



Scheme I