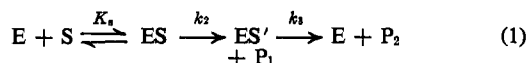the trypsin-catalyzed hydrolysis of p-nitrophenyl acetate[3]; *trans*-cinnamoyl-trypsin is seen in the trypsin-catalyzed hydrolysis of N-*trans*-cinnamoylimidazole[4]; a fast stoichiometric release of p-nitrophenol occurs in the trypsin-catalyzed hydrolysis of p-nitrophenyl α-N-benzyloxycarbonyl-L-lysinate hydrochloride[5] and the kinetics of the presteady-state and steady-state reactions in this system are satisfactorily described by eq. 1 involving an acyl-trypsin intermediate.[6]

$$E + S \underset{}{\overset{K_s}{\rightleftharpoons}} ES \overset{k_2}{\longrightarrow} \underset{+\ P_1}{ES'} \overset{k_3}{\longrightarrow} E + P_2 \qquad (1)$$

However, a recent report presents data which tend to disprove the compulsory formation of an acyl-enzyme intermediate in the trypsin-catalyzed hydrolysis of α-N-benzoyl-L-arginine ethyl ester.[7] Moreover, the evidence enumerated above may not be general if one postulates that: (1) the slowly reacting acetyl and cinnamoyl derivatives do not necessarily proceed *via* the same pathway as the fast arginine and lysine derivatives; (2) "activated" p-nitrophenyl esters do not necessarily proceed by the same pathway as the "nonactivated" methyl or ethyl esters and the amide; and (3) the small spread of reactivities expected in the series of esters of argument 1 above casts doubt on the validity of the argument. Therefore experiments were designed to determine whether an acyl-trypsin is formed in the trypsin-catalyzed hydrolysis of α-N-benzyloxy-carbonyl-L-lysine methyl and benzyl esters.

Table I. The Kinetics of the Trypsin-Catalyzed Hydrolysis of the Methyl, Benzyl, and p-Nitrophenyl Esters of α-N-Benzyloxycarbonyl-L-lysine[a]

Ester	pH	k_{cat}, sec.$^{-1}$	K_m(app), M
p-Nitrophenyl[b]	5.80	6.79	1.00×10^{-5}
	5.84	6.93	0.93×10^{-5}
	5.85	7.02	0.94×10^{-5}
Methyl[c]	5.80	6.40	2.98×10^{-4}
	5.80	6.55	2.72×10^{-4}
Benzyl[d]	5.80	6.18	$\sim 1 \times 10^{-4}$

[a] 25.0°, phosphate buffer, $I = 0.5$; $E_0 = (2.5-6) \times 10^{-7}$ M; 1.29% (v./v.) acetonitrile–water. [b] This compound was described previously.[5] [c] Hydrochloride purchased from Cyclo Chemical Co. as an oil; after two recrystallizations from methanol–diethyl ether, m.p. 75–76°. *Anal.* Calcd. for $C_{15}H_{13}N_2O_4Cl$: C, 54.46; H, 7.01; N, 8.47; Cl, 10.72. Found: C, 54.49; H, 7.19; N, 8.93; Cl, 10.80. The amount of D isomer must be less than 10% from its behavior with trypsin. [d] Tosylate salt, Cyclo Chemical Co. product, recrystallized from ethanol–diethyl ether, m.p. 111–112°. *Anal.* Calcd. for $C_{28}H_{34}N_2O_7S$: C, 61.98; H, 6.32; N, 5.16; S, 5.91. Found: C, 61.90; H, 6.36; N, 5.41; S, 5.76. No detectable amount of the D isomer was observed in its reaction with trypsin. [e] pH 5.8 was chosen since: (1) at this pH K_m of this reaction does not yet show the marked pH dependence occurring at lower pH values[5]; (2) the enzyme concentration is in a convenient range; (3) the rate of the reaction is not too fast and can be measured accurately; and (4) the nonenzymatic hydrolysis of the p-nitrophenyl ester is slow. The pH dependence of k_{cat} of the methyl and p-nitrophenyl ester reactions is identical from pH 3 to 7.5, and thus the equivalence seen at pH 5.8 is valid over this range.

(3) J. A. Stewart and L. Ouellet, *Can. J. Chem.*, 37, 751 (1959).
(4) M. L. Bender and E. T. Kaiser, *J. Am. Chem. Soc.*, 84, 2556 (1962).
(5) M. L. Bender, J. V. Killheffer, Jr., and R. W. Roeske, *Biochem. Biophys. Res. Commun.*, 19, 161 (1965).
(6) M. L. Bender, F. J. Kézdy, and J. Feder, *J. Am. Chem. Soc.*, 87, 4953 (1965).
(7) S. A. Bernhard and H. Gutfreund, *Proc. Natl. Acad. Sci. U. S.*, 53, 1238 (1965); T. E. Barman and H. Gutfreund, *ibid.*, 53, 1243 (1965).
(8) *Cf.* J. A. Stewart and J. E. Dobson, *Biochemistry*, 4, 1086 (1965).

The steady-state kinetics of the trypsin-catalyzed hydrolysis of the methyl, benzyl, and p-nitrophenyl esters of α-N-benzyloxycarbonyl-L-lysine were determined at pH 5.8, using Lineweaver–Burk plots of single experiments. The kinetic comparison shown in Table I indicates that the catalytic rate constants of the three esters are identical within $\pm 6\%$, whereas the K_m(app) values are markedly different. These data are quite similar to data on the α-chymotrypsin-catalyzed hydrolysis of the ethyl, methyl, and p-nitrophenyl esters of N-acetyl-L-tryptophan.[6] Like the chymotrypsin data, the trypsin data are inconsistent with the nucleophilic character of trypsin reactions since methyl, benzyl, and p-nitrophenyl esters have relative rates of 1, 1.1, and 50 toward the nucleophile hydroxide ion.[9,10] On the other hand, these data are completely consistent with the three-step mechanism (eq. 1) for all substrates, involving the rate-determining decomposition (k_3) of a common intermediate, α-N-benzyloxycarbonyl-L-lysyl-trypsin, k_2 values which reflect the reactivity of these esters toward nucleophiles, and closely similar K_s values. The slight trend in k_{cat} values ($k^{NPE} > k^{BE} = k^{ME}$) is in the expected nucleophilic order and indicates that deacylation is not completely rate controlling; the K_m(app) values are also in the expected order ($K_m^{NPE} \ll K_m^{BE} \sim K_m^{ME}$). However, the limited accuracy of the data and the lack of an estimated K_s prevents the calculation of k_2, as was done for chymotrypsin-catalyzed reactions.

The combination of the observation of the presteady state for the p-nitrophenyl ester[6] of α-N-benzyloxycarbonyl-L-lysine and the identity of the k_{cat} values for the methyl, benzyl, and p-nitrophenyl esters of α-N-benzyloxycarbonyl-L-lysine proves beyond any reasonable doubt that all three esters are hydrolyzed by trypsin *via* an acyl-enzyme intermediate.

(9) B. Zerner, R. P. M. Bond, and M. L. Bender. *J. Am. Chem. Soc.*, 86, 3674 (1964).
(10) The nucleophilic character of the reaction is seen in the relation between the k_{cat} values of α-N-benzoyl-L-arginine ethyl ester and amide which have values of 16 and 0.04 sec.$^{-1,11}$ respectively, and in the positive Hammett ρ constant of the deacylation of a series of *para*-substituted benzoyl-trypsins.[12]
(11) S. A. Bernhard, *Biochem. J.*, 59, 506 (1955).
(12) C. R. Gunter, unpublished experiments in this laboratory.

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Transient Formation of an Inactive Intermediate in the Reaction with α-N-Benzoyl-L-arginine Ethyl Ester and α-N-Benzoyl-L-arginine[1]

Sir:

In previous communications evidence that the trypsin-catalyzed hydrolysis of p-nitrophenyl, benzyl, and methyl esters of α-N-benzyloxycarbonyl-L-lysine proceed through an α-N-benzyloxycarbonyl-L-lysyl-trypsin intermediate was presented.[2,3] In this communication we present evidence that an enzymatically inactive but regenerable intermediate is formed in the trypsin-catalyzed hydrolysis of α-N-benzoyl-L-arginine ethyl

(1) This research was supported by grants from the National Institutes of Health.
(2) M. L. Bender, F. J. Kézdy, and J. Feder, *J. Am. Chem. Soc.*, 87, 4953 (1965).
(3) M. L. Bender and F. J. Kézdy, *ibid.*, 87, 4954 (1965).

Table I. Kinetics of Some Trypsin-Catalyzed Reactions[a]

Reactions	pH	k_{cat} $\times 10^2$,[b] sec.$^{-1}$	k_{accel} $\times 10^2$,[c] sec.$^{-1}$	k_{accel} $\times 10^2$,[d] sec.$^{-1}$
p-Nitrophenyl N-benzyloxycarbonyl-L-tyrosinate	3.21[g]	2.38		
p-Nitrophenyl α-N-benzyloxycarbonyl-L-lysinate	2.94	2.25		
	3.07	2.92		
α-N-Benzoyl-L-arginine ethyl ester	2.82	0.73		
	2.94	0.98[e]		
	3.07	1.4		
	3.24	2.0[f]		
α-N-Benzoyl-L-arginine ethyl ester + trypsin[h]	3.07		1.53	2.2
α-N-Benzoyl-L-arginine ethyl ester + trypsin[i]	2.94		1.07	1.21
α-N-Benzoyl-L-arginine + trypsin[j]	2.94		1.45	1.26

[a] 25.0°, Worthington 2× crystallized bovine trypsin, concentration determined by titration with p-nitrophenyl α-N-benzyloxycarbonyl-L-lysinate. [b] From turnover kinetics. [c] From first-order acceleration rate. [d] From $y_0 = (E_0 - E_{free})(1 - (k_3 CLyNPE/k_3 BAEE))$.[4] [e] Interpolation. [f] Extrapolation. [g] Citrate buffer (0.05 M). [h] Incubation in pH 4, 0.1 M acetate buffer, for 30 sec. before addition to p-nitrophenyl α-N-benzyloxycarbonyl-L-lysinate solution at pH 3.07. [i] A 50-μl. aliquot of a solution of trypsin, 1.09 × 10^{-3} M in pH 2.94, 0.05 M citrate buffer, incubated for 60 sec. with 5.39 × 10^{-2} M α-N-benzoyl-L-arginine ethyl ester, was added to 3.05 ml. of pH 2.94, 0.05 M citrate buffer, containing 4.05 × 10^{-3} M p-nitrophenyl α-N-benzyloxycarbonyl-L-lysinate. [j] A 50-μl. aliquot of a solution of trypsin, 2.58 × 10^{-3} M, equilibrated with 0.168 M α-N-benzoyl-L-arginine at pH 2.94, was added to 3.05 ml. of the above solution of p-nitrophenyl α-N-benzyloxycarbonyl-L-lysinate.

ester, the most common substrate of trypsin, and further that the same intermediate is formed in the reaction of α-N-benzoyl-L-arginine and trypsin.

At pH 3.24, the trypsin-catalyzed hydrolysis of p-nitrophenyl N-benzyloxycarbonyl-L-tyrosinate shows an initial burst of p-nitrophenol and also shows a
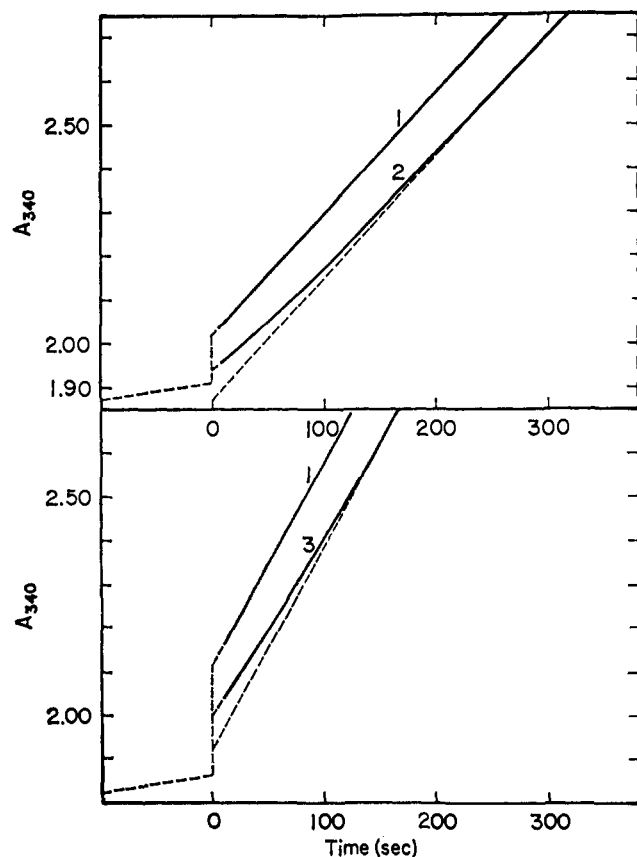
Figure 1. The reaction of p-nitrophenyl α-N-benzyloxycarbonyl-L-lysinate at pH 2.94, 0.05 M citrate buffer, 25.0°, with: (1) trypsin; (2) trypsin plus α-N-benzoyl-L-arginine ethyl ester, and (3) trypsin plus α-N-benzoyl-L-arginine. See Table I for conditions.

catalytic rate constant quite similar to that of the trypsin-catalyzed hydrolysis of α-N-benzoyl-L-arginine ethyl ester (Table I). Therefore, if the latter system involves the intermediate α-N-benzoyl-L-arginyl-tryp-

sin whose deacylation is rate limiting, addition of the reacting system to p-nitrophenyl N-benzyloxycarbonyl-L-tyrosinate should show the disappearance of the initial burst of p-nitrophenol, and also the maintenance of the zero-order rate of p-nitrophenol liberation.[4] This proved to be the case: when an aliquot of trypsin was added to the tyrosine ester, an initial burst followed by a linear turnover was observed. On the other hand, when the same trypsin solution was incubated with 10-fold excess of α-N-benzoyl-L-arginine ethyl ester for 30 sec. before adding an aliquot to the tyrosine solution, no initial burst of p-nitrophenol was observed within experimental error (10% of the original burst), but a linear turnover rate was observed. This zero-order rate was ca. 10% less than the original rate, which could account for approximately 25% decrease of the initial burst. Thus, these experiments show that in the presence of α-N-benzoyl-L-arginine ethyl ester about 75% of the enzyme is present as an inactive form, from which the enzyme is regenerated with a rate constant very similar to the turnover constant of the tyrosine ester.

At pH 2.94, the trypsin-catalyzed hydrolysis of p-nitrophenyl α-N-benzyloxycarbonyl-L-lysinate shows an initial burst of p-nitrophenol but has a catalytic rate constant 2.3-fold larger than the catalytic rate consant of the trypsin-catalyzed hydrolysis of α-N-benzoyl-L-arginine ethyl ester (Table I). If the latter system involves the intermediate α-N-benzoyl-L-arginyl-trypsin whose deacylation is rate limiting, addition of the indicator substrate, p-nitrophenyl α-N-benzyloxycarbonyl-L-lysinate, to the reacting system should show the disappearance of the initial burst of p-nitrophenol and also an acceleration of the liberation of p-nitrophenol preceding the zero-order liberation of p-nitrophenol.[4] An aliquot of trypsin solution added to the lysine ester solution produced a burst followed by a zero-order turnover. However, when an aliquot of the same enzyme solution, which had previously been incubated for 60 sec. with a 40-fold excess of α-N-benzoyl-L-arginine ethyl ester, was added the burst decreased to 29% of the blank experiment; furthermore, a marked acceleration, which was identical with the turnover in the absence of the arginine ester (Figure 1), was observed before reaching the zero-order turnover. By extrapolating the

(4) F. J. Kézdy and M. L. Bender, *Biochemistry*, **4**, 104 (1965).

zero-order turnover and measuring the difference between the experimental points and the extrapolated line, the first-order rate constant of the acceleration was obtained. An independent calculation of this rate constant using the intercept, y_0, of the P_1 vs. t plot at $t = 0$ and the equation, $y_0 = (E_0 - E_{free})(1 - (k_3 CLyNPE/k_3 BAEE))$,[5] gave good agreement. This rate constant which measures the regeneration of the enzyme from an inactive but regenerable form is slightly higher than the rate constant of the trypsin-catalyzed hydrolysis of α-N-benzoyl-L-arginine ethyl ester (Table I).

The only explanation of these results is that trypsin plus α-N-benzoyl-L-arginine ethyl ester form an appreciable amount of α-N-benzoyl-L-arginyl-trypsin in a dynamic equilibrium, and that the decomposition of this acyl-enzyme, seen in the acceleration,[6] is mainly but not completely rate determining in the over-all hydrolytic reaction. Using this hypothesis the value of k_2 at pH 2.94 may be calculated in several ways. The known values of k_3 (1.25×10^{-2} sec.$^{-1}$, from the average of four acceleration determinations) and k_{cat} (from the turnover reaction) and the equation $k_2 = k_{cat}k_3/(k_3 - k_{cat})$ yields 4.5×10^{-2} sec.$^{-1}$. The magnitude of the burst, the known k_3, and the equation $k_3/k_2 = (E_0/ES')/(1 + (K_m(app)/S)) - 1$ yields 2.4×10^{-2} sec.$^{-1}$. Thus, the k_2/k_3 ratio for the trypsin-catalyzed hydrolysis of α-N-benzoyl-L-arginine ethyl ester is about 3–4 at pH 2.94, and K_s is not far different from $K_m(app)$.

In a similar experiment, a trypsin solution was equilibrated with excess α-N-benzoyl-L-arginine, and then an aliquot of this mixture was added to the lysine ester solution. Again the burst decreased (to 55% of the blank experiment), and again an acceleration was observed before reaching a zero-order turnover identical with that in the absence of α-N-benzoyl-L-arginine (Figure 1). The first-order rate constant of this acceleration was similar to that produced from the corresponding ester, indicating that both α-N-benzoyl-L-arginine and α-N-benzoyl-L-arginine ethyl ester form the same inactive but regenerable enzyme derivative. The decrease in the initial burst of p-nitrophenol was dependent on the time of equilibration of trypsin with α-N-benzoyl-L-arginine (before addition to the lysine ester solution) with a rate constant of 2.1×10^{-2} sec.$^{-1}$. Both from the extent of the decrease in the burst and the rate constant of the decrease, the acylation/deacylation ratio of the acid (k_{-3}/k_3) was determined to be about 1.6. The absolute value of the k_2/k_3 of the ester and k_{-3}/k_3 of the acid and the relationship between them are closely similar to those found for the ester and acid of N-acetyl-L-tryptophan with α-chymotrypsin.[7]

The experiments reported in this paper[8] are consistent with the conclusion that the trypsin-catalyzed hydrolysis of α-N-benzoyl-L-arginine ethyl ester proceeds solely through an acyl-enzyme intermediate.

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Secondary Deuterium Isotope Effects in Asymmetric Syntheses and Kinetic Resolutions

Sir:

We wish to report that partial asymmetric alcoholysis of α-phenylbutyric anhydride in pyridine[1] with (+)-(S)-2-propanol-1-d_3 (1)[2] affords (after hydrolysis of unreacted anhydride) α-phenylbutyric acid (2) with detectable optical activity and thus provides the first example of the operation of a steric isotope effect in an asymmetric synthesis or kinetic resolution.[3]

In a typical experiment, a solution of α-phenylbutyric anhydride (1.619 g., 0.0052 mole) in 12.5 ml. of pyridine was added to 0.165 g. (0.0026 mole) of 1, and the mixture was allowed to stand at room temperature for 16 hr. The excess of unreacted anhydride was hydrolyzed by addition of 0.5 ml. of water in the cold. Titration of 2 with 1 N sodium hydroxide (0.0078 mole) in the presence of benzene established that the esterification reaction had been quantitative. The aqueous solution was extracted with chloroform and acidified; the acid solution was repeatedly extracted with benzene. The isolated acid 2 had $[\alpha]_D$ +0.46° before and after distillation, corresponding to a 0.48% optical yield based on the maximum rotation of 2 ($[\alpha]_D \pm 96.5°$ in benzene). Several repetitions of this experiment yielded essentially the same results. The observed rotations varied between +0.02 and +0.12° (depending on the conditions of measurement) and were thus well outside the limits of experimental error ($\pm 0.002°$). The optical yields of 2 varied between 0.4 and 0.5%.

Although the details of the mechanism of alcoholysis have not yet been fully elucidated,[1,5,6] it has been possible to show empirically[1] that the sign of rotation of recovered 2 is related to the absolute configuration of the inducing alcohol; partial alcoholysis with alcohols of configuration 3 affords, after hydrolysis of unreacted anhydride, acid 2 containing an excess of the (+)-(S)-enantiomer.[7]

(1) A. Horeau, *Tetrahedron Letters*, No. 15, 506 (1961); No. 21, 965 (1962); A. Horeau and H. B. Kagan, *Tetrahedron*, 20, 2431 (1964).

(2) K. Mislow, R. E. O'Brien, and H. Schaefer, *J. Am. Chem. Soc.*, 82, 5512 (1960); 84, 1940 (1962).

(3) It had previously been observed[2] that the partial asymmetric Meerwein–Ponndorf–Verley reduction of a racemic biphenyl ketone with 1 does not result in detectable asymmetric induction, and it was concluded that more highly crowded transition states might be required for the exhibition of steric isotope effects, *i.e.*, secondary deuterium isotope effects which could be ascribed to differences in the size of isotopic groupings. The first unambiguous kinetic evidence for the operation of steric isotope effects was subsequently provided by a study of the racemization of variously deuterated 9,10-dihydro-4,5-dimethylphenanthrenes.[4]

(4) K. Mislow, R. Graeve, A. J. Gordon, and G. H. Wahl, Jr., *J. Am. Chem. Soc.*, 85, 1199 (1963); 86, 1733 (1964).

(5) C. W. Bird, *Tetrahedron Letters*, No. 3, 117 (1962).

(6) H. Falk and K. Schlögl, *Monatsh. Chem.*, 96, 266, 276 (1965).

(7) This generalization is *not* equivalent to the statement[6] that partial alcoholysis with alcohols having the (R) configuration affords acid 2 containing an excess of the (+)-(S) enantiomer. Stereoformula 3 corresponds to (R) alcohol only if the large group (L) has priority over the medium-sized group (M) in the Cahn–Ingold–Prelog nomenclatural scheme (R. S. Cahn, *J. Chem. Educ.*, 41, 116 (1964)); however, there

(5) The intercept of eq. 4 of ref. 4.

(6) Only a single acceleration is observed. *A priori*, the acceleration could correspond to the decomposition of either the acylenzyme, the enzyme–substrate complex, or the enzyme–product complex. The two latter possibilities, however, are ruled out by the time scale of the acceleration.

(7) F. J. Kézdy, G. E. Clement, and M. L. Bender, *J. Am. Chem. Soc.*, 86, 3690 (1964).

(8) The k_2/k_3 ratio is independent of pH from 2.94 to 4 (Table I, footnote h), and the k_{cat} is dependent on a single ionizable group from pH 2 to 7. Therefore the k_2/k_3 ratio at pH 2.94 may be reasonably extrapolated to pH 7 (pK_a(acylation) = pK_a(deacylation) within experimental error).