$(TTF)_3BF_4$. The products of these reactions were not identified.

The single-crystal electrical conductivities and ESR spectra of these and other TTF salts are currently being determined[13] and will be the subject of future publications.

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(10) Correct elemental analysis was obtained.
(11) A dilute solution of $(TTF)_3(BF_4)_2$ in hot acetonitrile was filtered and to it was added an acetonitrile solution of the required anion as its tetrabutylammonium salt. Storage at room temperature overnight afforded crystals of the desired TTF salt.
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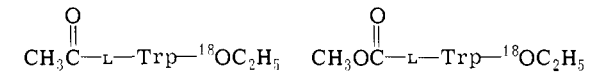
Kinetic Isotope Effects for the Chymotrypsin Catalyzed Hydrolysis of Ethoxyl-^{18}O Labeled Specific Ester Substrates[1]

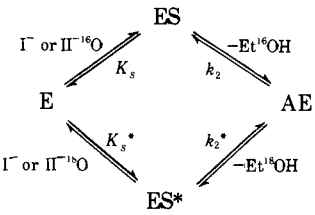
Sir:

We wish to report the results of oxygen-18 kinetic isotope effect measurements for the chymotrypsin-catalyzed hydrolysis of two esters, *N*-acetyl-L-tryptophan ethyl ester-*ethoxyl*-^{18}O (I) and *N*-carbomethoxy-L-tryptophan ethyl ester-*ethoxyl*-^{18}O (II). Kinetic isotope effect studies utilizing elements other than hydrogen are a relatively little used tool for the elucidation of enzyme mechanisms[2a] and yet offer unique insights into the nature of the transition state of many reactions. In a previous investigation with chymotrypsin, for example, O'Leary has determined a ^{15}N kinetic isotope effect of 1.006–1.010 for the chymotrypsin catalyzed hydrolysis *N*-acetyl-L-tryptophanamide, similar to the value 1.004–1.006 observed for the alkaline hydrolysis of amides.[2b]



I II

The accepted mechanism[3] for the chymotrypsin-catalyzed hydrolysis of esters proceeds by the initial rapid equilibrium formation of the Michaelis complex, followed by acylation of the enzyme with release of alcohol, and finally by deacylation to give the *N*-acylamino acid and the free enzyme. This pathway, when applied to a mixture of the ^{18}O- and ^{16}O-labeled forms of I or II is shown in eq 1, where ES and ES* are the Michaelis complexes with unlabeled and labeled substrate, AE is the acyl enzyme, and P is the *N*-acylamino acid. The kinetic isotope effect arising



(1)

from this mechanism in a competitive experiment is determined solely by the relative rates of formation of the acyl enzyme, even though the rate determining step is deacylation, a step not involving the isotope. With the assumption

$$\Delta = \frac{v}{v^*} = \frac{k_2 K_s^*}{k_2^* K_s}$$

that the binding constant, K_s, is the same for ^{18}O and ^{16}O substrates, $\Delta = k_2/k_2^*$.[4]

Ethanol-^{18}O was prepared as previously described.[5] L-Tryptophan ethyl ester hydrochloride-*ethoxyl*-^{18}O was prepared by the reaction of L-tryptophanyl chloride hydrochloride[6] with ethanol-^{18}O (65 atom %) in glyme, and N-acylated (in ethyl acetate over aqueous sodium carbonate) with acetic anhydride to give I, or with methyl chloroformate to give II. Racemization, as determined by measuring the unreacted substrate after chymotryptic hydrolysis, was less than 0.1%.

Enzymatic hydrolyses were done at pH 6.8 in potassium phosphate buffer, 0.05 *M* (or 0.01 *M* when $[S_0]$ = 0.1 m*M*), at 25°. The reaction was followed spectrophotometrically at 298 nm (or 265 nm when $[S_0]$ = 0.1 m*M*). Substrates used contained 10 or 25 atom % ^{18}O in the ethoxyl position. Aliquots were taken at preselected fractions of the complete reaction and quenched by extraction with methylene chloride. The substrate recovered from the organic layer was purified by preparative TLC on silica (ethyl acetate). For isotopic analysis, a substrate sample of 1–5 mg

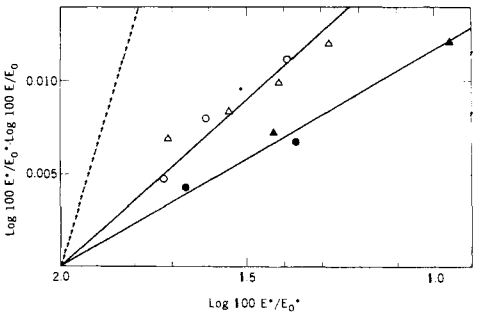


Figure 1. Oxygen-18 kinetic isotope effects for the chymotrypsin-catalyzed hydrolysis of ethoxyl-labeled *N*-acetyl-L-tryptophan ethyl ester: (○), (I) at S_0 = 0.1 m*M*, pH 6.8 in 0.01 *M* potassium phosphate buffer (△), (I) S_0 = 1.0 m*M*, pH 6.8 in 0.05 *M* potassium phosphate buffer; (●, ▲) (II) at S_0 = 0.5 m*M*, separate experiments at pH 6.8 in 0.05 *M* potassium phosphate buffer. The data are plotted according to eq 2, where the slope is $1 - (k_2/k_2^*)$. A horizontal line, therefore is indicative of no kinetic isotope effect, and the dashed line, given for reference, represents the isotope effect of 1.066 observed for hydrazinolysis of both esters under conditions of rate-determining breakdown of the tetrahedral intermediate, corresponding to a very late transition state with respect to the scission of the acyl–ethoxyl bond.

Table I. Summary of Oxygen-18 Kinetic Isotope Effects for Alkoxyl-Labeled *N*-Acyl-L-Tryptophan Ethyl Esters and Methyl Formate

Compound	Reaction	Kinetic isotope effect
II	Chymotrypsin catalyzed hydrolysis	1.0117 ± 0.0004[a]
I	Chymotrypsin catalyzed hydrolysis	1.0180 ± 0.0007[a]
I and II	Hydrazinolysis	1.066 ± 0.002[b,c]
Methyl formate	Hydrazinolysis at pH 7.85	1.0621 ± 0.0008[b,d]
Methyl formate	General base catalyzed hydrolysis	1.0115 ± 0.0002[d]
Methyl formate	Alkaline hydrolysis	1.0091 ± 0.0004[d]

[a] This work determined at pH 6.8. [b] For rate-determining breakdown of the tetrahedral intermediate. [c] C. B. Sawyer and J. F. Kirsch, unpublished results. [d] Reference 7.

was saponified in 3 μl of 6 *M* KOH in methanol for 5 min and the volatile products examined directly by mass spectroscopy. The ratio of ethanol-^{18}O to ethanol-^{16}O was determined as previously described.[5] The percent of total ester remaining at the time of taking of aliquots was determined from the least-squares fit of the integrated form of the Michaelis–Menten rate equation to the progress curve of [S] vs. time.

The kinetic isotope effect for chymotryptic hydrolysis of labeled substrate was determined for I at substrate concentrations of 1.0 m*M* ($12K_m$) and at 0.1 m*M* ($1.2K_m$), and for II at 0.5 m*M* ($2.5K_m$). The oxygen-18 content of unreacted substrate during hydrolysis is plotted as previously described[7] in Figure 1 according to eq. 2 where E, E_0, E^*, and E_0^* are the concentrations of unlabeled and labeled ester at time t and 0. The results are summarized in Table I. The kinetic isotope effect for chymotryptic hydrolysis of I is, within experimental error, independent of initial concentration, as shown in Figure 1.

$$\log (100E^*/E_0^*) - \log (100E/E_0) = (1 - \Delta) \log (100E^*/E_0^*) \quad (2)$$

The 1.2 and 1.8% ^{18}O kinetic isotope effects observed for the chymotrypsin catalyzed hydrolyses of esters I and II are remarkably close to that observed for the general base catalyzed hydrolysis of methyl formate[7] (Table I). This observation supports the proposed general base catalysis mechanism for the acylation of chymotrypsin.[8] We may also calculate the degree of cleavage of the ester bond in the transition state. Studies with methyl formate,[7] I and II,[9] have shown that the ^{18}O kinetic isotope effects for these esters range from 0 to 6.6%, the latter value corresponding to a very late transition state involving complete rate-determining scission of the ester bond. Thus in the chymotrypsin catalyzed reactions the bond order between the acyl carbon and the departing oxygen atoms is reduced about 1.2/6.6 = 18% and 1.8/6.6 = 27% in the transition state for II and I, respectively. This calculation is based on the assumption of a single rate determining transition state. Small kinetic isotope effects can also arise from a reaction pathway involving the formation of one (or more) intermediates whose rates of formation and decomposition exhibit differing sensitivities to the isotopic substitution in question and where both steps are partially rate determining.[10] A likely but by no means established intermediate for the acylation of chymotrypsin by specific *ester* substrates is the tetrahedral adduct formed by attack of Ser-195 on the acyl carbon atom. The available data do not permit differentiation among these two possibilities for this system; however, additional kinetic data employing linear free energy relationships or a second isotopic substitution may resolve this ambiguity.[11] The identical chemical reactivity of I and II (see below) does in our opinion constitute some evidence against different partitioning ratios of a tetrahedral adduct being the explanation for the different values of the observed kinetic isotope effects.

The small difference in observed kinetic isotope effects for I and II may indicate either a later transition state with the same mechanism for I as for II or, possibly, different mechanisms of acylation with the two esters. The latter possibility is intriguing; whereas the ester linkages in I and II are virtually identical (the rates of reaction with OH^- and N_2H_4 are equal within an experimental error of 5%),[9] I but not II can form an oxazoline as an additional intermediate en route to the acyl enzyme. Such an oxazoline has actually been isolated from the reaction of furylacryloyl-tryptophan methyl ester with chymotrypsin at low pH.[12]

Acknowledgment. We thank Professor M. H. O'Leary for a valuable discussion.

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Mechanisms of Grignard Reactions with Ketones. Polar vs. Single Electron Transfer Pathways

Sir:

In addition to the commonly accepted polar mechanism for the addition of Grignard reagents to ketones, evidence has been accumulating which supports a single electron transfer (SET) process in some cases[1] (reaction 1).[1c] We wish to report preliminary results of the role of ketyls in reactions of Grignard reagents with ketones and the conditions under which the polar or SET mechanisms operate.