About eight points were taken for each kinetic run. The rate constant was calculated from the slope of the best straight line, as determined by the method of least squares, through a plot of log [AD]/([AD] + [AH]) *vs.* time. If a single point deviated from the best straight line through the other points by four or more times the average deviation of the other points it was discarded. In no case were two points discarded from the same run. Altogether about ten points were discarded.

Runs 25–33 were particularly slow and were followed to only about 10% completion; even this required about a month. Most of the other runs were carried to about 50% reaction. A kinetic plot for run 32, Table II, one of the poorest runs, is shown in Figure 2.

Acknowledgment. We wish to acknowledge our indebtedness to the National Science Foundation for grants that permitted the purchase of the nuclear magnetic resonance spectrometers used in this investigation.

Carbonyl Oxygen Exchange in General Base Catalyzed Ester Hydrolysis[1a]

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Contribution from the Department of Chemistry, Northwestern University, Evanston, Illinois 60201. Received October 22, 1966

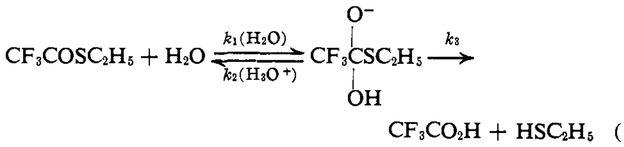
Abstract: The general base catalyzed hydrolyses of ethyl trifluorothiolacetate-O^{18} in 40% v/v tetrahydrofuran and of ethyl trifluoroacetate-*carbonyl*-O^{18} in 25% v/v acetonitrile are accompanied by carbonyl oxygen exchange with the solvent. The extent of oxygen exchange in the thiol ester experiments was quantitatively predicted from the hydrolysis kinetics, which were recently determined by Fedor and Bruice, and was consistent with an unsymmetrical partitioning of an addition intermediate. With the oxygen ester, partitioning of the intermediate is symmetrical since the partitioning is pH independent. The amount of oxygen exchange observed with the latter compound decreased significantly in the presence of deuterium oxide solvent. Temperature studies with both esters indicate that the activation energies are identical for breakdown of the intermediate to reactants or to products. The deacylations (hydrolyses) of cinnamoyl-α-chymotrypsin-*cinnamoyl-carbonyl*-O^{18} and *p*-nitrobenzoyl-α-chymotrypsin-*p-nitrobenzoyl-carbonyl*-O^{18} at neutral pH are not accompanied by carbonyl oxygen exchange with the solvent. However, in the alkaline hydrolysis of O-cinnamoyl-N-acetylserinamide-*cinnamoyl-carbonyl*-O^{18}, a model for an acyl-α-chymotrypsin intermediate, oxygen exchange takes place.

The hydrolysis of carboxylic esters often occurs simultaneously with carbonyl oxygen exchange with water.[2] Such exchange is strong evidence for the formation of a tetracovalent addition intermediate in the reaction, and provides the principal experimental justification for the two-step mechanism in ester hydrolysis.

It is well recognized, however, that oxygen exchange alone cannot prove that an intermediate lies on the reaction path. It is possible, for example, that ester hydrolysis proceeds *via* an SN2 type of substitution reaction, while oxygen exchange occurs through an addition mechanism which does not lead to the hydrolytic products.[3] Since carbonyl oxygen exchange reactions have been used widely in studies of reaction mechanism,[2] it would be of considerable interest to ascertain in a specific reaction whether the tetrahedral intermediate required for oxygen exchange is actually on the hydrolytic pathway.

Several kinetic arguments have been advanced recently requiring the presence of an intermediate on the pathway of an ester hydrolysis. Thus, the use of carbonyl oxygen exchange as a criterion for intermediate formation in ester hydrolysis can be tested by determining the amount of oxygen exchange in an ester hydrolysis which can be shown by independent kinetic evidence to involve an intermediate (presumably a tetrahedral addition compound). If the amount of exchange is in agreement with that which would be predicted from the kinetic experiments, one can conclude that the intermediate in which oxygen exchange occurs is the same intermediate which is observed kinetically.

The system chosen for investigation was ethyl trifluorothiolacetate-O^{18}. The kinetics of hydrolysis of the thiol ester have been thoroughly examined by Fedor and Bruice.[4] These authors presented strong evidence that the mechanism of hydrolysis is that outlined in eq 1. This mechanism involves a general base catalyzed attack of water at the carbonyl carbon of the thiol ester, forming a tetrahedral intermediate which decomposes spontaneously to products but reverts to reactants by acid catalysis.



$$\mathrm{CF_3COSC_2H_5 + H_2O} \underset{k_2(\mathrm{H_3O^+})}{\overset{k_1(\mathrm{H_2O})}{\rightleftharpoons}} \mathrm{CF_3}\underset{\mathrm{OH}}{\overset{\mathrm{O^-}}{\mathrm{C}}}\mathrm{SC_2H_5} \xrightarrow{k_3} \mathrm{CF_3CO_2H + HSC_2H_5} \quad (1)$$

By assuming a steady-state condition for the intermediate, the authors derived eq 2 for the hydrolytic rate constant, and showed, in accordance with eq 2,

$$k_\mathrm{h} = k_1k_3/(k_2a_\mathrm{H} + k_3) \quad (2)$$

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[1] (a) Taken from the Ph.D. dissertation of H. Heck, Northwestern University, 1967. Support of this work by a grant from the National Institutes of Health is gratefully acknowledged. (b) Predoctoral Fellow of the National Science Foundation.

[2] Oxygen exchange reactions of organic and organometallic compounds are reviewed by D. Samuel and B. L. Silver, *Advan. Phys. Org. Chem.*, 123 (1965).

[3] M. J. S. Dewar, "The Electronic Theory of Organic Chemistry," Oxford University Press, New York, N. Y., 1948, p 117.

[4] L. R. Fedor and T. C. Bruice, *J. Am. Chem. Soc.*, **87**, 4138 (1965).

that a plot of the inverse of k_h was proportional to the activity of hydrogen ion. From the slope and intercept of the plot, it was possible to calculate the k_2/k_3 ratio.

As k_2/k_3 ratios for ester hydrolyses can also be obtained using carbonyl oxygen exchange techniques,[5] the ethyl trifluorothiolacetate system can be employed to compare the partitioning ratios obtained by the two independent methods. This reaction system therefore provides an opportunity to determine precisely whether the intermediate required for carbonyl oxygen exchange is on the reaction path in ester hydrolysis. Experiments designed to solve this problem using the oxygen-18-labeled thiol ester are described in this paper.

It was demonstrated by Jencks and Carriuolo[6] that a number of simple oxygen esters which contain electronegative substituents in the acyl portion of the molecule undergo general base catalyzed hydrolysis in neutral or slightly acidic media. These reactions might be mechanistically similar to the deacylation of an acyl enzyme, since the latter process is thought to be a general base catalyzed hydrolysis of an acylserine ester.[7] It would therefore be of interest to determine the mechanism of a general base catalyzed ester hydrolysis in detail, particularly with regard to the role of acid or base catalysis in the partitioning of a tetracovalent intermediate. Information of the latter kind can be obtained by investigating the pH dependence of the carbonyl oxygen exchange reactions of a suitable general base catalyzed system. This paper reports experiments with ethyl trifluoroacetate-*carbonyl*-O^{18} designed to test whether the partitioning of the tetrahedral intermediate is catalyzed in an identical manner to reactants and to products.

Bender and Kézdy[7] proposed that the deacylation of an acyl-α-chymotrypsin involves a tetrahedral intermediate, and that the reaction is a symmetrical process, formation of the intermediate being catalyzed by an enzymatic general base, and its decomposition to products and reactants being catalyzed by the corresponding conjugate acid. The hypothesis of a two-step mechanism in acyl-enzyme deacylations is examined here using carbonyl oxygen exchange techniques, employing two acyl enzymes, cinnamoyl-α-chymotrypsin-*cinnamoyl-carbonyl*-O^{18} and *p*-nitrobenzoyl-α-chymotrypsin-*p-nitrobenzoyl-carbonyl*-O^{18}. Finally, this article reports studies of the alkaline hydrolysis of a compound chosen as a possible acyl-enzyme model, O-cinnamoyl-N-acetylserinamide-*cinnamoyl-carbonyl*-O^{18}, using oxygen exchange methods.

Experimental Section

Materials. Inorganic buffers, salts, and chemicals employed in the preparation of standard solutions were reagent grade quality. Acetate buffers were reagent grade. Other organic buffers were purified by recrystallization before use. Heavy water, approximately 99.8% D_2O, was obtained from Bio-Rad Laboratories. The per cent of oxygen-18 in the D_2O was nearly identical with that in water. Oxygen-18-labeled water of several different isotopic compositions was purchased from the Yeda Co., Ltd., Rehovoth, Israel. Tetrahydrofuran was Baker reagent grade, purified according to Wiberg.[8] Acetonitrile was either Baker reagent grade, purified by the method of O'Donnell, *et al.*,[9] or was unaltered Eastman Kodak Spectro grade. Iodine was Fisher reagent grade, dried by the method of Arndt.[10] The water employed in solution preparations was twice distilled, the second time either from alkaline permanganate or in a Corning all-glass distillation apparatus, Model AG-2, after passage of distilled water through a mixed-bed, ion-exchange column of Amberlite MB-3. Boiling points and melting points are uncorrected unless stated otherwise.

Ethyl trifluorothiolacetate (Eastman Kodak Co.) was distilled through a Vigreux column, bp 89.2–90.0° (lit.[11] bp 90.5°). Ethyl trifluoroacetate (Aldrich Chemical Co.) was distilled as above, bp 59.5–61.5° (lit.[12] bp 60.5°). Worthington three-times-recrystallized α-chymotrypsin was employed. Enzyme stock solutions were centrifuged at 15,000*g* for 30 min immediately before use. Prior to some experiments in which large quantities of enzyme were utilized, the centrifuged stock solution was eluted on a Sephadex G-25 column in order to remove small peptides.

Sodium trifluoroacetate-O^{18} was prepared by heating to reflux a solution of trifluoroacetic acid in a large excess of H_2O^{18} (approximately 1.5% oxygen-18) for 20 hr. After neutralization with solid sodium carbonate, the water-O^{18} was removed in a rotary evaporator with strong warming and the solid residue dried by prolonged heating under high vacuum over P_4O_{10}. The salt was then taken up in a minimal quantity of absolute ethanol and filtered. Ethanol was removed and the residue again dried under high vacuum by heating over P_4O_{10}. The resulting solid was powdery and appeared to be completely anhydrous.

Trifluoroacetyl chloride-O^{18} was synthesized from sodium trifluoroacetate-O^{18} and phosphorus trichloride, using the technique of Simons and Ramler.[13] Purification of the product was effected by distillation in a low-temperature column modeled after the design of von Bartal.[14]

Ethyl trifluorothiolacetate-O^{18} was prepared by allowing the trifluoroacetyl chloride-O^{18} to bubble through ethyl mercaptan, following the methods of Hauptschein and co-workers.[11] The ester was purified by distillation, bp 88–89°. Vapor phase chromatography (6-ft 15% silicone oil on Gas Pack-W, $T = 46°$) showed one large peak plus a very small peak attributed to ethyl mercaptan, constituting less than 0.01% of the larger.

Ethyl trifluoroacetate-*carbonyl*-O^{18} was synthesized by bubbling trifluoroacetyl chloride-O^{18} through a solution of absolute ethanol and dry pyridine in a narrow, vertical reaction tube above which was placed a dewar-type, Dry Ice condenser. During reaction the tube was cooled in ice. Pyridinium chloride precipitate was filtered off, and the filtrate was distilled. An azeotropic mixture of ethanol and ester was collected, boiling between 55 and 65°. The azeotrope was cooled in ice, then was rapidly extracted twice with cold, concentrated $CaCl_2$ solution. Solid $CaCl_2$ and P_4O_{10} were added to the organic layer, and the mixture was filtered. Distillation of the filtrate yielded the oxygen-18-labeled ester, boiling between 60.5 and 61.5°.

trans-Cinnamic acid-O^{18} and *p*-nitrobenzoic acid-*carboxyl*-O^{18} were prepared from the corresponding acyl chlorides by reaction with stoichiometric amounts of H_2O^{18} (~1.7 and 4% oxygen-18, respectively). The oxygen-18-labeled acyl chlorides were synthesized from the acids, using thionyl chloride.

N-*trans*-Cinnamoylimidazole-O^{18} was prepared from *trans*-cinnamoyl chloride-O^{18} using the method described by Schonbaum and co-workers.[15]

O-Cinnamoyl-N-acetylserinamide-*cinnamoyl-carbonyl*-O^{18} was made by adding *trans*-cinnamoyl chloride-O^{18} to a cooled suspension of N-acetylserinamide (Cyclo Chemical Corp.) in dry pyridine. Reaction was allowed to proceed for 2 hr. After recrystallizations from 50% methanol–water, the product melted at 193–194° (lit.[16] mp 195–196°).

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(5) M. L. Bender, *Chem. Rev.*, 60, 53 (1960).

(6) W. P. Jencks and J. Carriuolo, *J. Am. Chem. Soc.*, **83**, 1743 (1961).

(7) M. L. Bender and F. J. Kézdy, *Ann. Rev. Biochem.*, **34**, 49 (1965); M. L. Bender and F. J. Kézdy, *J. Am. Chem. Soc.*, **86**, 3704 (1964).

(8) K. B. Wiberg, "Laboratory Technique in Organic Chemistry," McGraw-Hill Book Co., Inc., New York, N. Y., 1960, p 246.

(9) J. F. O'Donnell, J. T. Ayres, and C. K. Mann, *Anal. Chem.*, **37**, 1161 (1965).

(10) F. Arndt, *Ber.*, **52**, 1131 (1919).

(11) M. Hauptschein, C. S. Stokes, and E. A. Nodiff, *J. Am. Chem. Soc.*, 74, 4005 (1952).

(12) A. L. Henne, M. S. Newman, L. L. Quill, and R. A. Staniforth, *ibid.*, **69**, 1819 (1947).

(13) J. H. Simons and E. O. Ramler, *ibid.*, 65, 389 (1943).

(14) C. J. Hansen in "Die Methoden der Organischen Chemie," Vol. I, J. Houben, Ed., 3rd ed, Verlag Georg Thieme, Leipzig, 1925, p 585. For details of the apparatus see the Ph.D. thesis of H. d'A. Heck.

(15) G. R. Schonbaum, B. Zerner, and M. L. Bender, *J. Biol. Chem.*, **236**, 2930 (1961).

(16) M. L. Bender, G. R. Schonbaum, and B. Zerner, *J. Am. Chem. Soc.*, **84**, 2540 (1962).

N-*p*-Nitrobenzoylimidazole-*carbonyl*-O^{18} was prepared byt he slow addition of a benzene solution of *p*-nitrobenzoyl chloride-*carbonyl*-O^{18} to a cooled suspension of imidazole in dry benzene. The reaction mixture was stirred for 30 min, imidazolium chloride was removed by filtration, and the pale yellow product was recrystallized from CCl_4, mp 121–122° (lit.[17] mp 120–122.5°).

Apparatus. Measurements of pH were made on a Radiometer Type PHM 4c pH meter. The meter was standardized for measurements of hydrogen ion concentration in solutions containing large amounts of organic solvent.[18] A series of solutions were prepared at constant ionic strength and containing known stoichiometric quantities of hydrogen ion and prescribed amounts of organic solvent. The pH of the solutions was then measured. With a given organic solvent, the measured pH differed from the stoichiometric pc_H by a constant, Δ, which depended on the amount of solvent used, over a wide range of pH. Equation 3 was therefore used to calculate pc_H from pH.

$$pc_H = pH + \Delta \tag{3}$$

At very low hydrogen ion concentrations, buffers were employed to find Δ. The apparent pK_a's of the buffers in the organic solvent–water mixtures were determined at hydrogen ion concentrations where the correction Δ was known. Then, at higher pH, the pc_H calculated from the apparent pK_a and the buffer ratio was compared with the measured pH. The same Δ was found at higher pH as at lower pH.

Kinetic runs were carried out in either a Cary Model 14 PM recording spectrophotometer equipped with a thermostated cell compartment or in a thermostated Radiometer Type TTT1c automatic titrator.

The analysis of the oxygen-18 contents of organic acids or their salts was performed by decarboxylation of the compounds and measurements of the per cent of oxygen-18 in the carbon dioxide, using a Consolidated Electrodynamics Corp. Model 21-130 mass spectrometer. Cinnamic acid-O^{18} and *p*-nitrobenzoic acid-*carboxyl*-O^{18} were decarboxylated over reduced copper turnings at elevated temperatures (700° for cinnamic acid, 400° for *p*-nitrobenzoic acid) in a helium train. Silver trifluoroacetate-O^{18} was decarboxylated in a helium train by warming the salt in the presence of iodine.[19] Carbon dioxide samples from the various decarboxylations were purified on a high vacuum line by distillations from Dry Ice–acetone. Measurements of the amount of gas produced showed that copper-catalyzed decarboxylations were quantitative. Iodine-catalyzed decarboxylations yielded approximately 10% more than the theoretical amount of gas; a mass spectrograph of a gas sample indicated that this was due to the presence of trifluoroiodomethane. The per cent of oxygen-18 excess, R_{meas}, was calculated using eq 4, where (46) and (44) signify peak heights at these values of *m*/*e*.

$$R_{meas} = \frac{1}{2}\frac{(46)/(44)}{[1 + (46)/(44)]}(100) - 0.204 \tag{4}$$

Kinetic and Oxygen-18 Exchange Investigations. In the following discussions, the statement of per cent of organic solvent in an organic solvent–water mixture will mean the per cent by volume. Expressions of pH signify the meter reading. Stated enzyme concentrations are values determined by titration with N-*trans*-cinnamoylimidazole.[15] Pseudo-first-order hydrolytic rate constants were calculated from semilogarithmic plots or by the method of Kèzdy, *et al.*[20]

The disappearance of ethyl trifluorothiolacetate was followed spectrophotometrically at 244 mμ. Stoppered cells were used for all kinetic runs. Reactions were allowed to proceed for a minimum of six half-lives. The pH of a reaction solution was measured immediately before the start of each run and after the completion of some runs to ensure constancy of pH. No pH changes were observed. Corrections to the glass electrode readings in terms of hydrogen ion concentration were determined at $I = 0.04$ *M* and 40% tetrahydrofuran. Solutions of hydrochloric acid were used from pc_H 1.48 to 2.97. From pc_H 2.57 to 4.65, chloroacetic acid–potassium chloroacetate buffers were employed. The apparent pK_a of chloroacetic acid at 25° under these conditions is 3.69 ± 0.02, and Δ is −0.16 pH unit.

The kinetics of hydrolysis of ethyl trifluoroacetate were determined by observing the disappearance of ester at 231 mμ or by following the amount of acid release on a pH-Stat. In the experiments carried out spectrophotometrically, the maximum pH drop was 0.1 unit; the same techniques were employed as in the thiol ester experiments. Beer's law was shown to be obeyed under the reaction conditions. The glass electrode was standardized for readings of hydrogen ion concentration in 25% acetonitrile from pc_H 1.20 to 6.05; hydrochloric acid solutions and benzoate buffers were employed. At 25°, $I = 0.060$ *M*, the apparent pK_a of benzoic acid in the organic solvent–water mixture is 4.86 ± 0.02 and Δ is 0.08 pH unit. Reactions in deuterium oxide solution were performed in an identical manner with the corresponding water reactions. Two measurements of pD in deuterium oxide containing 25% acetonitrile, $I = 0.060$ *M*, and known quantities of DCl indicated that the glass electrode registered 0.47 pD unit lower than the true pc_D value; pc_D values are calculated using this correction.

Oxygen exchange experiments with ethyl trifluorothiolacetate-O^{18} and ethyl trifluoroacetate-*carbonyl*-O^{18} were carried out in thermostated, 500-ml volumetric flasks. The initial concentration of ester was 5×10^{-3} *M* in the thiol ester experiments and 1×10^{-2} *M* in the oxygen ester experiments. Solutions were either buffered with acetate, or contained hydrochloric acid at concentrations sufficient to maintain an essentially constant pH. Ionic strength was held constant with NaCl or KCl. Carbonyl oxygen exchange of ethyl trifluoroacetate-*carbonyl*-O^{18} in deuterium oxide solution was carried out in the same manner as in the water reaction.

At completion of the ester hydrolyses (> seven half-lives), the solutions were neutralized with sodium hydroxide, and solvent was removed by lyophilization. The solid residue was dissolved in about 3 ml of redistilled water, and 7 ml of saturated silver nitrate was slowly added with stirring. Reduction of silver was avoided by working in a dark room or in the cold. Silver chloride and silver acetate precipitates were removed by filtration, and the precipitates were washed with saturated silver nitrate. Filtrates were combined and centrifuged. Solid silver nitrate was dissolved in the clear supernatant liquids, and the aqueous solutions were extracted with benzene, in which silver trifluoroacetate is readily soluble.[21] The benzene extract was dried with $CaSO_4$ and filtered, and benzene was removed on a rotary evaporator. The residue was dissolved in a minimal quantity of benzene, and crystals of silver trifluoroacetate-O^{18} were driven out by adding to the benzene solution approximately five times its volume of petroleum ether.[21] The yield was approximately 150 mg of the silver salt, about 20%.

The oxygen-18 contents of the carbonyl oxygens of the starting esters were assumed to be identical with the oxygen-18 contents of the sodium trifluoroacetate-O^{18} from which they had been synthesized. Strong support for this assumption will be demonstrated in the section on Results. In order to determine the oxygen-18 contents of the sodium trifluoroacetate-O^{18} starting materials, about 0.3 g of the salt was dissolved in a few drops of redistilled water, and approximately 1 ml of saturated silver nitrate was added. Extraction with benzene followed, and the silver salt was isolated as described above. *Anal.* Calcd for $C_2F_3O_2Ag$ (220.90): C, 10.88; F, 25.80. Found: C, 10.99; F, 26.50.

In order to measure the rates of oxygen exchange in the acid products resulting from the hydrolyses of ethyl trifluorothiolacetate-O^{18} and ethyl trifluoroacetate-*carbonyl*-O^{18}, oxygen exchange experiments with sodium trifluoroacetate-O^{18} were performed, using the same ionic strength and organic solvent concentrations as in the ester hydrolyses. In these reactions, 1.700 g of the sodium salt (12.5 mmoles) was dissolved in several 500-ml volumetric flasks containing the desired organic solvent–water solutions at various hydrogen ion concentrations. The rates of exchange were determined by withdrawing, at known times, 100-ml samples from the flasks and neutralizing the samples with NaOH. The products were worked up as silver trifluoroacetate-O^{18} in a manner similar to that noted above. The yield from each sample was approximately 250 mg, 50%.

The deacylation of cinnamoyl-α-chymotrypsin-*cinnamoyl-carbonyl*-O^{18} was carried out at 25.0° under turnover conditions by the addition of a number of small increments of a stock solution of N-*trans*-cinnamoylimidazole-O^{18} in dry acetonitrile to a 100-ml volumetric flask containing, initially, 2.00×10^{-4} *M* α-chymotrypsin in $^1/_{15}$ *M* phosphate buffer, pH 7.12, and 9% acetonitrile. The numerous substrate additions were made in order to accumulate a large amount of product without exceeding the solubility of the acylimidazole.

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(17) M. Caplow and W. P. Jencks, *Biochemistry*, 1, 883 (1962).

(18) L. G. Van Uitert and C. G. Haas, *J. Am. Chem. Soc.*, **75**, 451 (1953).

(19) R. N. Haszeldine, *J. Chem. Soc.*, 584 (1951).

(20) F. J. Kèzdy, J. Jaz, and A. Bruylants, *Bull. Soc. Chim. Belges*, **67**, 687 (1958).

(21) F. Swarts, *ibid.*, **32**, 102 (1923).

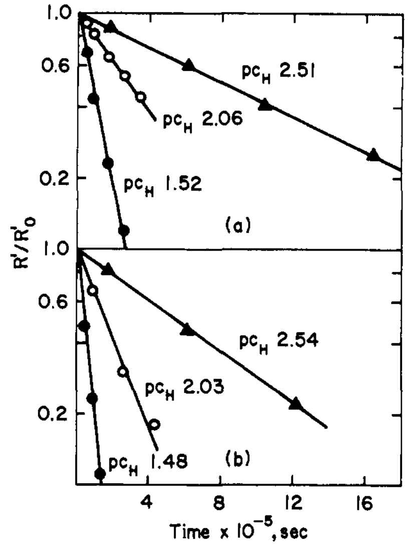

Figure 1. Oxygen exchange of trifluoroacetic acid-O^{18} with water at 25.0°, I = 0.065 M, in (a), 40% tetrahydrofuran, and (b), 25% acetonitrile.

The *p*-nitrobenzoyl-α-chymotrypsin-*p-nitrobenzoyl-carbonyl*-O^{18} deacylation was done in a 100-ml volumetric flask at 25.0° with the enzyme concentration always greater than substrate concentration, owing to the rapid spontaneous hydrolysis of *p*-nitrobenzoylimidazole-*carbonyl*-O^{18}. The initial enzyme concentration was 2.10 × 10^{-3} M, and the solution was buffered with $^1/_{15}$ M phosphate, pH 7.42. Several aliquots from a stock solution of the acylimidazole substrate in dry acetonitrile were added over a period of about 3 hr with thorough mixing, in order to accumulate a large amount of product.

In both of the enzyme experiments, times of addition of substrate were calculated from known deacylation rate constants[17,22] and from the rate constants for irreversible loss of enzyme activity under the reaction conditions determined in separate experiments by titration.

At completion of hydrolysis, the oxygen-18-labeled acid products were separated from the enzymes by gel filtration on a column of Sephadex G-25. The acids were obtained by precipitation from concentrated aqueous solutions in the cold. After recrystallization from hexane, cinnamic acid-O^{18} melted at 132–133.5°. *p*-Nitrobenzoic acid-*carboxyl*-O^{18} was recrystallized from benzene and chloroform, mp (cor) 241–241.5°. The oxygen-18 contents of the carbonyl oxygens of the starting acylimidazoles were assumed to be identical with the oxygen-18 contents of the acids from which they had been prepared. This assumption is borne out in the following section.

The rate of cinnamate release from O-cinnamoyl-N-acetylserinamide-*cinnamoyl-carbonyl*-O^{18} was measured in a disodium hydrogen phosphate–sodium hydroxide buffer (0.0175 M in total phosphate) containing 30% acetonitrile. The reaction was followed at 281.5 mμ. The final pH was 12.06. Oxygen exchange experiments were carried out by dissolving 0.415 g of the substrate (1.5 moles) in 30 ml of acetonitrile in a 100-ml volumetric flask, then diluting to the mark with the phosphate–hydroxide buffer. After 2 hr, the solution was neutralized with a few drops of concentrated HCl. The solvent was almost completely removed under reduced pressure, and the precipitate was filtered off; cinnamic acid-O^{18} was obtained from the clear filtrate by acid precipitation, as noted above. The hexane-recrystallized product melted at 133.5–134°. The amount of oxygen-18 in the cinnamoyl carbonyl oxygen atom of the starting material was assumed to be the same as in the cinnamic acid-O^{18} from which it had been synthesized.

Results

Trifluoroacetic Acid-O^{18}. The determination of the carbonyl oxygen exchange during the hydrolysis of ethyl trifluoroacetate and ethyl trifluorothiolacetate was carried out by measurement of the oxygen-18 content of the product, trifluoroacetic acid. Since the product itself can exchange its oxygen atoms with the solvent, the rate of its isotopic exchange was determined under the conditions of the ester hydrolyses. Experiments in 40% tetrahydrofuran–water and 25% acetonitrile–water are summarized in Table I where R' signifies the per cent of oxygen-18 excess in the acid at time t, and R_0' is the per cent of oxygen-18 excess in the starting material. The observed pseudo-first-order rate constants for exchange of oxygen atoms of trifluoroacetic acid-O^{18}, k_x, were calculated from the slopes of plots of log R'/R_0' *vs.* time (Figure 1).

Table I. Exchange of the Oxygen Atoms of Trifluoroacetic Acid-O^{18} with Water[a]

pc_H	Time × 10^{-5}, sec	R'	R'/R_0'	$k_x \times 10^6$, sec^{-1}
	40% Tetrahydrofuran–Water[b]			
1.52	0.432	1.02	0.68	8.67
	0.864	0.65	0.43	
	1.728	0.34	0.23	
	2.592	0.18	0.12	
2.06	0.432	1.36	0.91	2.43
	0.864	1.22	0.81	
	1.728	0.97	0.65	
	2.592	0.81	0.54	
	3.456	0.66	0.44	
2.51	1.728	1.30	0.87	0.847
	6.048	0.90	0.60	
	10.368	0.62	0.41	
	16.416	0.38	0.25	
	25% Acetonitrile–Water[c]			
1.48	0.432	0.66	0.47	17.1
	0.864	0.32	0.23	
	1.296	0.15	0.11	
2.03	0.864	0.86	0.66	4.44
	2.592	0.39	0.30	
	4.320	0.23	0.18	
2.54	1.728	1.16	0.81	1.39
	6.048	0.65	0.45	
	12.096	0.32	0.22	

[a] I = 0.065 M, 25.0°. [b] R_0' = 1.50 ± 0.02% excess. [c] R_0' varied with different samples.

From the work of Llewellyn and O'Connor,[23] the oxygen-18 exchange of trifluoroacetic acid-O^{18} under acidic conditions can occur by two paths (eq 5), with rate constants k_1' and k_2'. For the series of reactions shown in eq 5, the over-all k_x is given by eq 6.

$$\begin{tikzcd}
K_1 & H_2O + CF_3CO_2{}^*H_2{}^+ \arrow[r, "k_1'"] \arrow[d, shift right, "-H^+"'] & CF_3CO_2H_2{}^+ + H_2O^* \arrow[d, shift left, "-H^+"] & K_1 \\
& H_2O + CF_3CO_2{}^*H \arrow[u, shift right, "H^+"'] \arrow[r, "k_2'"] \arrow[d, shift right, "-H^+"'] & CF_3CO_2H + H_2O^* \arrow[u, shift left, "H^+"] \arrow[d, shift left, "-H^+"] & \\
K_2 & CF_3CO_2{}^{*-} \arrow[u, shift right, "H^+"'] & CF_3CO_2{}^- \arrow[u, shift left, "H^+"] & K_2
\end{tikzcd} \tag{5}$$

$$k_x = \frac{k_1'[H^+]^2 + k_2'K_1[H^+]}{[H^+]^2 + K_1[H^+] + K_1K_2} \tag{6}$$

If $[H^+]$ is sufficiently small, k_x becomes

$$k_x = (k_2'/K_2)[H^+] \tag{7}$$

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[22] M. L. Bender, G. R. Schonbaum, and B. Zerner, *J. Am. Chem. Soc.*, **84**, 2562 (1962). Acetonitrile at the concentration employed has virtually no effect on the rate constant of deacylation: G. E. Clement and M. L. Bender, *Biochemistry*, 2, 836 (1963).

[23] D. R. Llewellyn and C. O'Connor, *J. Chem. Soc.*, 4400 (1964).

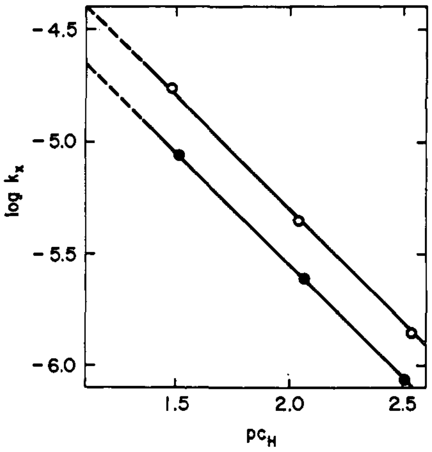

Figure 2. Plot of the logarithms of the pseudo-first-order rate constants for oxygen exchange of trifluoroacetic acid-O^{18} with water at 25.0°, $I = 0.065$ *M*, in 40% tetrahydrofuran, ●, and 25% acetonitrile, ○, *vs.* pc_H.

so that a plot of log k_x *vs.* pc_H would be linear with a slope of minus one. Such plots are shown in Figure 2; the slopes of the lines have the predicted value. (Strict linearity is not expected to hold in the upper left-hand corner of the plot since pK_2 is being approached. Although pK_2 is not known in 40% tetrahydrofuran or 25% acetonitrile at 25°, it has been measured in 40% dioxane at 15°, where it is 0.68.[24])

Ethyl Trifluorothiolacetate-O^{18}. The pH–rate constant profile for hydrolysis of the thiol ester is shown in Figure 3. Errors of approximately 6% in the rate constants are a result of the sensitivity of the reaction rate to slight differences in organic solvent concentration when large amounts of organic solvent are present. High organic solvent concentrations were required in the oxygen-18 exchange experiments owing to the low solubility of ethyl trifluorothiolacetate in water. Rate constants at the two highest values of pc_H were measured in buffered solutions, and were extrapolated to zero buffer concentration.

Equation 2 predicts a linear dependence of the inverse of k_h on $[H^+]$. The least-squares plot is shown in Figure 4. For the calculation of the least-squares line, the points were weighted according to the inverse squares of their probable errors. The percentage error in the slope is about five times larger than the percentage error in the intercept. From the slope and intercept of Figure 4, eq 8 was obtained, which was used to calculate the pc_H–rate profile of Figure 3. The

$$k_h = 1/(1.68 \times 10^3 + 2.3 \times 10^4[H^+]) \qquad (8)$$

rate profile is similar to that obtained by Fedor and Bruice[4] in water.

In eq 9, the mechanism proposed by Fedor and Bruice[4] for the hydrolysis of the thiol ester in acidic solution is applied to a simultaneous hydrolysis and carbonyl oxygen exchange reaction of ethyl trifluorothiolacetate-O^{18}. In this equation, E* and E signify labeled and unlabeled ester molecules, respectively, and T* and T represent tetrahedral intermediates produced from E* and E. A* and A are labeled and unlabeled acid product. The rate constant for exchange of the oxygen atoms of A* with water is signified by k_x.

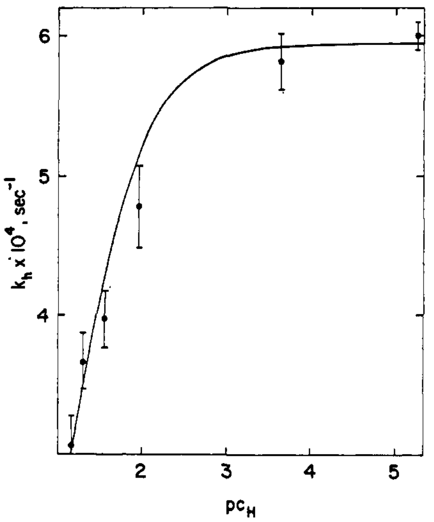

Figure 3. pc_H–rate constant profile for the hydrolysis of ethyl trifluorothiolacetate at 25.0° and 40% tetrahydrofuran, $I = 0.065$ *M*.

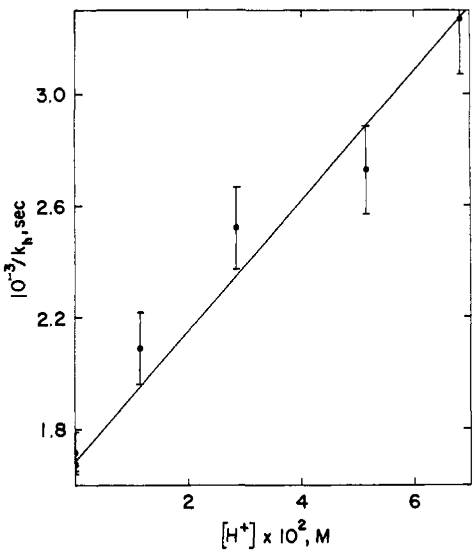

Figure 4. Plot of the reciprocals of the pseudo-first-order rate constants for the hydrolysis of ethyl trifluorothiolacetate at 25.0° in 40% tetrahydrofuran, $I = 0.065$ *M*, *vs.* hydrogen ion concentration.

Equation 9 neglects possible reaction of unlabeled ester or acid with labeled water owing to the high dilution of the latter, and neglects isotope effects since they should be small.

$$\begin{tikzcd}
H_2O + E^* \arrow[r, shift left, "k_1(H_2O)"] & T^* \arrow[l, shift left, "k_2/2(H_3O^+)"] \arrow[r, "k_3"] \arrow[d, "\frac{k_2}{2}(H_3O^+)"'] & A^* \arrow[r, "k_x{,}H_2O"] & A + H_2O^* \\
 & E + H_2O^* & + \; C_2H_5SH &
\end{tikzcd} \qquad (9)$$

$$\begin{tikzcd}
H_2O + E \arrow[r, shift left, "k_1(H_2O)"] & T \arrow[l, shift left, "k_2(HO_3^+)"] \arrow[r, "k_3"] & A + C_2H_5SH
\end{tikzcd}$$

Assuming a steady state in the tetrahedral intermediates, the integrated expressions for [A*] and [A] become

$$[A^*] = \frac{k_h}{k_h + k_e - k_x}[E^*]_0(e^{-k_xt} - e^{-(k_h + k_e)t}) \qquad (10)$$

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(24) C. A. Bunton and T. Hadwick, *J. Chem. Soc.* 3248 (1958).

Table II. Comparison of the Results of the Oxygen-18 Exchange Experiments of Ethyl Trifluorothiolacetate-O^{18} with Kinetic Predictions[a]

pc_H	Time × 10^{-4}, sec	k_h × 10^4, sec^{-1}	k_x × 10^5, sec^{-1}	R_{meas}	$(R/R_0)_f$[b] Obsd	$(R/R_0)_f$[b] Calcd
1.14	1.836	3.0	2.0	0.37	0.49 ± 0.04	0.48 ± 0.03
1.40	1.380	3.9	1.1	0.50	0.68 ± 0.05	0.69 ± 0.03
1.84	1.140	5.0	0.4	0.68	0.91 ± 0.05	0.88 ± 0.02
5.00[c]	1.440	5.9	0.00	0.79	1.05 ± 0.06	1.00 ± 0.00
5.10[d]	1.440	5.9	0.00	0.74	0.99 ± 0.06	1.00 ± 0.00
5.10[e]	1.440	5.9	0.00	0.76	1.01 ± 0.06	1.00 ± 0.00

[a] In 40% tetrahydrofuran, 25.0°, $I = 0.065\ M$. [b] $R_0 = 1.50 \pm 0.02\%$ excess. [c] Buffered with 0.030 *M* acetate. [d] Buffered with 0.048 *M* acetate. [e] Buffered with 0.065 *M* acetate.

$$[A] = [E]_{0,tot}(1 - e^{-k_h t}) - \frac{k_h}{k_h + k_e - k_x}[E^*]_0(e^{-k_x t} - e^{-(k_h+k_e)t}) \quad (11)$$

where $[E^*]_0$ is the initial concentration of labeled ester, and $[E]_{0,tot}$ is the initial concentration of total ester, both unlabeled and labeled. The rate constants k_h and k_e are defined below

$$k_h = k_1k_3/(k_2[H^+] + k_3) \quad (12)$$

$$k_e = k_1k_2[H^+]/2(k_2[H^+] + k_3) \quad (13)$$

k_e being the pseudo-first-order rate constant for oxygen exchange of E* with water. $[A]_{tot}$ is given by the sum of eq 10 and 11.

$$[A]_{tot} = [E]_{0,tot}(1 - e^{-k_h t}) \quad (14)$$

By comparing the per cent of acid molecules containing excess oxygen-18 at time *t* with the per cent of labeled ester molecules at zero time, one can determine the amount of oxygen exchange which has taken place. If the former per cent is represented by *R*, then at low excess oxygen-18

$$R = 2R_{meas} \quad (15)$$

since each carboxylic acid group contains two equivalent oxygen atoms. Denoting the latter per cent by R_0, it follows from eq 10 and 14 that

$$\frac{R}{R_0} = \frac{k_h}{k_h + k_e - k_x}\frac{(e^{-k_x t} - e^{-(k_h+k_e)t})}{(1 - e^{-k_h t})} \quad (16)$$

At sufficiently long times, corresponding to complete hydrolysis of the ester, but not to complete oxygen exchange of the acid product, eq 16 simplifies to

$$\left(\frac{R}{R_0}\right)_f = \frac{k_h}{k_h + k_e - k_x}e^{-k_x t} \quad (17)$$

From eq 17, a knowledge of the various rate constants permits the calculation of $(R/R_0)_f$ at a given pc_H. Values of k_x were taken from the plot of Figure 2. Values of k_e used in calculating $(R/R_0)_f$ are not known directly, but from eq 12 and 13

$$k_e/k_h = (k_2/2k_3)[H^+] \quad (18)$$

The k_2/k_3 ratio is equal to the slope of Figure 4 divided by its intercept. This quotient is 13.7 ± 1.4 M^{-1}. Thus, all rate constants are known and $(R/R_0)_f$ may be calculated. As shown in Table II, there is excellent agreement between the observed and calculated values of $(R/R_0)_f$.

Table II gives observed $(R/R_0)_f$ values in the hydrolysis of ethyl trifluorothiolacetate-O^{18}. At low pH considerable carbonyl oxygen exchange is evident, but at high pH no exchange occurs. This qualitative observation is consistent with the kinetic results of Fedor and Bruice.[4] Quantitative agreement between the oxygen exchange and kinetic results is shown by the use of eq 17.

The observed values of $(R/R_0)_f$ were based on an assumed R_0 of 1.50 ± 0.02%, which was the oxygen-18 excess of the sodium trifluoroacetate-O^{18} from which the ester had been synthesized. Values of R_{meas} determined at pc_H 5 bear out this assumption, since they are exactly one-half of the assumed R_0 (note eq 15). Thus, no label was lost in the preparation of the thiol ester-O^{18}.

The energetics of the partitioning of the tetrahedral intermediate formed in the hydrolysis of ethyl trifluorothiolacetate were examined. Since the k_2/k_3 ratio was most accurately measured kinetically, a number of runs were carried out at different hydrogen ion concentrations and at different temperatures, and plots of $1/k_h$ *vs.* $[H^+]$ were made for each temperature. The lines drawn in these plots were calculated by a least-squares analysis. The intercept and slope of each plot were used to calculate k_1 and the k_2/k_3 ratio according to the inverse of eq 12 at each temperature (Table III). The last column of Table III indicates that there is no discernible temperature effect on the k_2/k_3 ratio under the conditions of these experiments. Applying the Arrhenius equation to the k_2/k_3 ratio indicates that within an experimental error of 1 kcal/mole, there is no difference between the activation energies for the spontaneous loss of ethyl mercaptide and the acid-catalyzed loss of hydroxide from the tetrahedral intermediate. This is a surprising coincidence.

Table III. Temperature Dependence of Kinetic Parameters in the Hydrolysis of Ethyl Trifluorothiolacetate[a]

Temp, °C	k_1 × 10^3, sec	k_2/k_3, M^{-1}
9.0 ± 0.1	0.481	7.5 ± 0.4
25.0 ± 0.1	1.43	8.3 ± 1.3
38.9 ± 0.2	3.03	7.0 ± 1.1
50.7 ± 0.1	5.85	6.9 ± 0.6

[a] In 25% acetonitrile, $I = 0.065\ M$.

Using the data of Table III, activation parameters for the neutral hydrolysis of ethyl trifluorothiolacetate

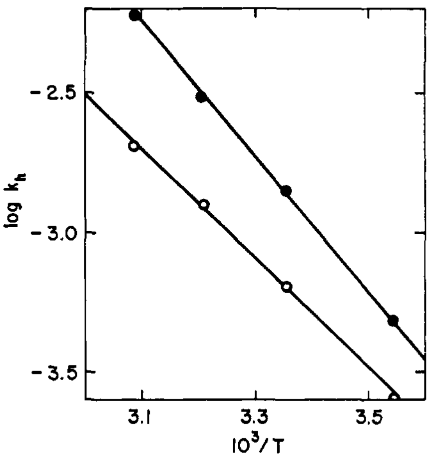

Figure 5. Temperature dependence of the hydrolysis of ethyl trifluorothiolacetate, ●, at neutral pH and of ethyl trifluoroacetate, ○, at pc_H = 2.38 in 25% acetonitrile, I = 0.065 M.

were calculated from a plot of log k_1 *vs.* $1/T$ (Figure 5 and Table IV). In Table IV, the values are compared with those obtained for the oxygen ester (see later). The calculated entropies of activation depend on the number of water molecules assumed to be involved in the transition state; calculations were made using concentration units of moles per liter and time in seconds. The activation parameters for the thiol ester agree well with values determined by Fedor and Bruice[4] under different reaction conditions.

Table IV. Comparison of Activation Parameters for the General Base Catalyzed Hydrolyses of Ethyl Trifluorothiolacetate and Ethyl Trifluoroacetate[a]

	CF_3CO-SC_2H_5[b]	CF_3CO_2-C_2H_5[c]
E_a, kcal/mole	10.9	8.92
$\Delta H^{\ddagger}$,[d] kcal/mole	10.3	8.33
$\Delta S^{\ddagger}$,[e] cal/deg mole	−44.5	−52.7
$\Delta S^{\ddagger}$,[f] cal/deg mole	−51.9	−60.1

[a] In 25% acetonitrile, I = 0.065 M. [b] On the flat of the pH–rate profile. [c] pc_H 2.38, essentially on the flat of the pH–rate profile. [d] T = 25°. [e] T = 25°, assuming one water molecule, acting as a nucleophile, in the transition state. [f] T = 25°, assuming two water molecules in the transition state, one acting as a nucleophile, the other as a general base.

Ethyl Trifluoroacetate-*carbonyl*-O^{18}. The rates of hydrolysis and deuterolysis of ethyl trifluoroacetate were measured under acidic conditions. The kinetic data are summarized in Table V. Errors of approximately 10% in the rate constants measured spectrophotometrically resulted from the small absorbance changes ($\Delta\epsilon_{231}$ 10 M^{-1} cm^{-1}). Errors in the rate constants determined on the titrator were about 15%, because the high organic solvent concentration limited the rate at which the solutions could be stirred without frothing. Rate constants were lowered by a factor of about three in deuterium oxide, indicating the expected general catalysis. The D_2O effect is in agreement with the findings of Jencks and Carriuolo[6] and of Bunton and co-workers[25] with similar compounds.

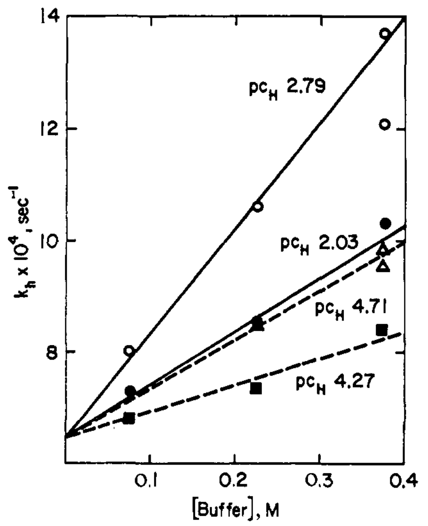

Figure 6. Plot of pseudo-first-order rate constants for the hydrolysis of ethyl trifluoroacetate at 25° in 25% acetonitrile, I = 0.375 M, *vs.* total phosphate buffer, ———, and total dimethylhydroxylamine buffer, - - - -, concentration.

In buffered systems, the rate constant shows a direct proportionality to the buffer concentration at constant ionic strength, increasing more rapidly in a given buffer at a higher pH (Figure 6), suggesting that the hydrolysis is catalyzed by the basic form of the buffer. The second-order catalytic constants calculated from the slopes of Figure 6 were used to obtain a rough approximation to the Brønsted β coefficient; the value of β was ~0.3. Unfortunately, the number of buffers available for use in the pH region of interest was severely limited by the unfavorable spectral characteristics of the ester.

Table V. Pseudo-First-Order Rate Constants for the Hydrolysis and Deuterolysis of Ethyl Trifluoroacetate[a]

pc_H	$k_h \times 10^4$, sec^{-1}	pc_D	$k_d \times 10^4$, sec^{-1}	k_h/k_d
1.20	7.1	1.23	2.3	3.2
1.37	6.9			
1.59	6.9			
2.00	5.9			
2.02	6.5			
2.27	6.0	2.10	1.8	3.4
3.08	5.6[b]			
3.15	7.0[c]			
3.38	5.5[b]			
5.21	6.8[b]			

[a] In 25% acetonitrile, I = 0.065 M, 25.0°. [b] Rate constants determined on an automatic titrator. [c] Extrapolation of rate constants in phosphoric acid–potassium dihydrogen phosphate buffer to zero buffer concentration.

The pseudo-first-order rate constants in strong acid medium are directly proportional to the hydrogen ion concentration at constant ionic strength, as other workers have shown,[26] indicating the importance of specific hydronium ion catalysis under these conditions. From pc_H = 2 to above 5, however, the water rate is virtually independent of the acid concentration (Table V and Figure 6).

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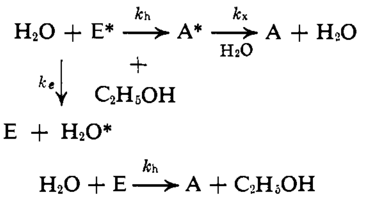
(25) C. A. Bunton, N. A. Fuller, S. G. Perry, and V. J. Shiner, *J. Chem. Soc.*, 2918 (1963).

(26) G. Gorin, O. R. Pierce, and E. T. McBee, *J. Am. Chem. Soc.*, 75, 5622 (1953).

The temperature dependence of the hydrolysis of ethyl trifluoroacetate was investigated at $pc_H = 2.38$. The Arrhenius plot was linear (Figure 5), and the activation parameters were calculated from the slope (Table IV). The activation energy so determined is in reasonably good agreement with values determined by other workers under different experimental conditions.[26,27] The similarities of Table IV are strong evidence that the rate-limiting step for both reactions is attack of water at the carbonyl carbon of the ester.

The simultaneous hydrolysis and carbonyl oxygen exchange of ethyl trifluoroacetate-*carbonyl*-O^{18} can be represented as shown in eq 19, where the symbols have the meanings defined for eq 9. A comparison of eq 19 with eq 9 establishes that eq 9 is simply a particular case of eq 19 involving the formation of tetrahedral intermediates and the pH dependencies of k_h and k_e. For the general scheme shown in eq 19, eq 17 states the relationship between R, the per cent of oxygen-18-labeled acid molecules at completion of the ester hydrolysis, and R_0, the initial per cent of oxygen-18-labeled ester molecules, and the various over-all rate constants describing the system. The k_e/k_h ratios at different values of pH can be calculated using eq 17 if k_h and k_x are known.



$$\begin{tikzcd} H_2O + E^* \arrow[r, "k_h"] \arrow[d, "k_e"'] & A^* \arrow[r, "k_x", "H_2O"'] & A + H_2O \\ E + H_2O^* & + C_2H_5OH & \end{tikzcd} \qquad (19)$$

$$H_2O + E \xrightarrow{k_h} A + C_2H_5OH$$

The oxygen-18 exchange experiments with ethyl trifluoroacetate-*carbonyl*-O^{18} at 25.0° are summarized in Table VI. Calculated values of k_e/k_h are depicted in the last column. It is evident from these values that the k_e/k_h ratios do not show a measurable hydrogen ion dependence in the pc_H range investigated. The k_e/k_h ratios reported in Table VI are approximately sevenfold larger than the ratio reported by Bunton and co-workers[25] for the hydrolysis of methyl trifluoroacetate in initially neutral 60% dioxane. The disparity in k_e/k_h in the two systems is in the expected direction for the different organic solvent concentrations.[28] Oxygen exchange experiments at the two highest values of pc_H were carried out in different concentrations of acetate buffer. Since the results are identical within experimental error, the k_e/k_h ratios do not exhibit a measureable buffer effect in this concentration range.

Table VI. Carbonyl Oxygen Exchange of Ethyl Trifluoroacetate-*carbonyl*-O^{18} with Water[a]

pc_H	Time × 10^{-3}, sec	$k_h \times 10^4$, sec^{-1}	$k_x \times 10^6$, sec^{-1}	R_{meas}	$(R/R_0)_f$[b]	k_e/k_h
1.17	7.80	7.4	34	0.33	0.49	0.6 ± 0.2
1.91	9.66	6.5	6.1	0.37	0.55	0.7 ± 0.2
4.89[c]	8.70	6.2	0.00	0.45	0.67	0.5 ± 0.1
4.85[d]	13.20	6.2	0.00	0.42	0.63	0.6 ± 0.1

[a] In 25% acetonitrile, $I = 0.065$ *M*, 25.0°. [b] $R_0 = 1.33 \pm 0.02\%$ excess. [c] Buffered with 0.035 *M* acetete. [d] Buffered with 0.060 *M* acetate.

In order to determine whether a deuterium isotope effect on the partitioning ratio occurs, an oxygen exchange experiment was carried out in deuterium oxide solution, buffered with 0.060 *M* acetate at $pc_D = 5.37$, containing 25% acetonitrile, $I = 0.065$ *M*. The buffer ratio was the same as in the water experiments at $pc_H = 4.85$. No oxygen exchange of the product was expected to occur under these conditions. $(R/R_0)_f$ in this experiment was 0.85, and k_e/k_h was 0.2 ± 0.1. A comparison with Table VI shows that in D_2O the amount of exchange is two- to threefold less than in water.

The temperature dependence of the k_e/k_h ratio was examined in 0.060 *M* acetate buffer, pc_H about 4.9. The results, presented in Table VII, show no discernible temperature effect on the partitioning ratio. Therefore, within an experimental error of approximately 1 kcal/mole, there is no difference in the activation energies for loss of alkoxide or hydroxide from ethyl trifluoroacetate under the conditions of these experiments.

Table VII. Temperature Dependence of k_e/k_h in the Hydrolysis of Ethyl Trifluoroacetate-*carbonyl*-O^{18} [a]

Temp, °C	Time, sec	R_{meas}	$(R/R_0)_f$[b]	k_e/k_h
4.6	24,300	0.42	0.63	0.6 ± 0.1
25.0	13,200	0.42	0.63	0.6 ± 0.1
54.8	2,100	0.46	0.68	0.5 ± 0.1

[a] In 25% acetonitrile, $I = 0.065$ *M*, pc_H 4.9 [b] $R_0 = 1.33 \pm 0.02\%$ excess.

Oxygen-18 Exchange Experiments with α-Chymotrypsin. The results of the enzyme experiments are compiled in Table VIII, which shows for each substrate the per cent of oxygen-18-labeled acid molecules at completion of hydrolysis. The per cent of labeled starting material for each reaction is stated in the final column. In no case was oxygen exchange observed.

Table VIII. Oxygen-18 Exchange Experiments with α-Chymotrypsin

Substrate	Time, hr	% O^{18}-labeled Acid product	 Starting material
N-*trans*-Cinnamoyl-imidazole-O^{18}	3.5	0.70 ± 0.02	0.70 ± 0.01
N-*p*-Nitrobenzoyl-imidazole-*carbonyl*-O^{18}	5.5	1.85 ± 0.02	1.83 ± 0.02

It was postulated in the Experimental Section that the synthesis of the acylimidazoles occurred without label dilution. This hypothesis is confirmed by the data of Table VIII, since the per cents of labeled acid product resulting from the enzyme-catalyzed hydrolyses of the substrates were identical with the assumed per cents of labeled starting material.

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(27) A. Moffat and H. Hunt, *J. Am. Chem. Soc.*, **79**, 54 (1957).

(28) M. L. Bender and R. D. Ginger, *Suomen Kemistilehti*, B33, 25 (1960).

Model Enzyme Studies. The alkaline hydrolysis of O-cinnamoyl-N-acetylserinamide-*cinnamoyl-carbonyl*-O^{18} was accompanied by carbonyl oxygen exchange with water. For this reaction, eq 17 reduces to eq 20, as oxygen exchange of the acid products with water is negligible. The results are shown in Table IX.

$$(R/R_0)_f = k_h/(k_h + k_e) \qquad (20)$$

Table IX. Carbonyl Oxygen Exchange in the Alkaline Hydrolysis of O-Cinnamoyl-N-acetylserinamide-*cinnamoyl-carbonyl*-O^{18}

$k_{OH} \times 10^2$, M^{-1} sec^{-1}	R_{meas}	$(R/R_0)_f{}^a$	k_e/k_h
1	0.57	0.81	0.23 ± 0.05
	0.61	0.87	0.15 ± 0.05

[a] $R_0 = 0.70 \pm 0.01\%$ excess.

The values of k_e/k_h for this compound fall within the range of known values for alkaline hydrolyses of several simple benzoate esters.[1]

Discussion

The general base catalyzed hydrolysis of ethyl trifluorothiolacetate has been investigated both by carbonyl oxygen exchange measurements and by kinetic measurements. A substantial agreement between the results obtained by these two approaches is seen in Table X, where the partitioning of the tetrahedral intermediate to reactants, k_e, and to products, k_h, is shown. Within experimental error the two methods show identical partitioning ratios. The agreement between the kinetic and oxygen exchange measurements may be seen in a more rigorous manner by a comparison of the observed $(R/R_0)_f$ values from oxygen exchange and those calculated from the kinetics (Table II). Here the limits of precision are higher and the agreement is quite good.

Table X. Comparison of the k_e/k_h Ratios Obtained from Oxygen-18 Exchange Experiments of Ethyl Trifluorothiolacetate-O^{18} with Kinetic Predictions[a]

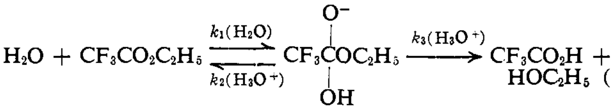
	k_e/k_h	
pc_H	Oxygen-18 exchange[b]	Kinetics[c]
1.14	0.4 ± 0.2	0.50 ± 0.06
1.40	0.3 ± 0.1	0.28 ± 0.04
1.84	0.07 ± 0.06	0.10 ± 0.02
5.00	0.00 ± 0.00	0.00 ± 0.00

[a] In 40% tetrahydrofuran, 25.0°, $I = 0.065$ M. [b] Calculated using eq 17. [c] Calculated using eq 18.

The excellent agreement between the oxygen exchange and kinetic results indicates that the same intermediate is being observed by both methods. The kinetic identification of the intermediate demands that it lie on the pathway of ester hydrolysis and not be a blind alley intermediate. Therefore, the intermediate observed by carbonyl oxygen exchange in this reaction also lies on the pathway of ester hydrolysis.

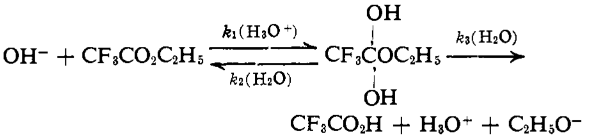
The carbonyl oxygen exchange measurements point directly to a tetrahedral intermediate. The kinetic experiments, while indicating an intermediate, do not rigorously demand that the intermediate be tetracovalent. The combination of the two experiments requires both that there be an intermediate and further that it be a tetrahedral addition compound. Furthermore, as pointed out above, this intermediate must lie on the reaction pathway.

Two mechanisms for the hydrolysis of ethyl trifluoroacetate in moderately acidic media are consistent with the kinetic and oxygen exchange data. The first mechanism is shown in eq 21. In this formulation, water assisted by a general base attacks the carbonyl carbon



$$H_2O + CF_3CO_2C_2H_5 \underset{k_2(H_3O^+)}{\overset{k_1(H_2O)}{\rightleftharpoons}} CF_3\overset{O^-}{\underset{OH}{C}}OC_2H_5 \xrightarrow{k_3(H_3O^+)} CF_3CO_2H + HOC_2H_5 \qquad (21)$$

atom of the ester, forming an intermediate which can decompose either to products or to reactants by acid catalysis. The second mechanism involves a general acid-catalyzed attack of hydroxide ion on the carbonyl carbon of the ester forming an intermediate which can decompose either to reactants or to products by general base catalysis (eq 22).



$$OH^- + CF_3CO_2C_2H_5 \underset{k_2(H_2O)}{\overset{k_1(H_3O^+)}{\rightleftharpoons}} CF_3\overset{OH}{\underset{OH}{C}}OC_2H_5 \xrightarrow{k_3(H_2O)} CF_3CO_2H + H_3O^+ + C_2H_5O^- \qquad (22)$$

In both of these mechanisms, partitioning of the intermediate is completely symmetrical, *i.e.*, decomposition of the intermediate to products and to reactants is a catalyzed process, the same type of catalysis being executed in both steps. This conclusion is indicated by the results of Table VI in which the k_e/k_h ratio shows no hydrogen ion dependence. In contrast, it is evident from Table X that in a general base catalyzed ester hydrolysis in which the partitioning of the intermediate is not symmetrical, the partitioning ratio depends directly on the hydrogen ion concentration. It is interesting to note here that the k_e/k_h ratios for the acidic and alkaline hydrolyses of ethyl benzoate differ by only about twofold, the ratio in acid being larger, whereas the second-order rate constant for alkaline hydrolysis is approximately 10^4 times greater than the corresponding rate constant for acid conditions.[29] In these latter reactions, the lack of a pH dependence of the k_e/k_h ratio can be explained readily by assuming that there is no acid or base catalysis of the breakdown of the intermediate either to products or to reactants. Thus, three types of partitioning of intermediates in ester hydrolysis are envisioned, depending on the compound studied and the reaction conditions used: uncatalyzed, unsymmetrically catalyzed, and symmetrically catalyzed.

Bender and Thomas[30] proposed that proton transfer within a tetrahedral intermediate might occur with rates comparable to the rate of breakdown of the intermediate to reactants or to products. For the mechanism depicted in eq 23 this proposal implies that the k_e/k_h ratio is given by eq 24, which takes account of proton transfers within the intermediate.

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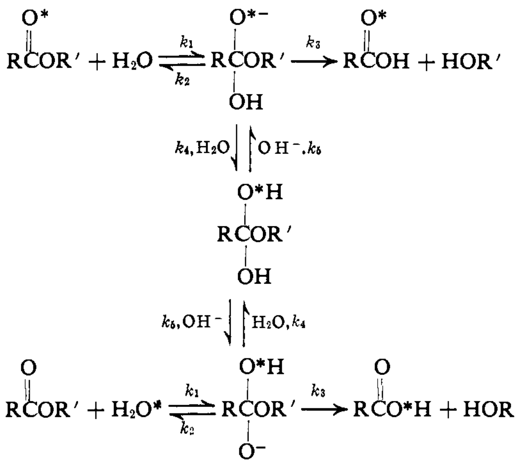
(29) M. L. Bender, R. D. Ginger, and J. P. Unik, *J. Am. Chem. Soc.*, **80**, 1044 (1958).

(30) M. L. Bender and R. J. Thomas, *ibid.*, **83**, 4189 (1961).



(23)

$$k_e/k_h = (k_2/2k_3)[k_4/(k_2 + k_3 + k_4)] \qquad (24)$$

If the proton transfer of step k_4 occurs much faster than the decomposition steps k_2 and k_3, one would not expect to observe a deuterium isotope effect on the k_e/k_h ratio. This follows from eq 24, since under these conditions,

$$k_e/k_h = k_2/2k_3 \qquad (25)$$

and, because k_2 and k_3 are catalyzed in an identical manner in ethyl trifluoroacetate, there should be a net cancellation of isotope effects on the k_2/k_3 ratio, resulting in no isotope effect for k_e/k_h.

On the other hand, from eq 24, a decrease in k_e/k_h would occur in deuterium oxide provided that the $(k_2 + k_3)/k_4$ ratio increases in that solvent. That $(k_2 + k_3)/k_4$ should increase in D_2O is shown by the following considerations: first, k_2 for the thiol ester is known to increase in D_2O by a factor of 1.7.[4] As the D_2O effect on the reverse step, k_1, is the same for the two esters, it is reasonable to suppose that k_2 (and hence k_3) would show the same increase for the oxygen ester as for the thiol ester in deuterium oxide. Second, since k_4 represents a proton transfer from water to an anionic intermediate, it can be expected to show a kinetic isotope effect, the magnitude of which will depend on the pK_a of the intermediate. A reasonable value for k_4^D/k_4^H is perhaps $^1/_3$. Consequently

$$[(k_2 + k_3)/k_4]^D/[(k_2 + k_3)/k_4]^H =$$
$$\frac{(k_2 + k_3)^D}{(k_2 + k_3)^H}\frac{k_4^H}{k_4^D} = 1.7 \times 3 = 5 \qquad (26)$$

The calculation of eq 26 and the observed two- to threefold decrease of k_e/k_h in D_2O indicate that $(k_2 + k_3)/k_4$ is approximately equal to unity. From the data of Eigen and co-workers,[31] k_4 is probably about 10^7 sec^{-1}, hence k_2 and k_3 should also be of this order of magnitude.

The lack of oxygen exchange in the deacylations of acyl-α-chymotrypsins can be interpreted in several ways. It is possible that deacylation is a concerted process not involving an addition intermediate. This explanation seems unlikely on the following grounds: first, the alcoholysis of an acyl enzyme is very probably a symmetrical process involving an intermediate.[7] A number of experiments suggest that the hydrolysis reaction is mechanistically similar to the alcoholysis.[32] Second, the hydrolysis of acyl-α-chymotrypsins appears to be a general base catalyzed cleavage of an ester bond.[7] The general base catalyzed hydrolysis of ethyl trifluoroacetate is a symmetrical process and proceeds through an addition intermediate. Third, a nonlinear Hammett plot was reported by Caplow and Jencks[17] for the deacylation of substituted benzoyl-α-chymotrypsins. A possible explanation for this phenomenon is that the reaction proceeds in two steps. Formation of the intermediate would generally be rate limiting, but with increasingly electronegative substituents in the acyl group, decomposition could become partially rate determining. This interpretation is supported by the work of Bender and Thomas[30] on the electronic effects of acyl group substituents in oxygen exchange reactions.

Assuming, therefore, that deacylation is a stepwise process, there are two possible explanations for the lack of simultaneous oxygen exchange. Either the intermediate breaks down too rapidly for exchange to occur, or, due to specific interactions with the asymmetric enzyme surface, the two oxygens never become equivalent in the addition intermediate, and thus are unable to undergo exchange. The former possibility does not seem likely in view of the oxygen exchange which was observed in the alkaline hydrolysis of O-cinnamoyl-N-acetylserinamide-*cinnamoyl-carbonyl*-O^{18}. There is no independent evidence to support or disprove the latter explanation at the present time. Since the preceding hypothesis appears, however, to be less likely, we tentatively favor the last interpretation for our results.

(31) M. Eigen and L. de Maeyer in "Technique in Organic Chemistry," S. L. Friess, E. S. Lewis, and A. Weissberger, Ed., Interscience Publishers, Inc., New York, N. Y., 1963, p 1035.

(32) M. L. Bender, G. E. Clement, C. R. Gunter, and F. J. Kézdy, *J. Am. Chem. Soc.*, **86**, 3697 (1964).