$$Cr(CO)_6 \xrightarrow[-CO]{h\nu\ (353\ nm)} Cr(CO)_5 \xrightarrow[k = 4 \pm 1 \times 10^9\ dm^3\ mol^{-1}\ s^{-1}]{+\ Cr(CO)_6} Cr_2(CO)_{11} \quad (1)$$

The reactivity of the pentacarbonyl suggests that it is at best only weakly coordinated to the perfluoro solvent. Indeed it is noteworthy that its wavelength of maximum absorption (620 ± 10 nm) is close to that (624 nm) reported for neon matrices,[2] where presumably it is "naked". In $Cr_2(CO)_{11}$, the coordination of the pentacarbonyl to the hexacarbonyl is presumably through a carbonyl oxygen of the latter compound.

The rate of decay of $Cr(CO)_5$ is accelerated by flushing the solution with carbon monoxide. Thus the half-life of the pentacarbonyl formed by irradiating a 2.6×10^{-3} M solution of $Cr(CO)_6$ is 13 ns when flushed with carbon monoxide compared with a half-life of 38 ns for an argon-flushed solution. Assuming that the concentration of carbon monoxide under these conditions is 1.2×10^{-2} M,[4] we may derive a rate constant of $3 \pm 1 \times 10^9$ dm^3 mol^{-1} s^{-1} for

$$Cr(CO)_5 + CO \rightarrow Cr(CO)_6 \quad (2)$$

This rate constant is a thousand times larger than that reported for chromium pentacarbonyl in cyclohexane.[1a] The rate of decay of $Cr(CO)_5$ is also enhanced in the presence of nitrogen, presumably owing to the formation of the complex $Cr(CO)_5(N_2)$.

It was previously reported that the species assigned as chromium pentacarbonyl in cyclohexane solution (λ_{max} 503 ± 5 nm) was formed within 50 ns of the excitation of the hexacarbonyl.[1a] The present study confirms this observation and shows that in cyclohexane the pentacarbonyl complex is formed within the 5-ns excitation pulse. Further we have been able to measure the rate constant of addition of cyclohexane to $Cr(CO)_5$ in perfluoromethylcyclohexane. Thus on excitation of a solution of $Cr(CO)_6$ in C_7F_{14} containing $\sim 10^{-2}$ M cyclohexane, a rapid decay of the 620-nm species is accompanied by the formation of a species with a broad band centered at 510 ± 10 nm, a spectrum similar to that reported for $Cr(CO)_5$ in cyclohexane.[1a,b] The observed reaction appears to be the production of a cyclohexane complex of $Cr(CO)_5$:

$$Cr(CO)_5 + c\text{-}C_6H_{12} \rightarrow Cr(CO)_5(c\text{-}C_6H_{12}) \quad (3)$$

From the dependence of the rate of decay of $Cr(CO)_5$ on the cyclohexane concentration (Figure 2b), a rate constant of 2.0 ± 0.5 × 10^9 dm^3 mol^{-1} s^{-1} has been derived. The formation of this complex is presumably the reason for the appreciably slower reaction of $Cr(CO)_5$ with carbon monoxide in cyclohexane solution compared with that in C_7F_{14} and for the lack of observation of the formation of $Cr_2(CO)_{11}$ in hydrocarbon solvents. It is also probable that formation of similar hydrocarbon complexes affects the rate of reaction of catalytically important coordinatively unsaturated species.

Short-lived transients are also observed with $W(CO)_6$ and $Mo(CO)_6$, and we are currently investigating these systems, as well as determining the stability constants for $Cr_2(CO)_{11}$ and $Cr(CO)_5(C_6H_{12})$ in perfluoromethylcyclohexane.

Acknowledgments. We thank Professor Jacques Joussot-Dubien for his encouragement and the C.N.R.S. (France) and the N.B.S.T. (Ireland) for support for J.M.K. under the Irish–French Scientific Exchange Scheme.

References and Notes

(1) (a) Kelly, J. M.; Hermann, H.; Koerner von Gustorf, E. *J. Chem. Soc., Chem. Commun.* **1973**, 105–106. (b) Kelly, J. M.; Bent, D. V.; Hermann, H.; Schulte-Frohlinde, D.; Koerner von Gustorf, E. *J. Organomet. Chem.* **1974**, *69*, 259–269. (c) Hughey, J. L.; Bock, C. R.; Meyer, T. J. *J. Am. Chem. Soc.* **1975**, *97*, 4440–4442.
(2) (a) Perutz, R. N.; Turner, J. J. *J. Am. Chem. Soc.* **1975**, *97*, 4791–4800. (b) Turner, J. J.; Burdett, J. K.; Perutz, R. N.; Poliakoff, M. *Pure Appl. Chem.* **1977**, *49*, 271–285
(3) At low concentrations of $Cr(CO)_6$ an additional reaction is responsible for the decay of the 620-nm transient. We are currently improving our purification and sample preparation procedures to determine whether this transient is formed by reaction of $Cr(CO)_5$ with impurities.
(4) This value is calculated for 22 °C using data in ref 5.
(5) Gjaldbaek, J. C.; Hildebrand, J. H. *J. Am. Chem. Soc.* **1949**, *71*, 3147–3150. Gjaldbaek, J. C. *Acta Chem. Scand.* **1952**, *6*, 623–633.

Roland Bonneau
Laboratoire de Chimie Physique
Université de Bordeaux I, Talence, France

John M. Kelly*
Chemistry Department, Trinity College
University of Dublin, Dublin 2, Ireland

Received August 29, 1979

A Novel ^{18}O Kinetic Isotope Effect in an α-Chymotrypsin Catalyzed Transesterification

Sir:

The ability of serine proteases to catalyze the transesterification of esters and amides is well known.[1] In studying the α-chymotrypsin catalyzed ethanolysis of *p*-nitrophenyl 2-(5-*n*-propyl)furoate we have observed a novel [^{18}O]ethanol kinetic isotope effect (KIE). Shortly after initiation of the reaction the KIE is normal ($k^{16}_{app} > k^{18}_{app}$), then decreases with time to become inverse ($k^{16}_{app} < k^{18}_{app}$), and levels off at a magnitude greater than any ^{18}O KIE reported heretofore.

Reactions were started by injecting the nitrophenyl ester into a stoppered vial immersed in a constant-temperature bath, and containing a mixture of [^{16}O]- plus [^{18}O]ethanol, α-chymotrypsin, and buffer. (See legend to Figure 1 for typical concentrations. Enzyme was purchased from Worthington Biochemical Corp. and used with no further purification; [^{18}O]-ethanol was synthesized by the procedure of Sawyer[2] and the ^{18}O enrichment determined as described below; substrate ester was synthesized as described previously.[3]) As the reaction proceeded, samples were withdrawn through the rubber cap into a Hamilton syringe, and injected promptly into a Hewlett-Packard Model 5840 gas chromatograph (GC) connected to a MP5985 quadrupole mass spectrometer (MS) controlled by an MP 21 MXE data acquisition and processing system. The GC was equipped with a 6-ft glass column packed with 10% AT-1000 on 80–100 mesh Chromosorb W/AW (Johns-Manville Co.). The MS was operated to scan a small number of preselected *m/e* ratios repetitively throughout the chromatographic separation. The intensities from individual ions were then displayed as a function of time to yield chromatographic peaks at specific *m/e*, usually chosen as 46 and 48 for the parent molecular ions of [^{16}O]- and [^{18}O]ethanol and as 182 and 184 for the corresponding ions of the reaction product ethyl 2-(5-*n*-propyl)furoate. Relative ^{18}O enrichments were computed from the ratios of the peak areas, and the apparent KIE then calculated from the ratio of enrichments in alcohol and ester.

Results obtained at 4 °C are presented in Figure 1. The first point after starting the reaction shows a normal KIE. Because of the very small amount of product formed at this time, the S/N is low, and the magnitude of the KIE is uncertain; however, a normal KIE shortly after reaction initiation has been observed in more than a dozen experiments conducted at various temperatures. The KIE then decreases to a plateau value, which in this experiment was 0.85, before the reaction stops. The values of the plateau KIE were ≤0.90 for all MS–GC experiments, while the estimated standard deviations varied from 0.01 to 0.06.

0002-7863/80/1502-1221$01.00/0

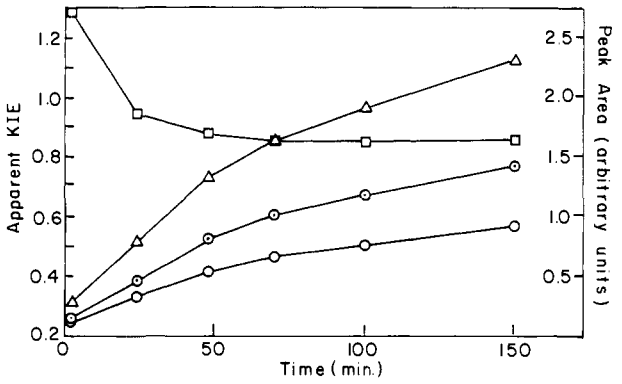


Figure 1. Time dependence of the apparent ^{18}O KIE during ethanolysis of 2-(5-*n*-propyl)furoylchymotrypsin. The procedure is given in the text; temperature = 4.0 °C; initial concentrations of reactants in approximately 55 μL of solution are 60–80 μM α-chymotrypsin, 1.5 M ethanol, 3 mM *p*-nitrophenyl 2-(5-*n*-propyl)furoate, 0.05 M phosphate, and 0.025 M borate with an adjusted pH (before addition of ethanol) of 9.0. Note that the apparent KIE as defined in the text is not identical with the true KIE until the former reaches a plateau value at ~70 min. Ester formation is complete when the total product reaches a peak area, of ~2.5. □, apparent KIE; △, total product; ⊙, ^{18}O product; ○, ^{16}O product.

We know of no previous report of a time dependent KIE, and the magnitude, 1.08, of the largest ^{18}O KIE in the literature is only 8%.[4] For two other enzymatic reactions reported, ^{18}O KIE's fall in the range of 1.01 to 1.02.[5,6] Because of the lack of precedents, we have carefully considered possible experimental artifacts.

The initial ethanol concentration was close to 500× greater than that of substrate in all the MS–GC experiments, and the reaction vial was closed to minimize evaporation; thus, depletion of [^{16}O]ethanol cannot explain the drop in KIE as the reaction proceeds. The same decrease was seen when [^{18}O]-ethanol was diluted either with redistilled commercial ethanol or with unenriched ethanol synthesized by the method used to make the enriched material. This suggests that possible contamination introduced with the ethanol is unimportant. Contamination of substrate and enzyme was also considered. No *m/e* peaks at 182 or 184 were observed when a 10-fold greater enzyme concentration (~600 μM) was used in the absence of substrate, nor were these peaks seen when ethanol was omitted; thus, there is little or no ethanol contamination of enzyme and substrate. Other reasons for ruling out an artifact due to contamination are (i) the reaction mixture was separated by GC prior to analysis; (ii) total mass spectra taken throughout the ester product peak indicated no composition change; (iii) peak integration with base-line subtraction compensated for both drift and column bleeding. (Peak integration also eliminated a possible error due to differences in retention times between ^{16}O and ^{18}O compounds, which in case of the ester product was ~0.2 s and was undetectable for ethanol.)

Nonenzymatic ethanolysis occurs concomitantly with the enzyme-catalyzed reaction. However, the former displays a small, or nonexistent, KIE (Table I) which is also time independent. Additionally, the enzyme concentration was sufficiently large to minimize the contribution of the nonenzymatic ethanolysis.

The KIE computation is based upon the independent measurements of ^{18}O enrichment in ethanol and ethyl ester product. This allows for potential errors from sources such as a machine artifact associated with measurements at widely different *m/e*, or a significant KIE associated with fragmentation in the MS. To eliminate this concern, ^{18}O enrichment was determined first in chemically synthesized ethyl 2-(5-*n*-propyl)furoate and then in the ethanol produced by mild alkaline hydrolysis of this ester. The two determinations were the same within experimental error.

Table I. ^{18}O KIE for the Ethanolysis of *p*-Nitrophenyl 2-(5-*n*-Propyl)furoate

enzyme	assay method	temp, °C	KIE
−	GC-MS[a]	2.0	0.99 (0.02)
		25.0	0.97 (0.02)
+	GC-MS[a]	4.0	0.85 (0.01)
		24.5	0.90 (0.02)
		31.5	0.86 (0.02)
+	steady state	22.0[b]	0.91 (0.09)
	p-nitrophenol release	25.0[c]	0.88 (0.06)
		32.0[c]	0.82 (0.04)

[a] Conditions are those of Figure 1. The KIE was computed using the formula $KIE = (A_{48}/A_{46})/[(A_{184}/A_{182}) - (A_{184}/A_{182})_N]$ where *A* refers to the integrated peak area for the *m/e* ion indicated in the subscript, and the ratio $(A_{184}/A_{182})_N$ is the natural abundance results obtained with nonenriched ethyl ester. [b] Initial concentrations of reactants: 80 μM α-chymotrypsin, 3.1 mM *p*-nitrophenyl 2-(5-*n*-propyl)furoate, 0.01 M $Na_2B_4O_7$ of pH 9.1, and 0–0.84 M ethanol (either natural abundance, or 75% ^{18}O enriched). The alcohol was added from stock solutions, the concentrations of which were determined using liver alcohol dehydrogenase and NAD.[11] Observed KIE was corrected for the 75% ^{18}O enrichment by the formula $KIE = [1 + 4(k^{18}/k^{16} - 1)/3]^{-1}$. [c] Initial concentrations of reactants: 5.3 μM α-chymotrypsin, 1.6 mM *p*-nitrophenyl 2-(5-*n*-propyl)furoate, 0.05 M phosphate, 0.025 M borate of pH 9.0, and 0–0.65 M ethanol (either natural abundance or 75% ^{18}O enriched.) The alcohol concentration was calculated from the known volumes of freshly distilled ethanol added to the stock solutions. Observed KIE was corrected as above. The Km for *p*-nitrophenyl 2-(5-*n*-propyl)furoate under the conditions of these experiments is <4 μM.[8]

A referee has suggested that the inverse KIE might arise when, near the end of the reaction, the ethyl ester product begins to compete as a substrate with the nitrophenyl ester. Were enzyme acylation to proceed with a normal KIE, and deacylation with no KIE, enrichment of ^{18}O into the ethyl ester might occur. This interpretation appears unlikely, since the ethyl ester is by far the poorer substrate. For example, at 25 °C the K_m of the nitrophenyl substrate in hydrolysis is ~0.7 μM. The K_i of the ethyl ester under the same conditions and measured as an inhibitor of the nitrophenyl ester reaction is 400 μM. Insofar as the ratio of substrate K_m to product K_i measures enzyme partitioning between the two, the ethyl ester cannot compete significantly for enzyme until the reaction is >97% complete. At all temperatures inversion of the apparent KIE occurs well before this point (e.g., Figure 1).

Finally, we determined the apparent KIE by an independent procedure. Addition of enzyme to substrate yields an initial burst release of *p*-nitrophenol (which was monitored by its absorbance at 400 nm) followed by a zero-order release due to the steady-state, rate-limiting breakdown of acylated enzyme.[7] The apparent first-order deacylation rate constant can be calculated from the magnitude of the initial burst and the slope of the zero-order absorbance increase.[8] This apparent rate constant varies linearly with the ethanol concentration permitting calculation of the second-order rate constant associated with formation of the ethyl ester product. Comparison of these rate constants obtained with natural abundance with ^{18}O-enriched ethanol yielded KIE values which were comparable with the plateau KIE results of the GC–MS experiments (Table I). The larger errors associated with the steady-state, spectrophotometric experiments make this procedure less reliable for establishment of the KIE. Nonetheless, independent support for the validity of the GC–MS results is provided.

Having found no experimental artifacts, we conclude that the observed kinetic isotope effects are real. An inverse KIE may be explained in one of two ways.[9] The first invokes a narrower potential energy profile in the transition state compared with reactants, but this cannot explain the time dependence observed by us. The second posits a preequilibrium iso-

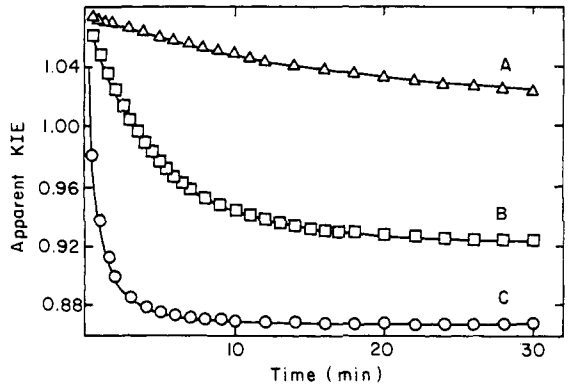
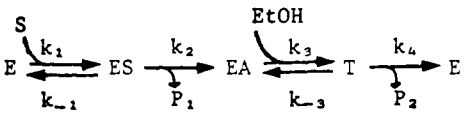


Figure 2. Simulated time dependence of apparent ^{18}O KIE. Simulated curves based on eq 1. $k_1 = 1.0 \times 10^8$, $k_{-1} = 1.0 \times 10^2$. $k_2 = 1.0$ values are identical in all cases, and associated KIE values are set to 1.0. A: $k_3^{16} = 0.005$, $k_{-3}^{16} = 0.05$, $k_4^{16} = 0.1$. B: $k_3^{16} = 0.12$, $k_{-3}^{16} = 0.5$, $k_4^{16} = 0.1$. C: $k_3^{16} = 0.001$, $k_{-3}^{16} = 5.0$, $k_4^{16} = 0.1$. $k_3^{16}/k_3^{18} = 1.075$, $k_{-3}^{16}/k_{-3}^{18} = 1.25$, $k_4^{16}/k_4^{18} = 1.0$ for all simulations.

tope effect in a multistep process. We assume a simple mechanistic model based upon that generally proposed for the serine proteases,[10] with the abbreviations E, enzyme; S, substrate;



(1)

ES, enzyme-substrate complex; P_1, *p*-nitrophenol; EA, acylated enzyme; T, a hypothetical species commonly believed to be the tetrahedral intermediate; and P_2, ethyl ester product. With substrate and ethanol in large excess, the rate equations for this scheme become linear, and the explicit integrated form of the equation for the production of P_2 can be obtained.[8] Using this equation, and assigning an arbitrary normal KIE to each of the constants k_3, k_{-3}, and k_4 and reasonable values to all six rate constants, one can generate simulated KIE data which resemble qualitatively the experimental results (Figure 2). The shape of the time-dependent curve depends upon the relative magnitudes of the rate constants, and two conditions are required for a switch from normal to inverse apparent KIE: $^{16}k_{-3}/^{18}k_{-3} > {}^{16}k_3/{}^{18}k_3$; k_4 not appreciably greater than k_{-3}. These simulated results (which are not intended to reproduce the experimental results quantitatively) support the interpretation that the inverse KIE observed by us is due to a kinetically significant intermediate between the acylated and free enzyme (eq 1). The simulation does not, however, account for the magnitude of the effect. If eq 1 is correct, then the shape of the experimental KIE curve may provide information about the relative magnitudes of the rate constants leading to and away from the hypothetical intermediate T.

Acknowledgments. We are grateful to The Ohio State University Instruction and Research Computer Center for providing computer time. M.H.K. is the recipient of a Research Career Development Award from the National Institutes of Health. Gifts from Dr. Henry Sonneborn III and Mrs. Marion Colwill toward the purchase of the GC–MS system are gratefully acknowledged.

References and Notes

(1) A. K. Balls and H. N. Wood, *J. Biol. Chem.*, **219**, 245 (1956); R. J. Foster *ibid.*, **236**, 2461 (1961); N. S. Issacs and C. Niemann, *Biochim. Biophys. Acta*, **44**, 196 (1960); R. M. Epand and I. B. Wilson, *J. Biol. Chem.*, **238**, 1718 (1963); P. W. Inward and W. P. Jencks, *ibid.*, **240**, 1986 (1965); M. L. Bender, G. E. Clement, C. R. Gunter, and F. J. Kezdy, *J. Am. Chem. Soc.*, **86**, 3697 (1964); F. Seydoux and J. Yon, *Eur. J. Biochem.*, **3**, 42 (1967); J. Fastrez and A. R. Fersht, *Biochemistry*, **12**, 2025 (1973); M. M. Werber and P. Greenzaid, *Biochim. Biophys. Acta*, **293**, 208 (1973).
(2) C. B. Sawyer, *J. Org. Chem.*, **37**, 4225 (1972).
(3) J. E. Baggott and M. H. Klapper, *Biochemistry*, **15**, 1473 (1976).
(4) H. L. Goering, J. T. Doi, and K. D. McMichael, *J. Am. Chem. Soc.*, **86**, 1951 (1964).
(5) C. B. Sawyer and J. F. Kirsch, *J. Am. Chem. Soc.*, **97**, 1963 (1975).
(6) S. Rosenberg and J. F. Kirsch, *Fed. Proc.*, **37**, 1296 (1978).
(7) B. S. Hartley and B. A. Kilby, *Biochem. J.*, **56**, 288 (1954); F. J. Kezdy and M. L. Bender, *Biochemistry*, **1**, 1097 (1962).
(8) Details to be published elsewhere.
(9) E. K. Thornton and E. R. Thornton in "Isotopes in Chemical Reactions", C. J. Collins and N. S. Bowman, Eds., Van Nostrand-Reinhold, New York (1970), p 213.
(10) M. L. Bender and J. V. Kilheffer, *Crit. Rev. Biochem.*, **1**, 149 (1973).
(11) N. O. Kaplan and M. Ciotti *Methods Enzymol.*, **3**, 253 (1955).

Chih-Lueh A. Wang, Charlotte M. Trout
K. C. Calvo, Michael H. Klapper*
Department of Chemistry, The Ohio State University
Columbus, Ohio 43210

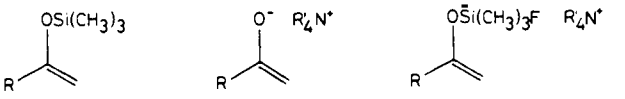
Lan K. Wong
Department of Pharmacology, College of Medicine
The Ohio State University, Columbus, Ohio 43210

Received October 16, 1978

Tris(dialkylamino)sulfonium Enolates

Sir:

Anion reactivity in aprotic solvents enhances greatly by crown ether complexation of the metal counter cation or replacement of the metal ion by an onium ion. Recently intermediacy of naked enolate **2** had been claimed as the highly reactive species in the reaction of enol silyl ether **1** and quaternary ammonium fluorides.[1,2] Rigorously, however, it has remained unclear whether the reactive intermediate is truly the ammonium enolate **2** or the alternative, pentacoordinate silicon species **3**.[3] In addition inevitable contamination of water molecules in the quaternary ammonium salts does not permit unambiguous judgment regarding the reaction mechanism. We disclose here that use of a tris(dialylamino)sulfonium difluorotrimethylsiliconate as a source of fluoride ion acting as a powerful siliconophile can remove this difficulty, providing direct evidence for generation of enolate species from enol silyl ethers and fluoride ion.



1 2 3

In order to examine this problem, we chose an enolate system which had been studied in detail by House.[4,5] The presence of a facile equilibrium outlined in Scheme I[6] allowed isolation of the enolate **8** utilizing a vacuum-line technique. An equimolar mixture of tris(diethylamino)sulfonium (TAS) difluorotrimethylsiliconate (**4**)[7] and the enol silyl ether **7** in THF solution was placed in a vessel connected with a vacuum line system. When this mixture was evacuated at 25 °C to 0.01 mmHg, 90% of the theoretical amount of fluorotrimethylsilane (**6**) was collected as THF solution in a cold trap (−78 °C). The residue obtained as extremely air-sensitive, yellowish crystals appeared

Scheme I

$$\underset{\mathbf{4}}{[(C_2H_5)_2N]_3S^+(CH_3)_3SiF_2^-} \rightleftarrows \underset{\mathbf{5}}{[(C_2H_5)_2N]_3S^+F^-} + \underset{\mathbf{6}}{(CH_3)_3SiF}$$

$$\underset{\mathbf{5}}{[(C_2H_5)_2N]_3S^+F^-} + \underset{\mathbf{7}}{(Z)\text{-}C_6H_5CH{=}C(CH_3)OSi(CH_3)_3} \rightleftarrows \underset{\mathbf{6}}{(CH_3)_3SiF} + \underset{\mathbf{8}}{[(C_2H_5)_2N]_3S^+(Z)\text{-}C_6H_5CH{=}C(CH_3)O^-}$$

0002-7863/80/1502-1223$01.00/0