Permeable Membrane/Mass Spectrometric Measurement of an Enzymatic Kinetic Isotope Effect: α-Chymotrypsin-Catalyzed Transesterification

K. C. Calvo, C. R. Weisenberger, L. B. Anderson, and Michael H. Klapper*

Contribution from the Department of Chemistry, The Ohio State University, Columbus, Ohio 43210. *Received October 18, 1982*

Abstract: The $^{16}O/^{18}O$ kinetic isotope effect (KIE) associated with an α-chymotrypsin-catalyzed transesterification was measured by using the technique of permeable membrane/mass spectroscopy. This almost in situ method is based upon the ability of the ester product, but not water and other polar compounds, to permeate through a dimethyl silicone membrane, which separates the aqueous reaction solution from the evacuated inlet of the mass spectrometer. The time course of both ^{16}O and ^{18}O product formation can be followed simultaneously, permitting rapid KIE measurements. With ^{18}O-substituted ethanol, the KIE for the formation of both ethyl 2-furoate and ethyl 5-*n*-propyl-2-furoate from the respective *p*-nitrophenyl esters is initially normal and decreases with time to reach within approximately 2 min at 25 °C, pH 8.5, a constant value of 1.009 ± 0.007 for the former and 0.90 ± 0.02 for the latter. The large inverse steady-state KIE associated with the formation of ethyl 5-*n*-propyl-2-furoate decreases when the temperature is raised or when the pH is lowered. The pH dependence can be fit with an apparent pK_a of 7.3. The $^{16}O/^{18}O$ KIE (ethyl ester oxygen) for the nonenzymatic, alkaline hydrolysis of ethyl 2-furoate was measured as 1.012 ± 0.010 at 25 °C.

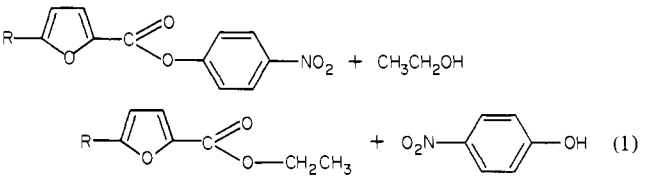
We undertook $^{16}O/^{18}O$ kinetic isotope effect (KIE) studies to learn more about the α-chymotrypsin deacylation pathway, having found that Arrhenius plots of k_{cat} are not linear in the temperature range of 5–40 °C for the enzyme-catalyzed hydrolysis and transesterification of *p*-nitrophenyl 5-*n*-alkyl-2-furoates.[1,2] These previous results are consistent with a proposal of two enzyme intermediates that, in rapid equilibrium with one another, can both turn over to release product. But the possibility that a temperature-dependent change in the rate-limiting step causes the observed nonlinearity could not be excluded. We, therefore, needed additional information concerning the number and nature of the elementary steps in the enzyme deacylation.

The substitution of one isotope for another perturbs a reacting system minimally, but can alter rates when the atom involved is at the reaction locus. This rate alteration, the KIE, may provide information about transition-state structures and reaction pathways.[3-5] When the substitution involves an atom other than hydrogen, the effect is small, and the direct comparison of rates obtained with the different isotopic reagents is difficult, although possible.[6-9] An alternate protocol is to mix the isotopic reagents together, thereby running the reactions simultaneously and minimizing experimental variability. The KIE can then be extracted by determining the difference in the isotopic ratios of reactants and products. This mixed isotope protocol may yield an observed KIE quite different from that obtained by direct measurement of rates in separate reactions. If the reaction is higher than first order overall, and if the isotopically separable compounds compete for the same reactant(s), then the mixed isotopic reactants are linked, affecting the observed KIE. Thus, both protocols may yield more information than either alone.[10]

The mixed protocol requires a method for detecting the isotopically separable compounds—commonly, mass spectrometry. Isotopic compositions of high precision can be determined with an isotope-ratio mass spectrometer.[11] Because of technical requirements, only volatile substances substances can be measured. Thus, the reaction under study must involve either a component that is, or that can be, converted to a small volatile analyte. A major advantage is that large isotope enrichments are not required, easing synthetic difficulties. For example, $^{13}C/^{12}C$ KIE measurements are possible with natural abundance reagents. If a small, volatile analyte is not available, reactants and/or products can be purified either in bulk[12] or by gas chromatography[13] and then introduced into a mass spectrometer. In any of these techniques, the time required for sample preparation before analysis precludes the measurement of many sample points over a small reaction interval. (Mass spectral measurements without prior separation of components have been performed, however, in the case of gas-phase reactions.[14])

An entirely different approach is based upon the perturbation observed when a reaction is started at chemical but not isotopic equilibrium.[15] If a fluorescing or light-absorbing compound is involved in the reaction, then the perturbation that follows the addition of isotopically substituted reagents can be measured spectrally, and the KIE can be determined with no need for a mass spectrometer. This method is limited, however, to those reactions in which there is a measurable equilibrium.

While studying α-chymotrypsin catalysis with the mixed isotope protocol, we had looked at the $^{16}O/^{18}O$ KIE (ethanol oxygen) in the transesterification of *p*-nitrophenyl 5-*n*-propyl-2-furoate (eq 1, R = $CH_3CH_2CH_2$). There was a large, >10%, inverse KIE



(1)

($^{18}k > {}^{16}k$) associated with the steady-state formation of the ethyl ester product.[13] Surprisingly, the initial product isotopic ratios (normalized to that of the starting ethanol) were normal, suggesting an initial normal KIE ($^{16}k > {}^{18}k$) that decreased with time to become inverse. The novelty of these results prompted us to attempt improvement in the precision of the data and to obtain better delineation of the time dependence for the apparent KIE.

To achieve these goals we have developed a new method based upon permeable membrane/mass spectroscopy (PM/MS). A description of the method together with its application to the

(1) Baggott, J. E.; Klapper, M. H. *Biochemistry* **1976**, *15*, 1473–1481.
(2) Wang, C.-L. A.; Calvo, K. C.; Klapper, M. H. *Biochemistry* **1981**, *20*, 1401–1408.
(3) Maggiora, G. M.; Schowen, R. L. In "Bioorganic Chemistry"; van Tamelan, E. E., Ed.; Academic Press: New York, 1977; Vol. 1, pp 173–229.
(4) Klinman, J. P. *Adv. Enzymol. Relat. Areas Mol. Biol.* **1978**, *46*, 415–494.
(5) Melander, L.; Saunders, W. H. "Reaction Rates of Isotopic Molecules"; Wiley: New York, 1980.
(6) Mitton, C. G.; Schowen, R. L. *Tetrahedron Lett.* **1968**, 5803–5806.
(7) Gorenstein, D. G. *J. Am. Chem. Soc.* **1972**, *94*, 2523–2525.
(8) Rosenberg, S.; Kirsch, J. F. *Anal. Chem.* **1979**, *51*, 1375–1379.
(9) Rosenberg, S.; Kirsch, J. F. *Anal. Chem.* **1979**, *51*, 1379–1383.
(10) Rosenberg, S.; Kirsch, J. F. *Biochemistry* **1981**, *20*, 3189–3196.
(11) O'Leary, M. H. *Methods Enzymol.* **1980**, *64*, 83–104.
(12) Sawyer, C. B.; Kirsch, J. F. *J. Am. Chem. Soc.* **1973**, *95*, 7375–7381.
(13) Wang, C.-L. A.; Trout, C. M.; Calvo, K. C.; Klapper, M. H.; Wong, L. K. *J. Am. Chem. Soc.* **1980**, *102*, 1221–1223.
(14) Kwart, H. *Acc. Chem. Res.* **1982**, *15*, 401–408.
(15) Cleland, W. W. *Methods Enzymol.* **1980**, *64*, 104–125.

0002-7863/83/1505-6935$01.50/0

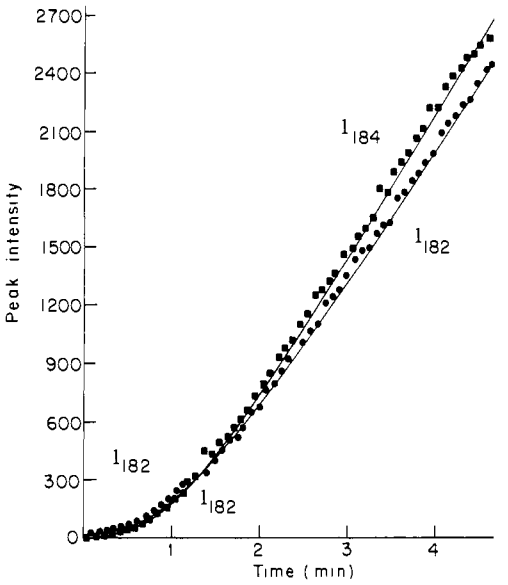


Figure 1. α-Chymotrypsin-catalyzed transesterification of *p*-nitrophenyl 2-(5-*n*-propyl)furoate to the ethyl ester: (●) mass spectral peak intensity of the ^{16}O ethyl ester parent ion; (■) mass spectral peak intensity of the ^{18}O ethyl ester: Conditions are described in Table II; R for this experiment was 0.94. The lines drawn through the points are arbitrary and are used only to aid in visualization.

measurement of reaction 1 has been published earlier.[16] We report here the PM/MS measurement of the KIE's for the α-chymotrypsin-catalyzed transesterification of *p*-nitrophenyl 2-furoate and *p*-nitrophenyl 5-*n*-propyl-2-furoate and the temperature and pH dependence of the KIE for the latter reaction. The time dependence of the apparent KIE that we reported previously is verified, as is the large steady-state value.

Methods and Materials

We have previously described the methodology for the measurement of reaction rates by PM/MS,[16] so that only a brief description is given here. A dimethyl silicone polymer membrane of nominal thickness 20 μm (General Electric, Schenectady, NY) was clamped between two stainless steel blocks, forming a reaction cell with the membrane as its bottom (area = 1 cm^2). This membrane excludes water almost totally, while the ethyl ester product can permeate across. The temperature was maintained by water pumped through the upper block, and the cell was connected through the lower block and via stainless steel tubing to the source of the MS, a Du Pont Model 21-490 single focusing, magnetic sector machine. The MS was operated in a selected ion mode by varying the accelerating voltage to scan two peaks, the parent (or base) and the corresponding ^{18}O-containing M + 2 peak, for approximately 3 s each.

The following procedure was typical for measuring reaction 1. Buffer (0.42 mL of 0.1 M K_2HPO_4), ethanol (13 μL of a mixture containing approximately 1/1 [^{16}O]-/[^{18}O]ethanol), and α-chymotrypsin (50 μL of enzyme in 0.001 M HCl; Worthington, Freehold, NJ) were allowed to come to temperature in the reaction cell; the reaction was initiated by addition of the *p*-nitrophenyl ester substrate in 20 μL of dimethylformamide. After an initial lag, the two signals corresponding to the ^{16}O and ^{18}O ethyl ester products rose linearly with time, e.g., Figure 1. We have shown that the time response for detection of the ethyl ester product is fit by the equation

$$I(t) = b_1\left[t - \frac{1 - e^{-b_2 t}}{b_2} - \frac{a_2(1 - e^{-a_3 t})}{a_3} - \frac{a_2(e^{-b_2 t} - e^{-a_3 t})}{b_2 - a_3}\right] \quad (2)$$

I is the signal intensity; a_1, a_2, and a_3 are constants from the time dependence of ethyl ester permeation across the membrane and can be determined in an independent experiment. The constant b_2 corresponds to the first-order rate constant for approach of the enzymatic reaction to steady state. A reliable value for this constant cannot be obtained by fitting data to eq 2, since under our present experimental conditions a_3 (a measure of the ethyl ester permeation rate across the membrane) is much smaller than b_2.[16] The constant b_1 is the slope of the linear portion of the response curve and is proportional to V_{max}.

$$b_1 = \alpha k_{ROH}(E_0)(CH_3CH_2OH) \quad (3)$$

where α, the proportionality constant, can be obtained by calibrating the instrumental response to known concentrations of the ester. It can be shown (see below) that when ^{16}O and ^{18}O reactions occur together, the ratio of steady-state slopes is

$$\frac{^{16}b_1}{^{18}b_1} = \frac{^{16}k_{ROH}}{^{18}k_{ROH}}\frac{^{16}\alpha(CH_3CH_2{}^{16}OH)}{^{18}\alpha(CH_3CH_2{}^{18}OH)} = \frac{^{16}k_{ROH}}{^{18}k_{ROH}}R \quad (4)$$

If the value of R is known, then the apparent steady-state KIE ($^{16}k_{ROH}/^{18}k_{ROH}$) can be calculated. All constants reported were obtained by least-squares fitting. The standard deviations reported refer to the fit of experimental points to the theoretical curve and are not meant as estimates of absolute error.

Since ethanol (0.5 M) is in large excess over substrate (0.8 mM) and enzyme (0.08 mM), the $^{16}O/^{18}O$ isotope ratio of the ethanol remains essentially constant during the reaction. Rather than measure this ratio, we chose to determine R directly, for a number of reasons: the difficulty in working with the ethanol mass spectrum; the mass discrimination that arises from scanning by varying the acceleration voltage;[17] possible isotope effects associated with fragmentation in the MS; a possible isotope effect in the permeation of ester across the membrane; the simplicity of the procedure we devised for determining R. The same ethanol mixture used in a transesterification reaction was quantitatively converted to the ester—either ethyl 2-furoate or ethyl 5-*n*-propyl-2-furoate—by the reaction with the acid chloride. Ethanol, 3 μL, was mixed with 300 μL of dry pyridine and with 50 μL of the appropriate acid chloride. The mixture was held at 40 °C for 10 min, when the reaction was complete. Gas–liquid chromatography through a 6 ft by $^1/_8$ in. o.d. stainless steel column packed with 3% OV-255 (Altech) on Chromosorb Q, 60–80 mesh (Johns-Manville), completely separates ethanol from all other components in the reaction mixture. No ethanol nor any other product other than the furoyl ethyl ester could be detected, indicating >99% reaction. An aliquot of this mixture was added to the MS cell, which contained the buffer solution for the transesterification reaction. The plateau intensities of either the parent or base peaks for both isotopic esters are the required measure of R.

The procedure just described is a one-step calibration of both the PM/MS system and the ethanol isotope ratio and was followed before each series of kinetic runs on any particular day. In support of the procedure's validity, we have shown that the measurement of R by GC/MS on the same mass spectrometer gave a value identical within experimental uncertainty with that determined by PM/MS. The ethanol/acid chloride reaction mixture was applied directly to the GC column, and the effluent was monitored in the single-ion mode for the two parent peaks (m/e = 140 and 142 for ethyl 2-furoate). The areas under the two chromatographic peaks were integrated numerically, and their ratio was taken as the measure of R. With ethyl 2-furoate, and one particular ethanol mixture, we obtained 0.855 ± 0.008 (GC/MS) and 0.861 ± 0.003 (PM/MS).

The acid chlorides of 2-furoic and 5-*n*-propyl-2-furoic acid were made by reaction with thionyl chloride. For the furoyl chloride, 200 mmol of the acid was dissolved in 20 mL of $SOCl_2$ and refluxed for 1 h. The excess $SOCl_2$ was boiled off, and the acid chloride was distilled at 172 °C (lit. bp 173–174 °C). The product was a clear liquid with a mass spectrum consistent with the proposed structure. The 5-*n*-propyl-2-furoyl chloride was synthesized in a similar manner. Both acid chlorides were stored at 4 °C in a desiccator. 5-*n*-Propyl-2-furoic acid was synthesized from *n*-propylfuran as described previously.[1] The ethyl esters of both acids were prepared by reaction of the acid chlorides with ethanol. The ethyl ester of furoic acid, which was recrystallized from water/methanol (70/30 v/v) at −78 °C, had a mp of 32–33 °C (lit. mp 34 °C). The ethyl ester of 5-*n*-propyl-2-furoic acid was isolated as an oil and was deemed pure by GC/MS. The *p*-nitrophenyl esters of the two acids were synthesized as described previously.[1] [^{18}O]Ethanol was synthesized by the method of Sawyer.[18] All other reagents were purchased from commercial sources and used with no further purification, except *p*-nitrophenol and 2-furoic acid, both recrystallized from ethanol.

Results

Kinetic measurement by PM/MS is based on the permeation of at least one component in a reacting system through a membrane barrier into the inlet of a mass spectrometer. When enzymatic reactions are studied, the advantage of the dimethyl

(16) Calvo, K. C.; Weisenberger, C. R.; Anderson, L. B.; Klapper, M. H. *Anal. Chem.* **1981**, *53*, 981–985.

(17) Coggeshall, N. D.; *J. Chem. Phys.* **1944**, *12*, 19–23.

(18) Sawyer, C. B. *J. Org. Chem.* **1972**, *37*, 4225–4226.

silicone polymer membrane is its impermeability to water, resulting in an efficient one-stage purification for sufficiently apolar analytes. Since the magnitude of the permeant component's mass spectrum is directly proportional to its concentration in solution, time-dependent changes in that concentration can be followed, when solute movement across the membrane is also taken into account.[16]

We consider a nonreacting system first. When ethyl 5-*n*-propyl-2-furoate is added to a buffer (0.1 M phosphate, pH 8.5) solution in the PM/MS cell, there is a rapid increase in the magnitudes of the parent (m/e = 182) and base (m/e = 154; loss of ethylene) ion peaks. The half-life for this increase, approximately 25 s at 25 °C under our experimental conditions, is related to l^2/D, the ratio of the membrane thickness squared and the diffusion coefficient of solute in the membrane. Subsequently, there is a slow first-order decline of the signal with $t_{1/2}$ in the range of 3–12 min between 18 and 38 °C ($t_{1/2}$ = 8.2 min at 25 °C). This decay, due to permeation loss of the ester, is so much slower than the initial buildup that it can be assigned a first-order rate constant to represent the permeation process. When a mixture of $^{16}O/^{18}O$ (ethyl) esters is added to the cell, the permeation loss of both can be monitored at either m/e = 182 and 184 or m/e = 154 and 156. Since first-order constants are concentration independent, the isotopic ratio of the starting mixture need not be known, and the permeation isotope effect is the ratio of first-order constants for ^{16}O and ^{18}O compounds. For ethyl 5-*n*-propyl-2-furoate, the isotope effect was 1.006 ± 0.006 over the temperature range 18–38 °C. A small or experimentally undetected effect would be expected.

We consider next the nonenzymatic saponification of ethyl 2-furoate, a first-order reaction. With the ester in dilute alkali, the first-order signal decay for parent (m/e = 140) and base (m/e = 112) ions results from two independent processes, hydrolysis and ester loss through the membrane. Thus, the hydrolysis rate constant can be obtained from the difference in decay times for alkaline and neutral (nonreacting) conditions. Saponification of the ethyl 2-[^{16}O,^{18}O]furoate mixture yields a hydrolysis rate constant for each isotopic species. Since the two reactions are first order, the isotopic ratio in the starting mixture need not be known, and the KIE is the ratio of these two constants. The observed KIE for ethyl 2-furoate is marginal at 1.012 ± 0.010 (pH 10.0, 25 °C, ^{16}k = 0.1244 ± 0.003 min^{-1}), consistent with reported results: 1.0091 ± 0.0004 for saponification of methyl formate[12] and 1.0062 ± 0.0006 for methyl benzoate.[19]

Finally, in the α-chymotrypsin-catalyzed transesterification of *p*-nitrophenyl furoyl esters, the ethyl ester is the product (eq 1), and the reaction is followed as an increase in signal intensity. (We have not been able to detect permeation of the *p*-nitrophenyl substrate nor of the acid formed in the competitive hydrolysis of the acyl enzyme. Under our experimental conditions, approximately 25% of the furoyl and 50% of the *n*-propylfuroyl reaction is diverted to hydrolysis.) Ethanol does pass through the membrane, although less readily than the ester; due to its low mass it does not interfere with detection of the ester. The results of one experiment with a $^{16}O/^{18}O$ mixture of ethanol is shown in Figure 1. The initial lag in both curves is due primarily to the transport of solute through the membrane.[16] The slopes of the linear portions of the curve are proportional to the enzymatic k_{cat}, and their ratio is proportional to the steady-state $^{16}O/^{18}O$ KIE (eq 4) as we show in the Appendix.

Note from the results in Figure 1 that steady state is achieved in less time (<2 min) than the half-life for loss of product by permeation into the mass spectrometer (approximately 8 min). In this instance permeation loss will contribute a negligible error to the calculated value of k_{cat}. Furthermore, permeation loss will not significantly affect the ratio of slopes, a measure of the KIE, since the permeation isotope effect is negligible. Under our conditions <80 μM ester is formed over the time of the experiment, which means that less than 20% of the substrate has been consumed (with approximately half of that lost to hydrolysis), and less than 0.02% of the ethanol has been converted to ester. Thus, the isotopic ratio of the alcohol changes negligibly during the reaction, and we require only an independent measurement of R (eq 4) in order to determine the steady-state KIE. As described under Methods and Materials, this is done by reacting the isotopic ethanol mixture quantitatively with the appropriate acid chloride and adding the unpurified product into a buffer solution in the PM/MS cell. The ratio of $^{16}O/^{18}O$ signals after maximum intensities have been reached is R.

(19) O'Leary, M. H.; Marlier, J. F. *J. Am. Chem. Soc.* **1978**, *100*, 2582–2583.

Table I. Steady-State $^{16}O/^{18}O$ KIE for α-Chymotrypsin-Catalyzed Transesterification of *p*-Nitrophenyl 2-Furoate[a]

m/e[b]	R[c]	$^{16}k_{obsd}/^{18}k_{obsd}$[d]	KIE[e]
140/142	0.860 (0.003)	0.863 (0.004)	1.004 (0.005)
140/142	0.594 (0.011)	0.593 (0.007)	1.00 (0.02)
112/114	0.904 (0.008)	0.920 (0.004)	1.02 (0.01)
112/114	0.366 (0.004)	0.372 (0.003)	1.02 (0.01)
112/114	8.95 (0.077)	9.11 (0.06)	1.02 (0.01)
		weighted av:	1.009 (0.007)

[a] Reaction conditions: 0.10 M phosphate, pH 8.5; (E_0) = 40–60 μM; ($CH_3CH_2{}^{16}OH$ plus $CH_3CH_2{}^{18}OH$) = 0.5 M; (S_0) = 0.8 mM; T = 25 °C. All measured or calculated values are presented with estimated standard deviations in parentheses. [b] m/e = 140 is the parent ion peak of ethyl 2-[^{16}O]furoate; m/e = 112 is the corresponding base peak (loss of ethylene). [c] R as defined in the text is $^{16}\alpha(Et^{16}OH)/^{18}\alpha(Et^{18}OH)$. The variation of R is obtained by varying ratios of the two isotopic species. R is measured experimentally as described in the text. [d] Obtained from the zero-order slopes as described in the text. [e] Calculated as column 3 divided by column 2.

Table II. Steady-State $^{16}O/^{18}O$ KIE for α-Chymotrypsin-Catalyzed Transesterification of *p*-Nitrophenyl 5-*n*-Propyl-2-furoate[a]

m/e[b]	R	$^{16}k_{obsd}/^{18}k_{obsd}$	KIE
182/184	0.940 (0.011)	0.85 (0.003)	0.90 (0.011)
154/156	0.932 (0.036)	0.81 (0.004)	0.87 (0.030)
154/156	0.917 (0.013)	0.81 (0.003)	0.88 (0.013)
154/156	0.467 (0.005)	0.413 (0.002)	0.88 (0.011)
182/184	0.917 (0.013)	0.833 (0.005)	0.91 (0.014)
182/184	0.917 (0.013)	0.843 (0.004)	0.92 (0.014)
182/184	1.073 (0.018)	1.001 (0.006)	0.93 (0.014)
		weighted av:	0.090 (0.02)

[a] Conditions identical with those reported in Table I except (S_0) = 60–80 μM. [b] m/e = 182 is the parent ion peak of ethyl 5-*n*-propyl-2-[^{16}O]furoate; m/e = 154 is the corresponding base peak (loss of ethylene).

Although the pre-steady-state rate constants cannot be extracted from results such as those of Figure 1, at any point the M to M + 2 ion ratio is proportional to the isotopic ratio of the ethyl ester formed at some earlier time in the aqueous reaction mixture. This is true because the negligibly small isotope effect for permeation is taken into account in the determination of R. The time dependence of the M to M + 2 ratio is shown for the formation of ethyl 5-*n*-propyl-2-furoate in Figure 2 and ethyl 2-furoate in Figure 3. Early in these reactions, when little product has been formed, the precision of the points is poor, as evidenced by the scatter. Nonetheless, the trend is clear. There is an initial normal mass ratio, which means that the reaction begins with a normal KIE. Within 1–2 min at 25 °C the mass ratio approaches the value of the KIE for the steady state. These results confirm our earlier report, in which the same trend was suggested on the basis of less data.[13]

We have also examined the effect of pH and temperature on the *n*-propylfuroate steady-state KIE. The KIE increases from

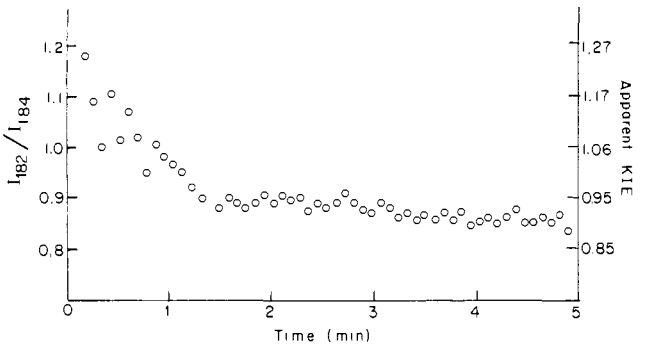


Figure 2. Time dependence of the apparent KIE associated with the α-chymotrypsin-catalyzed transesterification of *p*-nitrophenyl 5-*n*-propyl-2-furoate: the results of Figure 1 have been replotted as the measured peak intensity ratio on the left and as the apparent KIE on the right.

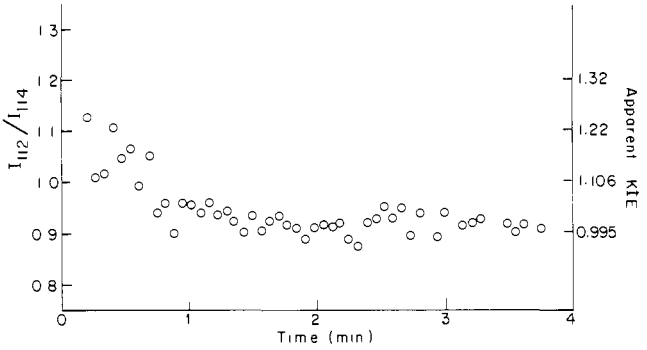


Figure 3. Time dependence of the apparent KIE associated with the α-chymotrypsin-catalyzed transesterification of *p*-nitrophenyl 2-furoate. Conditions are described in Table II; R for this experiment was 0.90. The measured peak intensity ratio for m/e = 112 and m/e = 114 is plotted on the left and the apparent KIE on the right.

0.90 to 0.97 when the pH is lowered from 8.5 to 6.5 (Figure 4). While the estimated uncertainties are large, these pH results are fit well if we assume that the KIE is controlled by a single acid/base titration with pK_a = 7.3. α-Chymotrypsin-catalyzed reactions are all controlled by a process with a pK_a near 7, presumed to be the titration of a histidine side chain in the active site. For the hydrolysis of *p*-nitrophenyl 5-*n*-propyl-2-furoate the pK_a is 7.1 at 25 °C,[20] and for the esterification of this compound with ethanol the pK_a is 7.2 (unpublished results). There is also a marked temperature effect on the KIE (Figure 5). The KIE rises from a low value of 0.87 at 18 °C to a high of 0.96 at 38 °C. (Previous data[13] are not consistent with this trend. However, the earlier results, obtained at other than room temperatures, are less reliable, since we did not control the temperature during the brief time of sampling.) This change, measured at pH 8.5, is well outside experimental uncertainty.

Discussion

Since the PM/MS method is new to the measurement of small kinetic isotope effects, we shall consider some of its advantages and disadvantages. With the mixed isotope protocol, both isotopic species react concurrently in the same solution, minimizing experimental error. Unlike other mass spectrometric mixed isotope procedures, the PM/MS method permits both the measurement of mass ratios and the direct determination of rate constants for each isotopic reaction. One result, seen in the case of nonenzymatic ester hydrolysis, is that two reactions which occur independently, and are detected independently, can be treated as independent even though they are housed in the same solution. Thus, a first-order KIE can be obtained with no knowledge of the initial reactant isotope ratio, which helps to reduce experimental

(20) Baggott, J. E. Master of Science Thesis, The Ohio State University, 1975.

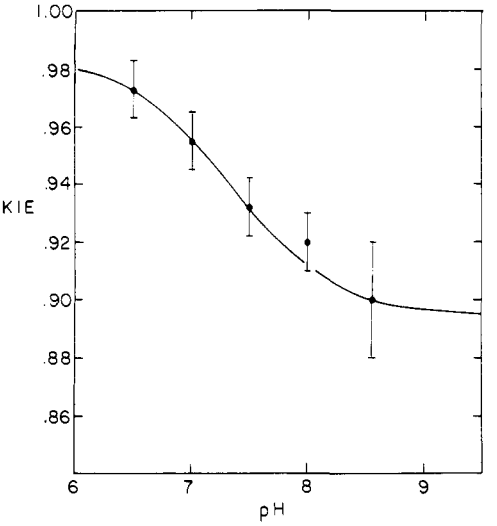


Figure 4. Dependence on pH of the steady-state KIE for the transesterification of *p*-nitrophenyl 5-*n*-propyl-2-furoate. Reaction conditions are those given in the text. The temperature was 25 °C. Each point represents two determinations, except at pH 8.5, which is the weighted average taken from Table II. The curve is calculated on the basis of the best fit of the data to a single proton acid/base titration model.

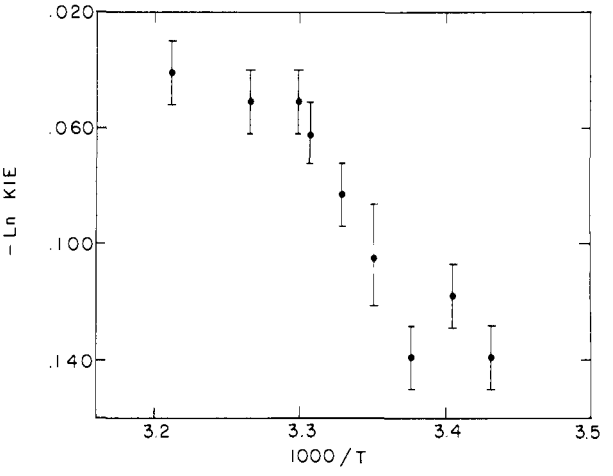


Figure 5. Dependence on temperature of the steady-state KIE for the transesterification of *p*-nitrophenyl 5-*n*-propyl-2-furoate. The reactions were run at pH 8.5. Each point represents duplicate determinations, except for the 25 °C result, which is the weighted average taken from Table II.

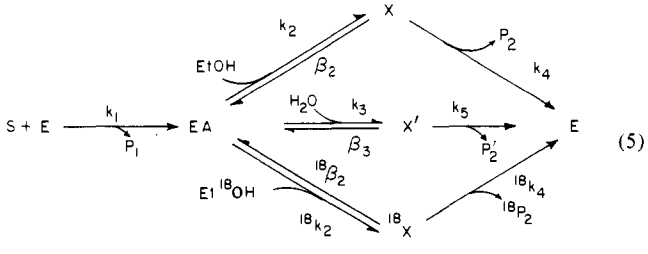
uncertainty. Because of the mass spectrometer's sensitivity, analyte concentrations in the experiments reported here were usually <20 μM. Improvement of signal-to-noise characteristics, which should come with a better instrument and utilization of commercially available, thinner membranes, will allow us to work at much lower concentrations. This sensitivity also permits the use of very small samples, an important factor when isotopically substituted compounds are expensive or difficult to obtain. Perhaps the major advantage is that analyte measurement is almost in situ—almost, since some sample is constantly removed through the membrane. In common with all in situ measurements, there is minimal experimental manipulation required for analysis, with resulting ease and speed. In the experiments reported here the scan time over two mass ion peaks was approximately 6 s, a 2–3 order increase in the point-to-point time resolution over other mass spectrometric methods. Because of this measurement speed, the decline in the product mass ratio during the first minute of the α-chymotrypsin-catalyzed transesterification could be observed unambiguously. In a different comparison, the equilibrium perturbation method for KIE measurements, which is an in situ technique,[15] is limited to reaction systems with a measurable equilibrium point; PM/MS can be used for both reversible and irreversible reactions.

As to the method's disadvantages, the cost and technology of the mass spectrometer may keep the PM/MS method out of normal enzymology projects, a practical limitation to its usefulness. Presently, we are not confident with a measured KIE smaller than 1–1.5%, a range in which we would expect a number of the heavier atoms to fall. With commercially available instruments having better signal-to-noise characteristics than our own, this region of unreliability should be reduced substantially, although we do not anticipate achieving the precision possible with an isotope-ratio mass spectrometer.[11] Finally, the basis of the method's success is also the cause of its major limitation. The method works well because the membrane is so poorly permeable to water and other polar compounds. But the majority of enzymatic reactions involve charged or polar reactants and products, thus making them inaccessible to PM/MS. This limitation may be circumvented should it prove possible to use a coupling reaction in which a permeable compound is produced.

We turn next to the novel time dependence of the transesterification KIE (Figures 2 and 3), first to consider possible sources of error and second to propose a plausible explanation. So little of the ethanol was converted to ester (<0.02%) that the [^{16}O]-/[^{18}O]ethanol ratio was effectively constant throughout the reaction. An artifact due to contamination is unlikely, since similar time dependencies were obtained with two different substrates (although the final KIE's did differ), with variation of ethanol isotopic ratios, and when either parent or base ions were monitored. We also have evidence for the same ratio decline when the ethyl ester was purified from the reaction mixture prior to analysis.[13] Analytical errors can arise from sources such as mass discrimination due to the scanning method employed and isotope effects in ion fragmentation and membrane permeation. The last of these is undetectable with our current precision, the measurement of the ethanol isotopic ratio was designed to correct for these possible errors, and finally, we would expect errors of this type to be time independent.

Recently, Northrop,[21] in commenting on our earlier GC/MS results, argued that the observed decline is a distortion, arising because of (i) isotopic discrimination, presumably by an enzyme-catalyzed equilibration between ethyl ester and ethanol, and (ii) ethyl ester hydrolysis, which, having a normal KIE, would enrich the unhydrolyzed ester in ^{18}O. He proposed that the latter effect would be especially severe as hydrolysis of the ethyl ester approaches completion. However, we cannot accept his arguments. The K_i for ethyl 5-*n*-propyl-2-furoate is 0.4 mM, while the K_m of the *p*-nitrophenyl substrate is 0.7 μM.[13] After 1 min, when the mass ratio decline is largely complete (at 25 °C), little product has accumulated. Given these results, it is unlikely that there is a significant, let alone complete, hydrolysis of, or additional ethanol exchange into, the ethyl ester product that is free in solution. For during the first minute there is less product than substrate. Additionally, the large pH and temperature sensitivities of the steady-state KIE are inconsistent with an argument based upon an equilibrium isotope effect for partitioning between ethanol and an ethyl ester. Such an equilibrium effect would be relatively insensitive to the pH and temperature changes we studied. One might still wish to argue for equilibration between ethanol and ethyl ester prior to the latter's release from the enzyme, but this cannot be called distortion and would be kinetically similar to the explanation we are about to offer.

Consider the minimal reaction mechanism



(5)

Compounds containing ^{18}O, and the rate constants of their reactions, are indicated by a superscript 18. No superscript is used for the ^{16}O reactions. Reaction of enzyme, E, with *p*-nitrophenyl ester substrates to form acyl enzyme, EA, is essentially irreversible, and sufficiently rapid in comparison with subsequent deacylation, that EA formation can be represented as a single irreversible reaction. More complicated schemes for the formation of EA would yield functionally equivalent equations. In the presence of isotopically mixed ethanol the acyl enzyme can move into one of three paths: two lead to the ethyl esters P_2 and $^{18}P_2$; the third is competitive hydrolysis to the acid P_2'. We propose a model in which there is at least one reaction intermediate along each path, a widely held assumption, and in which product release is functionally irreversible. We have derived for this model the time dependence of the mass spectral intensities for the two ethyl esters (eq A6 in the Appendix). It is easily shown that

$$\lim_{t\to\infty}\frac{I(t)}{^{18}I(t)} = \frac{\lim_{t\to\infty} I(t)}{\lim_{t\to\infty} {}^{18}I(t)} = \frac{a_1k_2k_4(^{18}k_4 + {}^{18}\beta_2)(\mathrm{EtOH})}{^{18}a_1\,{}^{18}k_2\,{}^{18}k_4(k_4+\beta_2)(\mathrm{Et}{}^{18}\mathrm{OH})} \quad (6)$$

Equations 4 and 6 are equivalent, and hence

$$\mathrm{KIE(steady\ state)} = \frac{k_2k_4(^{18}k_4 + {}^{18}\beta_2)}{^{18}k_2\,{}^{18}k_4(k_4+\beta_2)} \quad (7)$$

It is also shown in the Appendix that the KIE at time zero falls within the range

$$\frac{k_2k_4}{^{18}k_2\,{}^{18}k_4} \leftarrow \mathrm{KIE}(t=0) \rightarrow \frac{k_2k_4(^{18}k_4 + {}^{18}\beta_2 + k_5 + \beta_3)}{^{18}k_2\,{}^{18}k_4(k_4+\beta_2+k_5+\beta_3)} \quad (8)$$

Comparison of eq 7 and 8 suggests an explanation for the early decline of the isotope ratio. If normal isotope effects are associated with primary steps (e.g., $k_2 > {}^{18}k_2$, $\beta_2 > {}^{18}\beta_2$), then the apparent KIE during steady state (eq 7) could be either normal or inverse. For example, were $k_4 \gg \beta_2$, then the steady-state KIE would become $k_2/{}^{18}k_2$ and normal; were $\beta_2 \gg k_4$, then the observed KIE could be inverse depending on the magnitudes of $^{18}\beta_2/\beta_2$ and $k_2k_4/(^{18}k_2\,{}^{18}k_4)$. The KIE associated with breakdown of the intermediate X, $k_4/{}^{18}k_4$, should be close to 1, since this reaction does not involve bond cleavage at ^{18}O. Thus, an inverse steady-state KIE most probably corresponds to an inverse equilibrium isotope effect for the reaction of EA with ethanol to form X.

A similar range of values is possible for the initial KIE (eq 8). But at one extreme the KIE($t \rightarrow 0$) would be normal, since $k_2k_4 > {}^{18}k_2\,{}^{18}k_4$. As a qualitative explanation we propose that initially the measured KIE depends largely upon the one-way flow of substrate to product. As the reaction approaches steady state there would be a smooth change to a KIE governed largely by the equilibrium between EA and X.

The observed large difference in steady-state KIE between furoyl and propylfuroyl reactions is consistent with this proposal. Addition of a three-carbon side chain at a distance from the reaction site could only have a large effect if the apparent KIE were a balanced collection of primary reaction constants. The reaction scheme of eq 5 requires a hyperbolic dependence of turnover rate on ethanol concentration. The reaction rate, however, is linear with ethanol concentration up to 5 M at 25 °C (unpublished results) with the enzyme becoming unstable at higher ethanol levels. This either is an inconsistency or more likely means that the apparent ethanol K_M is above 5 M.

The largest previously reported ^{16}O/^{18}O KIE's of which we know are 1.08 in the nonenzymatic solvolysis of *cis*-5-methyl-2-cyclohexenyl *p*-nitrobenzoate[22] and 1.07 in the enzymatic conversion of malate to fumarate.[23] Were our interpretation of the inverse ethyl 5-*n*-propyl-2-furoate KIE correct, then a normal KIE $\geq$ 11% would be inferred for the release of ethanol and the reformation of acyl enzyme ($\beta_2/{}^{18}\beta_2 \geq 1.11$). Although such a large

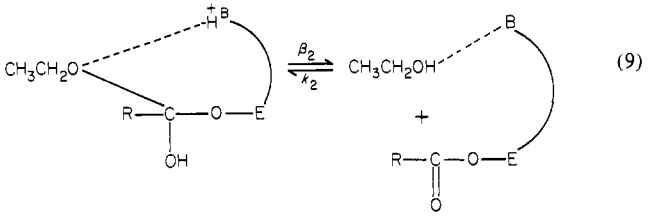
(21) Northrop, D. B. *Annu. Rev. Biochem.* **1981**, *50*, 103–131.

(22) Goering, H. L.; Doi, J. T.; McMichael, K. D. *J. Am. Chem. Soc.* **1964**, *86*, 1951–1957.

(23) Blanchard, J. S.; Cleland, W. W. *Biochemistry* **1980**, *19*, 4506–4513.

$^{16}O/^{18}O$ KIE is unprecedented, there is little likelihood that it arises as an artifact of our experimental procedure. First, we have obtained with the PM/MS method a marginal, perhaps normal, KIE for the alkaline hydrolysis of ethyl 2-furoate, 1.012 ± 0.010. Similar results have been reported with other esters.[12,19] Second, the α-chymotrypsin-catalyzed transesterification shows a much smaller KIE with a different substrate, *p*-nitrophenyl 2-furoate (1.009), and when the reaction conditions are altered (0.97 at pH 6.5 and 25 °C; 0.96 at pH 8.5 and 38 °C). Finally, similar results were obtained by two other methods.[13] With GC/MS, in which the ethyl ester was purified prior to mass spectral analysis, the KIE was 0.90 ± 0.02 (at 25 °C). With the separated isotope, spectrophotometric measurement of steady-state *p*-nitrophenolate release, the KIE was 0.88 ± 0.06 (at 25 °C). The PM/MS value reported here is 0.90 ± 0.02.

We can only offer suggestions to explain this apparently large inverse KIE. The minimal formulation of eq 5 may be incomplete in that the formation of the intermediate X could involve two elementary reactions, both of which have an $^{16}O/^{18}O$ KIE. Were there a multiplicative contribution of the two sets of rate constants, then a large overall KIE would result. A second suggestion is based on the reports of Pinchas[24,25] that with 0.336 M H_2O in dioxane the measured ratio of the dimerization constants for $H_2{}^{16}O$ and $H_2{}^{18}O$ is 1.61. At higher water concentrations the ratio approaches 1. He proposed that this very large equilibrium isotope effect is due to the decreased ability of ^{18}O to form the water dimer hydrogen bond. With this in mind, and if we assume that the conversion of X to EA is the acid-assisted reaction



occurring in an environment shielded from bulk water, then a large inverse KIE might result. Reaction 9 is commonly seen in discussions of chymotrypsin catalysis, with B the active site imidazole.

Because of its magnitude in the *n*-propylfuroyl case we were able to investigate the pH and temperature dependencies of the steady-state KIE. The relative changes are large, results not unknown in other systems. For example, Blanchard and Cleland[23] reported an $^{16}O/^{18}O$ KIE for the enzymatic conversion of malate to fumarate, which is close to unity at neutral pHs and increases to 1.07 as the pH is lowered; O'Leary and Piazzi[26] reported a temperature dependence for the $^{12}C/^{13}C$ KIE associated with arginine decarboxylase that is qualitatively similar to that described here. Northrop[21] has emphasized the difficulty in assigning rate-limiting steps for enzyme-catalyzed reactions on the basis of KIE magnitudes. Our results are an example of this problem. When the pH is raised, the reaction turnover increases, and the KIE becomes more inverse (Figure 4). When the temperature is raised, the reaction turnover increases, but the KIE becomes less inverse (Figure 5). Thus, a simple explanation in terms of a switch in rate-limiting steps may not be warranted. The KIE dependence on pH may be controlled by a pK_a close to that of the enzyme active site histidine, but with our present precision this has not been established unambiguously. Over the temperature range studied, the KIE for a primary reaction would be expected to change only slightly. The large change observed may arise from a shift in the partitioning of one or more intermediates within a complex reaction path, or from some other physical source such as a structural change at the active site. The former is consistent with the KIE results reported here, and the latter with our previous observation of Arrhenius plot nonlinearities, which occur over the same temperature region. This is a distinction that is of some interest to us but must await further results.

In closing, we have developed a new procedure for investigating small kinetic isotope effects. Because of the simplicity in experimental manipulation, PM/MS may become the method of choice in those systems to which it is applicable, currently reactions with at least one apolar constituent. We have applied PM/MS to the α-chymotrypsin-catalyzed transesterification of *p*-nitrophenyl 5-*n*-alkyl-2-furoates. Our results confirm earlier observations on the same reaction obtained with different procedures. These results are unexpected in light of previous experience with, and predictions of, $^{16}O/^{18}O$ KIE's. If substantiated by further experimentation, these findings should serve to widen our understanding of heavy atom isotope effects. The data of this report, together with our earlier temperature studies, suggest that (depending upon one's inclinations, fortunately or unfortunately) the mechanism of α-chymotrypsin esterification is more complex than heretofore understood.

Appendix

The following equations apply to the reaction scheme of eq 5.

$$(E_0) = (E) + (EA) + (X) + (^{18}X) + (X')$$

$$d(EA)/dt = k_1(S)(E) + \beta_2(X) + {}^{18}\beta_2(^{18}X) + \beta_3(X') - [k_2(EtOH) + {}^{18}k_2(Et^{18}OH) + k_3](EA)$$

$$d(X)/dt = k_2(EtOH)(EA) - (k_4 + \beta_2)(X)$$

$$d(^{18}X)/dt = {}^{18}k_2(Et^{18}OH)(EA) - ({}^{18}k_4 + {}^{18}\beta_2)(^{18}X)$$

$$d(X')/dt = k_3(EA) - (k_5 + \beta_3)(X')$$

$$d(P_2)/dt = k_4(X)$$

$$d(^{18}P_2)/dt = {}^{18}k_4(^{18}X)$$

$$d(P_2')/dt = k_5(X') \qquad \text{(A1)}$$

If we assume that substrate and ethanol concentrations remain constant during the course of the reaction, then eq A1 becomes linear and can be solved by using LaPlace-Carson transforms.[27] The solution for the hypothetical intermediate X is

$$(X) = k_1k_2(EtOH)(E_0)\{({}^{18}k_4 + {}^{18}\beta_2) \times (k_5 + \beta_3)/\gamma_1\gamma_2\gamma_3\gamma_4 - \sum_{i=1}^{4}[\gamma_i{}^2 - \gamma_i({}^{18}k_4 + {}^{18}\beta_2 + k_5 + \beta_3) + ({}^{18}k_4 + {}^{18}\beta_2)(k_5 + \beta_3)]e^{-\gamma_i t}/[\gamma_i\prod_{i\neq j}(\gamma_j - \gamma_i)]\}$$

$$(^{18}X) = k_1{}^{18}k_2(Et^{18}OH)(E_0)\{(k_4 + \beta_2) \times (k_5 + \beta_3)/\gamma_1\gamma_2\gamma_3\gamma_4 - \sum_{i=1}^{4}[\gamma_i{}^2 - \gamma_i(k_4 + \beta_2 + k_5 + \beta_3) + (k_4 + \beta_2)(k_5 + \beta_3)]e^{-\gamma_i t}/[\gamma_i\prod_{i=j}(\gamma_j - \gamma_i)]\} \qquad \text{(A2)}$$

The γ_i, pseudo-first-order constants, which are the negative values of the roots of a biquadratic equation, are constructed from all the rate constants, and include the substrate and both isotopic ethanol concentrations. Since there is no experimental evidence for more than one first-order process in the α-chymotrypsin pre-steady state, it is reasonable to assume that one of the four γ_i's is sufficiently small that on the experimental time scale one of the four exponential terms in the summation dominates and

$$(X) \approx k_1k_2(EtOH)(E_0)\{({}^{18}k_4 + {}^{18}\beta_2) \times (k_5 + \beta_3)/\gamma_1\gamma_2\gamma_3\gamma_4 - [\gamma_i{}^2 - \gamma_i({}^{18}k_4 + {}^{18}\beta_2 + k_5 + \beta_3) + ({}^{18}k_4 + {}^{18}\beta_2)(k_5 + \beta_3)]e^{-\gamma_i t}/\gamma_1\gamma_2\gamma_3\gamma_4\}$$

$$(^{18}X) \approx k_1{}^{18}k_2(Et^{18}OH)(E_0)\{(k_4 + \beta_2)(k_5 + \beta_3)/\gamma_1\gamma_2\gamma_3\gamma_4 - [\gamma_i{}^2 - \gamma_i(k_4 + \beta_2 + k_5 + \beta_3) + (k_4 + \beta_2)(k_5 + \beta_3)]e^{-\gamma_i t}/\gamma_1\gamma_2\gamma_3\gamma_4\} \qquad \text{(A3)}$$

(24) Pinchas, S. *Nature (London), Phys. Sci.* **1973**, *242*, 46–47.
(25) Pinchas, S. *J. Inorg. Nucl. Chem.* **1977**, *39*, 459–462.
(26) O'Leary, M. H.; Piazzi, G. J. *Biochemistry* **1981**, *20*, 2743–2748.
(27) Rodiguin, N. M.; Rodiguina, E. N. "Consecutive Chemical Reactions, Mathematical Analysis and Development: Schneider, R. F., Translator; D. van Nostrand: Princeton, NJ, 1964.

This approximation makes the remainder of the derivation look less complex, but the conclusion arrived at is closely similar to that obtained had we not introduced this simplification.

To determine (P_2) and ($^{18}P_2$) we multiply the two eq A3 by k_4 and $^{18}k_4$, respectively, and integrate. For our purpose we do not need these two explicit solutions. What we are interested in is the time dependence of the signals in the mass spectrometer due to the time-dependent appearance of P_2 and $^{18}P_2$. We have shown earlier[16] that the appearance of a signal due to the instantaneous addition of solute to the PM/MS cell is given by

$$J(t) = a_1(1 - a_2e^{-a_3t}) \tag{A4}$$

The constants a_1 to a_3 can be obtained experimentally. The first, a_1, is the measured signal intensity once steady-state permeation of solute across the membrane has been attained (under the assumption that the solute concentration in the cell does not change with time). The third, a_3, is the rate constant associated with the approach to steady state, and the second, a_2, is a correction factor introduced to account for higher order terms that were dropped in the derivation of eq A4.

The explicit expression of signal intensity as a function of time for the case of the enzyme-catalyzed reaction is the convolution

$$I(t) = \int_0^t [\mathrm{d}(P_2)/\mathrm{d}\lambda]J(t - \lambda)\mathrm{d}\lambda \tag{A5}$$

Substituting eq A3 and A4 into A5 and integrating

$$\begin{aligned} I(t) = [a_1k_1k_2k_4(\mathrm{EtOH})/\gamma_1\gamma_2\gamma_3\gamma_4(E_0)]\{(^{18}k_4 + {}^{18}\beta_2) \times \\ (k_5 + \beta_3)t - a_2(1 - e^{-a_3t})/a_3 - \\ [\gamma_i^2 - \gamma_i(^{18}k_4 + {}^{18}\beta_2 + k_5 + \beta_3) + (^{18}k_4 + {}^{18}\beta_2)(k_5 + \\ \beta_3)](1 - e^{-\gamma_it})/\gamma_i - \\ [a_2\gamma_i^2 - \gamma_i(^{18}k_4 + {}^{18}\beta_2 + k_5 + \beta_3) + (^{18}k_4 + {}^{18}\beta_2) \times \\ (k_5 + \beta_3)](e^{-a_3t} - e^{-\gamma_it})/(a_3 - \gamma_i)\} \end{aligned} \tag{A6}$$

Equation A6 is the time-dependent intensity for the ^{16}O product. A similar expression is obtained for the ^{18}O product by interchanging the appropriate rate constants (e.g., $^{18}k_4$ for k_4 and vice versa), the permeation constants (e.g., $^{18}a_1$ for a_1), and the ethanol terms.

It should be noted that the constants γ_i are identical for the ^{16}O and ^{18}O cases in eq A3 and A6. Because the reactions occur simultaneously with competition for the common intermediate EA, the three reactions are linked, and thus the exponential terms in each are identical. Presently we cannot measure γ_i, but the model predicts that this parameter will not display a KIE.

By inspection it is obvious that

$$\lim_{t\to\infty} I(t) = a_1k_1k_2k_4(\mathrm{EtOH})(E_0)(^{18}k_4 + {}^{18}\beta_2)(k_5 + \beta_3)t/\gamma_1\gamma_2\gamma_3\gamma_4$$

$$\lim_{t\to\infty} {}^{18}I(t) = {}^{18}a_1k_1{}^{18}k_2{}^{18}k_4(\mathrm{Et}^{18}\mathrm{OH})(E_0)(k_4 + \beta_2)(k_5 + \beta_3)t/\gamma_1\gamma_2\gamma_3\gamma_4 \tag{A7}$$

which is an expression of the zero-order, steady-state increase seen in Figure 1. The ratio of these two equations is given in eq 7. From a consideration of eq A6 it can also be shown that

$$\lim_{t\to\infty} [I(t)/{}^{18}I(t)] = a_1k_2k_4(\mathrm{EtOH})(^{18}k_4 + {}^{18}\beta_2)/[{}^{18}a_1{}^{18}k_2{}^{18}k_4(\mathrm{Et}^{18}\mathrm{OH})(k_4 + \beta_2)] \tag{A8}$$

and hence

$$\lim_{t\to\infty} I(t)/\lim_{t\to\infty} {}^{18}I(t) = \lim_{t\to\infty} [I(t)/{}^{18}I(t)] \tag{A9}$$

Thus, the apparent steady-state KIE can be obtained either from the product mass ratio observed in the steady-state portion of the reaction or from the ratio of the steady-state rate constants.

To obtain the limit of $I(t)$ as t approaches 0 requires L'Hopital's rule. Thus

$$\begin{aligned} \lim_{t\to0} \mathrm{d}I(t)/\mathrm{d}t = a_1(1 - a_2)k_1k_2k_4(E_0)(\mathrm{EtOH}) \times \\ [\gamma_i(^{18}k_4 + {}^{18}\beta_2 + k_5 + k_3) - \gamma_i^2]/\gamma_1\gamma_2\gamma_3\gamma_4 \\ \lim_{t\to0} \mathrm{d}{}^{18}I(t)/\mathrm{d}t = {}^{18}a_1(1 - a_2)k_1{}^{18}k_2{}^{18}k_4(E_0)(\mathrm{Et}^{18}\mathrm{OH}) \times \\ [\gamma_i(k_4 + \beta_2 + k_5 + k_3) - \gamma_i^2]/\gamma_1\gamma_2\gamma_3\gamma_4 \end{aligned} \tag{A10}$$

Since we have detected no isotope effect for permeation, assuming that $^{18}a_1 \neq a_1$ is a nicety not required by our results. On the other hand, the constant a_2 is an empirical constant introduced to account for an approximation, as mentioned above. There is no reason to believe that a_2 will have any isotope dependence, and none has been assumed for eq A10. We are interested in the $t = 0$ limit for the ratio of intensities. Perusal of eq A10 suggests two extreme possibilities

$$\lim_{t\to0} [I(t)/{}^{18}I(t)] = a_1k_2k_4(\mathrm{EtOH})/[{}^{18}a_1{}^{18}k_2{}^{18}k_4(\mathrm{Et}^{18}\mathrm{OH})] \tag{A11}$$

when $\gamma_i \gg k_4 + \beta_2 + k_5 + \beta_3$.

From eq A11 we infer that the apparent KIE at time zero is given by

$$\mathrm{KIE}(t = 0) = k_2k_4/(^{18}k_2{}^{18}k_4) \tag{A12}$$

If, however, $\gamma_i \ll k_4 + \beta_2 + k_5 + \beta_3$, then

$$\lim_{t\to0} [I(t)/{}^{18}I(t)] = a_1k_2k_4(\mathrm{EtOH})(^{18}k_4 + {}^{18}\beta_2 + k_5 + \beta_3)/[{}^{18}a_1{}^{18}k_2{}^{18}k_4(\mathrm{Et}^{18}\mathrm{OH})(k_4 + \beta_2 + k_5 + \beta_3)] \tag{A13}$$

so that

$$\mathrm{KIE}(t = 0) = k_2k_4(^{18}k_4 + {}^{18}\beta_2 + k_5 + \beta_3)/[{}^{18}k_2{}^{18}k_4(k_4 + \beta_2 + k_5 + \beta_3)] \tag{A14}$$

It is interesting to note that the KIE expressed in eq A14 contains the rate constants k_5 and β_3, which refer to the hydrolytic pathway through intermediate X′ to free acid. This arises from the fact that the hydrolytic pathway is competitive with transesterification, and the three reactions are linked. Thus, the apparent KIE at time zero may be dependent on the hydrolysis reaction for which no isotope effect is possible.

Registry No. α-Chymotrypsin, 9004-07-3; ethyl 2-furoate, 614-99-3; ethyl 5-*n*-propyl-2-furoate, 87395-49-1; *p*-nitrophenyl 5-*n*-propyl-2-furoate, 59212-59-8; 2-furoic acid, 88-14-2; 5-*n*-propyl-2-furoic acid, 14497-25-7; furoyl chloride, 527-69-5; 5-*n*-propyl-2-furoyl chloride, 87395-50-4; *p*-nitrophenyl 2-furoate, 14967-86-3.