Thermodynamics of the Hydrolysis of *N*-Acetyl-L-phenylalanine Ethyl Ester in Water and in Organic Solvents

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Equilibrium measurements have been performed on the α-chymotrypsin-catalyzed hydrolysis reaction of *N*-acetyl-L-phenylalanine ethyl ester to (*N*-acetyl-L-phenylalanine + ethanol) with carbon tetrachloride, dichloromethane, toluene, and aqueous phosphate buffer as solvents for the reactants and products. Apparent equilibrium constants were measured as a function of temperature for this reaction in all four solvents. Calorimetric measurements were also performed for this reaction in aqueous phosphate buffer. The principal reaction occurring in the aqueous phosphate buffer at pH = 6−7 is *N*-acetyl-L-phenylalanine ethyl ester(aq) + H_2O(aq) = *N*-acetyl-L−phenylalanine$^-$(aq) + ethanol(aq) + H^+(aq). Therefore, to compare the results for the reaction in water with those for the reaction in the organic solvents where it is assumed only neutral species are present, it was necessary to adjust the experimental results to the reaction involving neutral species: *N*-acetyl-L-phenylalanine ethyl ester(sln) + H_2O(sln) = *N*-acetyl-L-phenylalanine(sln) + ethanol-(sln), where sln denotes either aqueous media, carbon tetrachloride, dichloromethane, or toluene. The values of the equilibrium constant for this latter reaction, with the concentration of water included in the expression for the equilibrium constant, ranged from 0.057 to 0.20 at T = 298.15 K for the four solvents. This rather limited range of values for the equilibrium constants is significant. The very limited amount of information available from the literature is also suggestive of the rule that equilibrium constants for hydrolysis reactions in different solvents are comparable if the reaction refers to neutral species and the concentration of water is included in the formulation of the equilibrium constant. Also, the standard molar enthalpy of reaction was found to be a linear function (slope = 313 K) of the standard molar entropy of reaction. This is indicative of an enthalpy−entropy compensation effect.

Introduction

There is substantial interest in carrying out biochemical reactions in organic solvents.[1−4] Much of this interest arises from the desire to produce new substances by novel synthetic pathways as well as to enhance product yields. The several advantages to carrying out biochemical reactions in organic solvents are summarized in the aforementioned reviews.[1−4] One obvious advantage is the improvement of product yield for an ester hydrolysis reaction where, if the concentration of water is kept low, the position of equilibrium is reversed by simple mass action from what it normally is in aqueous solutions. Thus, the ester can be produced in substantial amounts. Clearly, both thermodynamics and kinetics play a role in the development of this science and technology. Surprisingly, the literature in the area of thermodynamics is very limited. There are only a few experimental studies[5−7] as well as some theoretical discussion[8−11] about the effects on the position of equilibrium due to a change of the solvent in which the reaction is performed. Also, there do not appear to be any data on the standard transformed enthalpies or entropies of biochemical reactions in different solvents. Clearly, this information is needed to gain a more complete understanding of the thermodynamics of these reactions. Consequently, we have undertaken a study of a representative reaction, namely, the hydrolysis of *N*-acetyl-L-phenylalanine ethyl ester. The reaction was carried out in aqueous phosphate buffer and in carbon tetrachloride, dichloromethane, and toluene. Equilibrium measurements were also performed as a function of temperature to obtain standard molar enthalpies and entropies of reaction. Microcalorimetry was used to measure the standard molar enthalpy of this hydrolysis reaction in an aqueous phosphate buffer solution.

Experimental Section

The principal substances used in this study, their respective Chemical Abstracts Services registry numbers, empirical formulas, and molar masses are *N*-acetyl-L-phenylalanine, 2018-61-3, $C_{11}H_{13}NO_3$, 207.23 g mol^{-1}; *N*-acetyl-L-phenylalanine ethyl ester, 2361-96-8, $C_{13}H_{17}NO_3$, 235.28 g mol^{-1}; phosphoric acid, 7664-38-2, H_3PO_4, 98.00 g mol^{-1}; potassium phosphate (dibasic), 7758-11-4, K_2HPO_4, 174.18 g mol^{-1}; carbon tetrachloride 56-23-5, CCl_4, 153.82 g mol^{-1}; ethanol, 64-17-5, C_2H_6O, 46.069 g mol^{-1}; dichloromethane, 75-09-2, CH_2Cl_2, 84.93 g mol^{-1}; and toluene, 108-88-3, C_7H_8, 92.14 g mol^{-1}.

Reagent grade chemicals were used throughout this study. The α-chymotrypsin (Enzyme Commission number 3.4.21.1) from bovine pancreas was in the form of a lyophilized powder and was obtained from Sigma.[12] The *N*-acetyl-L-phenylalanine, *N*-acetyl-L-phenylalanine ethyl ester, and K_2HPO_4 were also from Sigma. The ethanol and concentrated phosphoric acid were from Baker. The carbon tetrachloride, dichloromethane, and toluene were obtained from Mallinckrodt. The *N*-acetyl-L-phenylalanine and *N*-acetyl-L-phenylalanine ethyl ester were reported by the vendor to have purities > 99 mol % as determined by thin-layer chromatography. These two substances were also found to be "pure" with the chromatographic procedures described below. The vendor also reported the following angles of optical rotation: α(589.3 nm, *N*-acetyl-L-

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phenylalanine, 293.15 K, 10 g dm^{-3} in methanol, 10-cm path) = +39.2° and α(589.3 nm, *N*-acetyl-L-phenylalanine ethyl ester, 298.15 K, 10 g dm^{-3} in ethanol, 10-cm path) = +14.2°. All substances were used without further purification. The moisture contents were determined by Karl-Fischer analysis (see below) to be 0.32 ± 0.03 mass %[13] for *N*-acetyl-L-phenylalanine, 0.37 ± 0.02 mass % for *N*-acetyl-L-phenylalanine ethyl ester, and 0.09 ± 0.02 mass % for ethanol.

A Metrohm Model 633 automatic Karl Fischer titrator and a Model 665 dosimat were used for the determinations of the amounts of water in the various samples, which were dissolved in a mixture of formamide and "Hydranal Composite 2" solution. This latter solution was also used for the titration, the end point of which was determined coulometrically with appropriate application of corrections for drift. The Karl Fischer apparatus was enclosed in a Plexiglas box, which was maintained under a continual positive pressure of dry nitrogen gas. Calibration was done with a solution consisting of 1-octanol saturated with water. The calculated moisture contents are based on the solubility of water in 1-octanol at T = 298.15 K determined by Leo and Hansch.[14]

The concentrations of *N*-acetyl-L-phenylalanine and *N*-acetyl-L-phenylalanine ethyl ester were measured with a Hewlett-Packard 1090 HPLC with a Zorbax ODS column thermostated at 311 K and a UV detector set at 220 nm. The mobile phase was a gradient formed from two solutions. At time t = 0, the gradient was 100 volume % of a phosphate solution (KH_2PO_4, c = 0.02 mol dm^{-3}, pH = 4.65), and at t = 20 min, the gradient was 100% methanol. The flow rate was 0.012 cm^3 s^{-1}. The retention times were 13.4 min for *N*-acetyl L-phenylalanine and 19.9 min for *N*-acetyl L-phenylalanine ethyl ester. The column was flushed with water and then with phosphate solution (KH_2PO_4, c = 0.02 mol dm^{-3}, pH = 4.65) following each measurement. The solutions used for the determination of the response factors of *N*-acetyl-L-phenylalanine and of *N*-acetyl-L-phenylalanine ethyl ester were prepared by dissolving these two substances in a small amount of ethanol and then adding the appropriate solvent. The concentrations were comparable to those found in the solutions used in the equilibrium experiments.

Analysis of ethanol in the equilibrium experiments was done with a Varian 6000 gas chromatograph equipped with a flame ionization detector and a fused silica capillary column (0.25 mm i.d. × 60 m long) coated with a 5% (w/w) phenyl-substituted methylpolysiloxane phase (2.5 × 10^{-7} m film thickness) (DB-5 column, J&W Scientific, Folsom, CA). The temperatures of the column oven, injector port, and detector were 308 K, 553 K, and 573 K, respectively. The carrier gas (helium) head pressure was 2.8 bar. One-microliter injections of each sample were done at a split ratio of 25:1. Under these conditions, the retention times for ethanol and butanol (the internal standard) were 3.0 and 5.5 min, respectively. For the analysis of ethanol samples in chloroform, dichloromethane, and toluene, 0.5 cm^3 of sample was combined with 0.5 cm^3 of an internal standard solution (butanol at a known concentration in dichloromethane) and 2 cm^3 of dichloromethane. For the ethanol samples in water, 0.5 cm^3 of sample was combined with 0.5 cm^3 of an internal standard solution (butanol at a known concentration in methanol) and 10 cm^3 of methanol. The concentration of the butanol in the internal standard solution was always kept comparable to the concentration of ethanol in the respective sample. Two standard solutions of ethanol in each solvent were prepared gravimetrically and combined with the internal standard solution and solvent in the same way as for the samples. Thus, the calculation of the concentration of ethanol in the samples was based upon the known composition of these standard solutions.

The procedure used for the immobilization of the α-chymotrypsin is now described. Controlled-pore glass (CPG) beads having a pore diameter of (5.5 ± 0.3) × 10^{-8} m were silanized with 3-aminopropyltriethoxysilane according to the procedure described by Weetall.[15] Then, 10 g of the silanized CPG was suspended in 15 cm^3 of distilled water and kept in a desiccator under vacuum for 1 h to open the glass pores. The CPG was then collected by filtration and suspended in 15 cm^3 of a solution consisting of 2.5% (w/v) glutaraldehyde in phosphate buffer solution "A" (K_2HPO_4, c = 0.1 mol dm^{-3}, adjusted to pH = 7.2 with H_3PO_4). This suspension was then shaken in a bath thermostated at 298.15 K for 3−4 h. The CPG was collected by filtration and washed with phosphate buffer solution A several times to remove glutaraldehyde. The CPG was then suspended in an enzyme solution (1 g of α-chymotrypsin dissolved in 6 cm^3 of phosphate buffer solution A) and allowed to stand for ≈16 h at 277 K. The suspension was then shaken in a bath thermostated at 298.15 K for 1 h and then filtered and washed with phosphate buffer solution A. When not in use, the immobilized enzyme was stored in phosphate buffer solution A at 277 K.

Equilibrium measurements were performed by approaching equilibrium from both directions of reaction. In the studies done with the organic solvents, the solutions used for the study of the reaction starting with *N*-acetyl-L-phenylalanine ethyl ester (the forward direction) were prepared by dissolving ≈0.08 g of *N*-acetyl-L-phenylalanine ethyl ester in ≈2 cm^3 of ethanol. Approximately 35 cm^3 of water-saturated organic solvent was then added to this solution followed by ≈2.4 g of immobilized enzyme. The solutions used for the study of the reaction starting with *N*-acetyl-L-phenylalanine and ethanol (the reverse direction) were prepared in the same way except that ≈0.08 g of *N*-acetyl-L-phenylalanine was used instead of its ester. In general, ≈7 days was allowed for equilibration of the reaction in carbon tetrachloride, dichloromethane, and toluene. Only 1 day was required for the reaction in aqueous phosphate buffer. During the equilibrations, the flasks in which the samples were contained were kept in a thermostated shaker bath (≈60 cycles min^{-1}). Great care was taken in withdrawing samples of the organic phase from the reaction flasks with a syringe. Specifically, it was necessary to avoid sampling the region near the immobilized enzyme, which contains water in excess to that found in the bulk organic phase. Since the amounts of *N*-acetyl-L-phenylalanine ethyl ester and of *N*-acetyl-L-phenylalanine were determined with the HPLC, their units of composition are identical and cancel in the equation for the apparent equilibrium constant. The unit of composition in which the water and ethanol were respectively determined by Karl Fischer and GC is mol (kg of solution)$^{-1}$. Thus, these units also cancel in the equation for the apparent equilibrium constant. Note that different measures of solution composition [mol kg^{-1}, mol (kg solution)$^{-1}$, and mol dm^{-3}] were used in this study. The choice of units depended upon the nature of the application and the actual experimental situation.

The microcalorimeters were of the heat-conduction type. The sample vessels, which were fabricated from high-density polyethylene, contained two compartments holding approximately 0.55 and 0.45 cm^3 of solution. In these experiments, the substrate solution (placed in the 0.55-cm^3 compartment) consisted of *N*-acetyl-L-phenylalanine ethyl ester dissolved in a phosphate buffer (m = 0.158 mol kg^{-1}, pH = 6.28) containing ethanol (m = 2.65 mol kg^{-1}). The ethanol was needed to dissolve the *N*-acetyl-L-phenylalanine ethyl ester. The enzyme solution (placed in the 0.45-cm^3 compartment) consisted of the

soluble α-chymotrypsin dissolved in the same (phosphate buffer + ethanol) solution used for the substrate solution. The mass fraction of the enzyme in this solution was 0.0039. The vessels and their contents were allowed to equilibrate in the microcalorimeters for ≈1 h before the solutions were mixed. Calibration of the calorimeters was done electrically with a calibrated digital voltmeter, standard resistor, and time-interval counter. Descriptions of the calorimeters and their performance characteristics, the data-acquisition system, and the computer programs used to treat the results are given in refs 16 and 17. Following reaction in the calorimeter for ≈22 min (the time required for a nearly complete hydrolysis of the *N*-acetyl-L-phenylalanine ethyl ester), the reaction vessels were removed from the calorimeters and their contents were analyzed with the HPLC. It was found that 2.9−3.8 mol % of the *N*-acetyl-L-phenylalanine ethyl ester had not reacted. Appropriate corrections were applied for this unreacted ester. The average of the "blank" enthalpy changes accompanying the mixing of the substrate solution and the enzyme solution with the buffer was 0.3 ± 0.4 mJ. The measured enthalpies of reaction ranged from −16.8 to −20.5 mJ.

The ionization constant and enthalpy of ionization of *N*-acetyl-L-phenylalanine(aq) were measured with a Microcal MCS isothermal titration calorimeter.[18,19] In these experiments, the molality of the *N*-acetyl-L-phenylalanine(aq) was 0.003 91 mol kg^{-1}. The *N*-acetyl-L-phenylalanine was dissolved in aqueous KCl (m = 0.011 mol kg^{-1}). This solution was then adjusted to pH = 6.0 with dilute KOH. Hydrochloric acid (c = 0.10 mol dm^{-3}) was used for the titration. On the basis of two replicate experiments it was found that, at T = 298.15 K and ionic strength I_m = 0.015 mol kg^{-1}, the ionization constant for *N*-acetyl-L-phenylalanine(aq) was (3.55 ± 0.10) × 10^{-4} and the standard enthalpy of ionization was −2.50 ± 0.02 kJ mol^{-1}.

The pH of the reaction mixtures was measured with a combination glass microelectrode and an Orion Model 811 pH meter. All measurements were done at the temperature at which the reactions occurred. Calibration of the pH meter was performed with a standard buffer prepared from potassium dihydrogen phosphate (m = 0.009 695 mol kg^{-1}) and disodium hydrogen phosphate (m = 0.030 43 mol kg^{-1}). These phosphates are standard reference materials 186-Id and 186-IId, respectively, from the National Institute of Standards and Technology. Intercomparisons of this "physiological" buffer against Fisher buffers certified at pH = 7.00, 8.00, and 9.00 were also done with satisfactory agreement (±0.03) in the pH of these solutions.

Results and Discussion

The reaction that is the principal subject of this study is

N-acetyl-L-phenylalanine ethyl ester(sln) + H_2O(sln) =

N-acetyl-L-phenylalanine(sln) + ethanol(sln) (1)

Here, sln denotes either aqueous media, carbon tetrachloride, dichloromethane, or toluene solvents. The equilibrium constant K for reaction 1 in the nonaqueous solvents used in this study is

$$K = c(N\text{-acetyl-L-phenylalanine})c(\text{ethanol})/c(N\text{-acetyl-L-phenylalanine ethyl ester})c(H_2O) \quad (2)$$

It is important to note that the concentration c of water is included in eq 2. This equilibrium constant is symmetrical and thus its value is independent of the choice of standard state. In writing the above equilibrium constant, it was assumed that each of the reactants and products exists in only one form; i.e., none of the substances ionizes in solution. This assumption seems reasonable since the relative permittivities of carbon tetrachloride, dichloromethane, and toluene are all less than 10 and substantially less than that of water.[20] If this were not the case, it would be necessary to use an apparent equilibrium constant K' involving sums of concentrations of pseudoisomer species.[21] In discussing the thermodynamics of reaction 1 in aqueous solutions, the apparent equilibrium constant K'_m is used:

$$K'_m = m(N\text{-acetyl-L-phenylalanine})m(\text{ethanol})/m(N\text{-acetyl-L-phenylalanine ethyl ester})m^\circ \quad (3)$$

where m° = 1 mol kg^{-1}. This quantity is used to make the apparent equilibrium constant dimensionless. Equation 3 is based on the convention that the activity of water a_w = 1. For the reaction in water, one of the reactants (*N*-acetyl-L-phenylalanine) exists in ionized form. Thus, the molalities in eq 3 are the total molalities of the various charged and uncharged species that are formed from the ionization of the various substances in solution. The subscript m in K'_m indicates that the apparent equilibrium constant K'_m was calculated on a molality basis. In this case, the value of the apparent equilibrium constant depends on the choice of standard state. Thus, if concentration c is used instead of molality in eq 3, the value of the apparent equilibrium constant K'_c will differ slightly (<1% for dilute aqueous solutions at T = 298.15 K) from the value of the apparent equilibrium constant K'_m.

The results for the equilibrium constants for reaction 1 in the organic solvents and the apparent equilibrium constants in water are given in Tables 1 and 2, respectively. The results for the calorimetrically determined enthalpy $\Delta_r H$(cal) for the reaction in aqueous phosphate buffer are given in Table 3. The uncertainties assigned to the measured quantities in these tables are equal to two estimated standard deviations of the mean with the exception of the uncertainties assigned to the apparent equilibrium constants K' (combined) given in column 8 in Table 1. In this case, each of these uncertainties has two components. The first is equal to $^1/_2|K'(\text{forward}) - K'(\text{reverse})|$. This is an estimate of the systematic error in the measurements due to a possible failure to reach equilibrium. The second component is equal to the statistical uncertainty for the unweighted average of K'(forward) and K'(reverse). These two components are then combined in quadrature to arrive at the final uncertainties given for K'(combined) in Table 1.

In order to relate the results for the reaction in water, which were obtained in terms of an apparent equilibrium constant involving sums of species, to a chemical reaction involving specific chemical species, it is necessary to have some information on the ionization behavior of these substances. The only substance which has an ionization in the pH range of interest is *N*-acetyl-L-phenylalanine. In the absence of a result for the ionization constant of this substance in the literature, we used titration calorimetry[18,19] to measure the ionization constant and the standard molar enthalpy of ionization of *N*-acetyl-L-phenylalanine. Thus, for the ionization reaction

N-acetyl-L-phenylalanine(aq) =

N-acetyl-L-phenylalanine$^-$(aq) + H^+(aq) (4)

we measured K_m = (3.55 ± 0.10) × 10^{-4}, pK = 3.45 ± 0.02, and $\Delta_r H^\circ$ = −2.50 ± 0.02 kJ mol^{-1} at T = 298.15 K and I_m = 0.015 mol kg^{-1}. With the extended Debye−Hückel equation and an estimated "ion-size" parameter of 1.6 kg$^{1/2}$ mol$^{-1/2}$, we calculate[22] K_m = (2.79 ± 0.15) × 10^{-4}, pK = 3.55 ± 0.02, and

TABLE 1: Results of Equilibrium Measurements for Reaction 1 in Dichloromethane, Carbon Tetrachloride, and Toluene[a]

T, K	direction	$c'(C_{11}H_{13}NO_3) \times 10^4$, mol (kg soln.)$^{-1}$	$c'(C_{13}H_{17}NO_3) \times 10^3$, mol (kg soln.)$^{-1}$	$c'(H_2O)$, mol (kg soln.)$^{-1}$	$c'(C_2H_6O)$, mol (kg soln)$^{-1}$	K	K(combined)
			Dichloromethane				
283.15	forward	1.198 ± 0.009	6.303 ± 0.047	0.1087 ± 0.0018	0.234 ± 0.014	0.0409 ± 0.0026	
							0.0377 ± 0.0037
283.15	reverse	0.747 ± 0.006	4.452 ± 0.023	0.1118 ± 0.0038	0.229 ± 0.011	0.0344 ± 0.0021	
288.15	forward	1.513 ± 0.044	6.497 ± 0.032	0.1180 ± 0.0083	0.213 ± 0.011	0.0420 ± 0.0039	
							0.0437 ± 0.0029
288.15	reverse	1.300 ± 0.022	5.795 ± 0.13	0.1127 ± 0.0009	0.228 ± 0.012	0.0454 ± 0.0027	
293.25	forward	1.227 ± 0.016	5.375 ± 0.012	0.1275 ± 0.0053	0.289 ± 0.013	0.0517 ± 0.0032	
							0.0513 ± 0.0022
293.25	reverse	1.111 ± 0.024	5.045 ± 0.027	0.1222 ± 0.0042	0.283 ± 0.013	0.0510 ± 0.0031	
298.25	forward	2.040 ± 0.10	7.340 ± 0.27	0.1729 ± 0.0067	0.363 ± 0.019	0.0584 ± 0.0052	
							0.0572 ± 0.0037
298.25	reverse	1.761 ± 0.034	5.920 ± 0.33	0.1762 ± 0.0047	0.332 ± 0.017	0.0561 ± 0.0046	
			Carbon Tetrachloride				
283.15	forward	0.2168 ± 0.048	0.8078 ± 0.0040	0.0141 ± 0.0006	0.272 ± 0.013	0.520 ± 0.11	
							0.38 ± 0.15
283.15	reverse	0.0408 ± 0.009	0.3378 ± 0.0057	0.0151 ± 0.0009	0.296 ± 0.015	0.237 ± 0.053	
288.15	forward	1.481 ± 0.035	3.949 ± 0.011	0.0258 ± 0.0014	0.234 ± 0.012	0.340 ± 0.027	
							0.30 ± 0.04
288.15	reverse	1.504 ± 0.019	4.127 ± 0.076	0.0288 ± 0.0011	0.211 ± 0.010	0.268 ± 0.017	
293.15	forward	0.6350 ± 0.024	2.810 ± 0.011	0.0204 ± 0.0008	0.230 ± 0.010	0.255 ± 0.018	
							0.23 ± 0.03
293.15	reverse	0.3674 ± 0.026	1.872 ± 0.021	0.0213 ± 0.0004	0.226 ± 0.011	0.209 ± 0.019	
298.25	forward	1.467 ± 0.056	4.203 ± 0.028	0.0278 ± 0.0022	0.156 ± 0.010	0.196 ± 0.021	
							0.197 ± 0.046
298.25	reverse	0.8735 ± 0.39	3.075 ± 0.006	0.0220 ± 0.0021	0.152 ± 0.008	0.197 ± 0.090	
			Toluene				
283.15	forward	1.203 ± 0.044	5.301 ± 0.017	0.0609 ± 0.0018	0.460 ± 0.023	0.172 ± 0.012	
							0.182 ± 0.013
283.15	reverse	1.726 ± 0.054	6.442 ± 0.015	0.0673 ± 0.0022	0.483 ± 0.025	0.192 ± 0.013	
288.15	forward	2.349 ± 0.035	6.633 ± 0.18	0.0736 ± 0.0002	0.353 ± 0.020	0.170 ± 0.011	
							0.201 ± 0.033
288.15	reverse	2.010 ± 0.077	5.796 ± 0.041	0.0648 ± 0.0029	0.435 ± 0.024	0.233 ± 0.019	
293.25	forward	1.720 ± 0.028	5.831 ± 0.064	0.0570 ± 0.0026	0.286 ± 0.017	0.148 ± 0.011	
							0.144 ± 0.009
293.25	reverse	1.285 ± 0.054	4.241 ± 0.008	0.0524 ± 0.0023	0.243 ± 0.013	0.141 ± 0.011	
298.25	forward	1.630 ± 0.053	5.156 ± 0.025	0.1027 ± 0.0083	0.336 ± 0.017	0.104 ± 0.010	
							0.107 ± 0.007
298.25	reverse	1.672 ± 0.041	5.132 ± 0.057	0.1021 ± 0.0030	0.346 ± 0.018	0.110 ± 0.007	

[a] The equilibrium constants K were calculated with eq 2. The quantities c' (defined as amounts of substance divided by mass of solution) for these substances in solution at equilibrium are given in columns 3–6. Measurements of c' for water *N*-acetyl-L-phenylalanine, and *N*-acetyl-L-phenylalanine ethyl ester were done in triplicate; the measurements of c' for ethanol were done in duplicate. *N*-Acetyl-L-phenylalanine is $C_{11}H_{13}NO_3$, *N*-acetyl-L-phenylalanine ethyl ester is $C_{13}H_{17}NO_3$, and ethanol is C_2H_6O. K(combined) was calculated from the equilibrium constants, which were measured from both directions of the reaction. The basis of the uncertainties is discussed in the text.

TABLE 2: Results of Equilibrium Measurements for Reaction 1 in Water[a]

T, K	direction	pH	$m(K_2HPO_4)$, mol kg^{-1}	$m(H_3PO_4)$, mol kg^{-1}	$m(C_{11}H_{13}NO_3) \times 10^2$, mol kg^{-1}	$m(C_{13}H_{17}NO_3) \times 10^5$, mol kg^{-1}	$m(C_2H_6O)$, mol kg^{-1}	I_m, mol kg^{-1}	$K'_m \times 10^{-3}$	$K_m \times 10^3$
288.15	forward	6.09	0.090 45	0.019 62	6.492 ± 0.087	5.249 ± 0.14	2.87 ± 0.16	0.11	3.55 ± 0.22	2.25 ± 0.15
288.15	reverse	6.29	0.088 15	0.019 12	5.116 ± 0.053	1.474 ± 0.045	2.30 ± 0.14	0.11	7.98 ± 0.55	3.19 ± 0.22
293.15	forward	6.17	0.088 59	0.019 21	6.550 ± 0.13	6.817 ± 0.21	2.36 ± 0.15	0.11	2.27 ± 0.17	1.19 ± 0.10
293.15	reverse	6.39	0.089 97	0.019 51	5.929 ± 0.13	3.834 ± 0.12	2.77 ± 0.15	0.13	4.28 ± 0.28	1.34 ± 0.10
298.15	forward	6.19	0.093 19	0.020 21	6.895 ± 0.12	6.161 ± 0.072	3.61 ± 0.19	0.12	4.04 ± 0.23	2.01 ± 0.12
298.15	reverse	6.44	0.090 74	0.019 68	5.945 ± 0.083	3.351 ± 0.071	2.98 ± 0.15	0.13	5.29 ± 0.30	1.47 ± 0.09
308.15	forward	5.98	0.095 89	0.020 80	6.254 ± 0.074	16.78 ± 0.23	4.38 ± 0.20	0.11	1.63 ± 0.08	1.32 ± 0.08
308.15	reverse	6.23	0.095 57	0.020 73	5.486 ± 0.010	11.93 ± 0.50	4.33 ± 0.18	0.12	1.99 ± 0.12	0.90 ± 0.07

[a] The apparent equilibrium constants K'_m were calculated with eq 3. The molalities of these substances in solution at equilibrium are given in columns 6–8. Measurements of the molalities of water, *N*-acetyl-L-phenylalanine, and *N*-acetyl-L-phenylalanine ethyl ester were done in triplicate; the measurements of the molality of ethanol were done in duplicate. *N*-acetyl-L-phenylalanine is $C_{11}H_{13}NO_3$, *N*-acetyl-L-phenylalanine ethyl ester is $C_{13}H_{17}NO_3$, and ethanol is C_2H_6O. The apparent equilibrium constant K'_m is given in column 10. The ionic strength I_m and the equilibrium constant K_m for the chemical reference reaction 5 at the indicated temperature and at $I = 0$ are calculated quantities. The basis of the uncertainties is discussed in the text.

$\Delta_r H° = -2.80 \pm 0.04$ kJ mol^{-1} at $I = 0$. The uncertainties in these quantities have been increased to allow for possible systematic errors in the adjustment to $I = 0$. The only other result in the literature for reaction 4 is $\Delta_r H°$ ($T = 298.15$ K, $I_m \approx 0.05$ mol kg^{-1}) $= -2.46 \pm 0.09$ kJ mol^{-1} reported by Rekharsky et al.[23] We adjust this result to $I = 0$ in the same way as above and obtain $\Delta_r H° = -2.95 \pm 0.15$ kJ mol^{-1} at $I = 0$. This is in agreement with the result obtained in this study.

The assignment of the charge on *N*-acetyl-L-phenylalanine is based upon an examination of its structure. The absence of additional ionizations having a p$K \leq 9$ from *N*-acetyl-L-phenylalanine and any ionizations from *N*-acetyl-L-phenylalanine

TABLE 3: Results of Calorimetric Measurements for Reaction 1 in Water at $T = 298.15$ K and pH = 6.28[a]

experiment	$m(K_2HPO_4)$, mol kg^{-1}	$m(H_3PO_4)$, mol kg^{-1}	$m(C_2H_6O)$, mol kg^{-1}	$m(C_{13}H_{17}NO)_3 \times 10^3$, mol kg^{-1}	$\Delta_r H$(cal), kJ mol^{-1}
1	0.1017	0.056 69	2.649	3.023	−8.61
2	0.1017	0.056 69	2.649	2.954	−7.70
3	0.1017	0.056 70	2.648	3.057	−8.26
4	0.1017	0.056 70	2.649	2.868	−8.52
5	0.1017	0.056 69	2.649	2.956	−8.54
					av −8.33 ± 0.34

[a] $\Delta_r H$(cal) is the calorimetrically determined enthalpy of reaction. The molality of the *N*-acetyl-L-phenylalanine ethyl ester ($C_{13}H_{17}NO_3$) is that after mixing of the enzyme and substrate solutions and prior to any reaction. Ethanol is denoted by C_2H_6O. The ionic strength is 0.13 mol kg^{-1}.

ethyl ester was confirmed by a potentiometric titration which we performed on these substances. Clearly, there are no ionizations from either ethanol or water that occur in the pH range 6−6.5 used in this study. Therefore, the predominant hydrolysis reaction occurring in aqueous solution in this pH range is

N-acetyl-L-phenylalanine ethyl ester(aq) + H_2O(aq) =

N-acetyl-L-phenylalanine$^-$(aq) + ethanol(aq) + H^+(aq) (5)

This is selected as a chemical reference reaction for the overall biochemical reaction 1 occurring in water. The choice of reference reaction is not unique. Later, a reference reaction involving only neutral species will be used for purposes of comparison with the results obtained for the hydrolysis reaction in the organic solvents.

Since phosphate buffer was used in both the equilibrium and calorimetric measurements, information on the ionization of $H_2PO_4^-$(aq) is also needed for the equilibrium calculations. Specifically, while $H_2PO_4^-$(aq) does not participate directly in the hydrolysis reaction, it does affect the ionic strength. Thus, we have calculated the ionization constant ($K_m = 6.23 \times 10^{-8}$) and standard enthalpy of ionization of $H_2PO_4^-$(aq) ($\Delta_r H° = 4.2$ kJ mol^{-1}) from the standard molar Gibbs energies and enthalpies of formation given in the "NBS Tables of Chemical Thermodynamic Properties".[24] The standard molar heat capacity of reaction $\Delta_r C_p^\circ = -220$ J K^{-1} mol^{-1} was calculated from the standard apparent molar heat capacities reported by Larson et al.[25]

The results given in Tables 2 and 3 for reaction 1 in water can be used together with the ionization constants and standard molar enthalpies of ionization of *N*-acetyl-L-phenylalanine(aq) and of $H_2PO_4^-$(aq) in an equilibrium model calculation[22] to obtain thermodynamic quantities for the chemical reference reaction 5. In performing these calculations, we used an estimated "ion-size" parameter of 1.6 kg$^{1/2}$ mol$^{-1/2}$ in the extended Debye−Hückel equation to estimate the activity coefficients of the aqueous species in solution. From the equilibrium results given in Table 2 and with the equilibrium model,[22] we calculate the values of the equilibrium constants K_m for reaction 5 given in column 11 in Table 2. The values of the ionic strengths given in column 9 are also calculated. From Table 3, we obtain an average value of $K_m = 0.0017$ for reaction 5 at $T = 298.15$ K and $I = 0$. From the temperature dependence of the equilibrium constants given in column 11 in Table 2, we calculate $\Delta_r H° = -27 \pm 22$ kJ mol^{-1} for reaction 5 at $T = 298.15$ K and $I = 0$. The uncertainty in this result is large because of the large amount of scatter in the values of the equilibrium constants for reaction 5. This scatter is attributable to the fact that the measurement of the concentration of *N*-acetyl-L-phenylalanine ethyl ester was performed near the limit of detection of the HPLC. A more accurate and precise result for the standard molar enthalpy of reaction will be obtained from the calorimetrically determined reaction enthalpy.

From the calorimetric results given in Table 3 and with the equilibrium model, we obtain the standard transformed enthalpy of reaction[26] $\Delta_r H'° = -2.8$ kJ mol^{-1} for the biochemical reaction 1 occurring in water at $T = 298.15$ K, pH = 6.28, and $I = 0.13$ mol kg^{-1}. The standard enthalpy of reaction $\Delta_r H°$ for the chemical reference reaction 5 is -3.5 kJ mol^{-1} at $T = 298.15$ K and $I = 0$. The uncertainty in this result is substantially less than the uncertainty in the value of $\Delta_r H°$ calculated from the temperature dependence of K_m for reaction 5. The change in binding of the hydrogen ion $\Delta_r N(H^+)$ for the biochemical reaction 1 in water is also obtained from this calculation: $\Delta_r N(H^+) = -0.999$ at $T = 298.15$ K, pH = 6.19, and $I = 0.13$ mol kg^{-1}.

We now wish to obtain error estimates for the thermodynamic quantities for the chemical reference reaction 5. To do this, we assume that the pKs and standard molar enthalpies of ionization of *N*-acetyl-L-phenylalanine(aq) and of $H_2PO_4^-$(aq) are uncertain by ±0.02 and ±0.1 kJ mol^{-1}, respectively. We also assume that the "ion-size" parameter in the extended Debye−Hückel equation is uncertain by ±0.3 kg$^{1/2}$ mol$^{-1/2}$. These assumed uncertainties were then individually used to perturb the parameters in the model. The effects of these individual perturbations were then combined in quadrature with each other and with the estimates of random error given for the apparent equilibrium constants in column 10 in Table 2 to calculate the uncertainties given for the equilibrium constants for the chemical reference reaction in column 11. A similar treatment was used for the calorimetrically determined reaction enthalpy. The uncertainty assigned to the equilibrium constant for the chemical reference reaction 5 has been expanded to include the differences between the two results obtained from the forward and reverse directions of reaction at $T = 298.15$ K. Thus, we have $K_m = (1.7 \pm 0.3) \times 10^{-3}$, $\Delta_r G° = 15.8 \pm 0.5$ kJ mol^{-1}, $\Delta_r H° = -3.5 \pm 0.4$ kJ mol^{-1}, and $\Delta_r S° = -65 \pm 2$ J K^{-1} mol^{-1} for the chemical reference reaction 5 at $T = 298.15$ K and $I = 0$.

Some comparisons of these results can be made with data from the literature. From equilibrium measurements, Antonov et al.[27] obtained K_c' ($T = 293.15$ K, pH = 5.5) = 588 for the overall biochemical reaction

N-acetyl-L-phenylalanine methyl ester(aq) + H_2O(aq) =

N-acetyl-L-phenylalanine(aq) + methanol(aq) (6)

From an equilibrium model calculation similar to that described above, we obtain $K_m = 1.4 \times 10^{-3}$ at $T = 298.15$ K and $I = 0$ for the chemical reference reaction

N-acetyl-L-phenylalanine methyl ester(aq) + H_2O(aq) =

N-acetyl-L-phenylalanine$^-$(aq) + methanol(aq) + H^+(aq) (7)

In performing this calculation, we used $\Delta_r H°$ ($T = 298.15$ K,

TABLE 4: Equilibrium Constants *K*, Standard Molar Gibbs Energies of Reaction $\Delta_r G°$, Standard Molar Enthalpies of Reaction $\Delta_r H°$, and Standard Molar Entropies of Reaction $\Delta_r S°$ for the Chemical Reference Reaction 8 at $T = 298.15$ K and $T = 0$[a]

solvent	K or K_m	$\Delta_r G°$, kJ mol^{-1}	$\Delta_r H°$, kJ mol^{-1}	$\Delta_r S°$, J K^{-1} mol^{-1}
dichloromethane	0.0572 ± 0.0037	7.09 ± 0.17	20 ± 5	43 ± 17
carbon tetrachloride	0.197 ± 0.046	4.03 ± 0.66	−28 ± 22	−107 ± 74
toluene	0.107 ± 0.007	5.54 ± 0.17	−26 ± 14	−106 ± 47
water[b]	6.1 ± 1.1	−4.5 ± 0.5	−0.7 ± 0.4	13 ± 2
water[c]	0.11 ± 0.02	5.5 ± 0.5	−0.7 ± 0.4	−21 ± 2

[a] The equilibrium constant for this reaction in the organic solvents is independent of the choice of standard state. The equilibrium constant $K_m = 6.1 \pm 1.1$ for this reaction in water is based upon a molality standard state ($m° = 1$ mol kg^{-1}). Two different conventions have been used to calculate an equilibrium constant for the reaction occurring in water. [b] Based upon the convention $a_w = 1$ (see eq 10). [c] Based upon the use of eq 9 and $m(H_2O) = 55.508$ mol kg^{-1}.

$I = 0) = -3.5$ kJ mol^{-1} for the chemical reference reaction 7 based on its chemical similarity with reaction 5. Also, we assumed that the ionic strength used by Antonov et al.[27] was ≈0.1 mol kg^{-1} and neglected the small difference (<1 percent) between equilibrium constants based on a concentration and a molality scale. The value of the calculated equilibrium constant (1.4×10^{-3}) is comparable to the result obtained in this study for the chemical reference reaction 5, namely, $K_m = (1.7 \pm 0.3) \times 10^{-3}$ at $T = 298.15$ K and $I = 0$.

Rekharsky et al.[23] measured $\Delta_r H(\text{cal}) = -4.81 \pm 0.1$ kJ mol^{-1} for reaction 6 at $T = 298.15$ K, pH = 7.0, and with phosphate buffer ($c = 0.05$ mol dm^{-3}). With this result and our equilibrium model, we calculate $\Delta_r H° = -0.1$ kJ mol^{-1} at $T = 298.15$ K and $I = 0$ for the chemical reference reaction 7. In this study, we obtained $\Delta_r H°$ ($T = 298.15$ K, $I = 0$) $= -3.5 \pm 0.4$ kJ mol^{-1} for the chemical reference reaction 5. The difference between these results seems larger than one would expect on the basis of the chemical similarity of the substances involved in these reactions.

The results obtained in this study can be used in conjunction with the chemical equilibrium model to calculate the apparent equilibrium constant, the standard transformed Gibbs energy of reaction $\Delta_r G'°$,[28] and the standard transformed enthalpy of reaction $\Delta_r H'°$ for the biochemical reaction 1 in water under a wide variety of conditions. At $T = 298.15$ K, pH = 7.0, and $I_m = 0.25$ mol kg^{-1}, $K' = 2.3 \times 10^4$, $\Delta_r G'° = -24.9$ kJ mol^{-1}, and $\Delta_r H'° = -2.7$ kJ mol^{-1}. At $T = 310.15$ K, pH = 7.0, and $I_m = 0.25$ mol kg^{-1}, $K' = 2.2 \times 10^4$, $\Delta_r G'° = -25.8$ kJ mol^{-1}, and, assuming that $\Delta_r C_p^\circ = 0$ for the chemical reference reaction 5, $\Delta_r H'° \approx -2.5$ kJ mol^{-1}.

An alternative choice to the chemical reference reaction 5 is

$$N\text{-acetyl-L-phenylalanine ethyl ester}^0(\text{sln}) + H_2O(\text{sln}) = N\text{-acetyl-L-phenylalanine}^0(\text{sln}) + \text{ethanol}^0(\text{sln}) \quad (8)$$

The appearance of reaction 8 is similar to that of reaction 1 but differs from it in that it is a reference reaction involving specific chemical species which, in this case, are all electrically neutral. This is the "convention" suggested by Carpenter[29] for the comparison of the thermodynamics of hydrolysis reactions. The equilibrium constant for this reaction is

$$K = m(N\text{-acetyl-L-phenylalanine}^0)m(\text{ethanol}^0)/m(N\text{-acetyl-L-phenylalanine ethyl ester}^0)m(H_2O) \quad (9)$$

When considering reaction 8 in water, the usual convention is to use the equilibrium constant

$$K_m = m(N\text{-acetyl-L-phenylalanine}^0)m(\text{ethanol}^0)/m(N\text{-acetyl-L-phenylalanine ethyl ester}^0)m° \quad (10)$$

Here, the activity of water $a_w = 1$ and $m° = 1$ mol kg^{-1}. We now use the standard thermodynamic quantities for reaction 5 together with the standard molar thermodynamic quantities for the ionization reaction 4 to calculate $K_m = 6.1 \pm 1.1$, $\Delta_r G° = -4.5 \pm 0.5$ kJ mol^{-1}, $\Delta_r H° = -0.7 \pm 0.4$ kJ mol^{-1}, and $\Delta_r S° = 13 \pm 2$ J K^{-1} mol^{-1} for the chemical reference reaction 8 in water at $T = 298.15$ K and $I = 0$. However, a better comparison of the result obtained for the equilibrium constant in water with the results obtained for the hydrolysis reaction in the organic solvents should include the molality of water in the formulation of the equilibrium constant for the reaction in water. Therefore, we use eq 9 with $m(H_2O) = 55.508$ mol kg^{-1} and calculate the standard thermodynamic quantities for reaction 8 in water: $K = 0.11 \pm 0.02$, $\Delta_r G° = 5.5 \pm 0.5$ kJ mol^{-1}, $\Delta_r H° = -0.7 \pm 0.4$ kJ mol^{-1}, and $\Delta_r S° = -21 \pm 2$ J K^{-1} mol^{-1} at $T = 298.15$ K and $I = 0$.

The standard molar thermodynamic quantities for reaction 8 at $T = 298.15$ K and $I = 0$ are given in Table 4. The standard molar enthalpies of reaction $\Delta_r H°$ given in this table were calculated from the temperature dependence of the apparent equilibrium constants given in Table 1. In this calculation, we assumed that the standard molar enthalpies of reaction were constant over the temperature range used in this study, i.e., $\Delta_r C_p^\circ = 0$.

Examination of the results given in column 2 in Table 4 shows that the equilibrium constants for reaction 8 in the three organic solvents and the equilibrium constant for this reaction in water calculated with eq 9 and $m(H_2O) = 55.508$ mol kg^{-1} have comparable values. This suggests the possibility of a simple and useful rule for estimating equilibrium constants for a reaction occurring in various solvents from the known value of the equilibrium constant in another solvent (most typically water). Clearly, additional comparisons of such data are needed to assess the validity and limits of this rule.

The fact that the values of the equilibrium constants in Table 4 are comparable and that the standard molar enthalpies and entropies of reaction are quite different is indicative of an enthalpy−entropy compensation effect. This is shown in Figure 1 where the standard molar enthalpy of reaction is given as a function of the standard molar entropy of reaction. The straight line drawn through the points has a slope of 313 ± 15 K and an intercept ($\Delta_r S° = 0$) of 6.2 ± 1.2 kJ mol^{-1}. The slope is typical of such plots.[30] It is particularly interesting that this effect is found for a reaction which has been studied both in water and in organic solvents. Although Figure 1 is based upon a very limited set of data, it indicates that enthalpy−entropy compensation is not limited to aqueous solutions and may be an attribute of the reaction itself. While a discussion of the extensive literature dealing with this effect is beyond the scope of this paper, a simple qualitative explanation for this effect for reactions involving polymers was given by Pimentel and McClellan:[31] "a higher value of ($-\Delta_r H°$) implies stronger

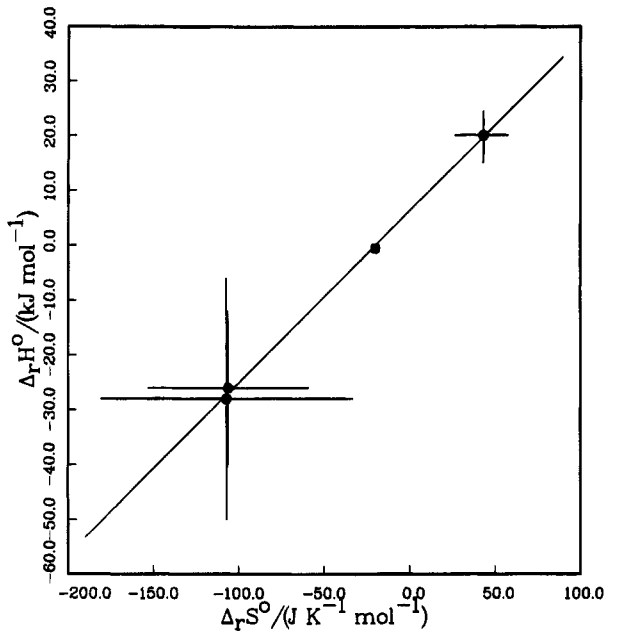

Figure 1. Standard molar enthalpy of reaction $\Delta_r H^\circ$ as a function of the standard molar entropy of reaction $\Delta_r S^\circ$ for the chemical reference reaction 8 at $T = 298.15$ K. The result for water is based upon the use of eq 9 with $m(H_2O) = 55.508$ mol kg^{-1} (see Discussion and Table 4). The straight line is the least-squares fit to the results. The error bars for the reaction in water are too small to be seen.

bonding, with a more restricted configuration in the polymer, hence greater order, leading to a larger value of $(-\Delta_r S^\circ)$". Going further than this qualitative argument, Larson and Hepler[32] used thermodynamic arguments and some reasonable extrathermodynamic assumptions to derive equations which predict a linear relationship between $\Delta_r H^\circ$ and $\Delta_r S^\circ$.

Compared with the rather extensive information available on the thermodynamics of biochemical reactions in aqueous media, there is relatively little information available on these reactions in neat organic solvents. In the most extensive work to date, Valivety et al.[5] studied the reaction

$$n\text{-decanoic acid } n\text{-dodecanyl ester(sln)} + H_2O(\text{sln}) = n\text{-dodecanol(sln)} + n\text{-decanoic acid(sln)} \quad (11)$$

in 20 different organic solvents. They reported the quantity

$$A = \{c(n\text{-dodecanol})c(n\text{-decanoic acid})/c(n\text{-decanoic acid } n\text{-dodecanyl ester})\}^{-1} \quad (12)$$

The quantity A given by Valivety et al.[5] is useful for calculating the product yield of the ester. However, Valivety et al. did not measure the concentration of water in the reaction mixtures. Therefore, it is not possible to relate directly the quantity A to the equilibrium constant

$$K = c(n\text{-dodecanol})c(n\text{-decanoic acid})/c(n\text{-decanoic acid } n\text{-dodecanyl ester})c(H_2O) \quad (13)$$

To obtain values for these equilibrium constants, we have used the solubilities of water in the various organic solvents given in Valivety et al.'s Table II to estimate $c(H_2O)$ in eq 13. The quantity A is then used with $c(H_2O)$ to calculate *approximate* values of the equilibrium constants for reaction 11. These range from 0.012 with nitromethane as the solvent to 0.78 with n-hexadecane as the solvent. Nitromethane has a substantially higher relative permittivity ($\epsilon_r = 35.87$)[20] than the other solvents used by Valivety et al.[5] Thus, the value of the equilibrium constant for the reaction in nitromethane may be anomalous due to the fact the n-decanoic acid has ionized in this solvent and the reaction as measured does not pertain to only neutral species. However, most of the approximate values of the equilibrium constants fall in the range 0.1−0.4. This is consistent with the finding of this study that the equilibrium constants for a hydrolysis reaction have comparable values in different solvents when the reaction refers to neutral species and the concentration of water is included in the equilibrium constant. It is seen from eqs 12 and 13 that $A = \{Kc(H_2O)\}^{-1}$. Thus, if the values of the equilibrium constants for reaction 11 in the various organic solvents were indeed comparable, the quantity A should correlate well with the solubility of water in these various solvents. This was the finding of Valivety et al.[5]

Blanco et al.[6] studied the reaction

$$N\text{-acetyl-L-tryptophan phenylethyl ester(sln)} + H_2O(\text{sln}) = N\text{-acetyl-L-tryptophan(sln)} + \text{phenylethanol(sln)} \quad (14)$$

They, like Valivety et al.,[5] did not measure $c(H_2O)$ in the five different solvents used in their study. Again, we used the mole fraction solubilities of water (given in Blanco et al.'s Table I) with densities from the literature[20] to estimate $c(H_2O)$ and then to calculate approximate values of the equilibrium constants

$$K = (N\text{-acetyl-L-tryptophan})c(\text{phenylethanol})/c(N\text{-acetyl-L-tryptophan phenylethyl ester})c(H_2O) \quad (15)$$

The approximate values of the equilibrium constants range from 0.62 with 2-butanone as the solvent to 2.1 with trichloroethane as the solvent and, again, are seen to be comparable for the various solvents used in the study.

Janssen et al.[7] studied the lipase-catalyzed esterification of glycerol with octanoic acid, decanoic acid, and oleic acid to form the monoesters, diesters, and triesters. The equilibrium constants they report pertain to the organic phase consisting of these substances without the addition of any organic solvent.

Clearly, the amount of information currently available is very limited and there is a need for additional thermodynamic data on biochemical reactions in organic solvents. Such data could provide further tests of the hypothesis that equilibrium constants for hydrolysis and perhaps other classes of reactions in different solvents are comparable if the reaction refers to neutral species and the concentration of water is included in the formulation of the equilibrium constant.

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(12) Certain commercial materials and products are identified in this paper to specify adequately the experimental procedure. Such identification does not imply recommendation or endorsement by the National Institute of Standards and Technology.

(13) All uncertainties given in this paper are, unless indicated otherwise, based on two estimated standard deviations of the mean.

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