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# Thermodynamics of the hydrolysis reactions of nitriles

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### Abstract

Microcalorimetry and high-performance liquid chromatography (h.p.l.c) have been used to conduct a thermodynamic investigation of the following nitrilase catalyzed reactions: (1) benzonitrile(aq) + 2H<sub>2</sub>O(l) = benzoic acid(aq) + ammonia(aq), (2) benzylcyanide(aq) + 2H<sub>2</sub>O(l) = benzeneacetic acid(aq) + ammonia(aq), (3) 3-phenylpropionitrile(aq) + 2H<sub>2</sub>O(l) = 3-phenylpropanoic acid(aq) + ammonia(aq), (4) 4-phenylbutyonitrile(aq) + 2H<sub>2</sub>O(l) = 4-phenylbutyric acid(aq) + ammonia(aq), (5)  $\alpha$ -methylbenzyl cyanide(aq) + 2H<sub>2</sub>O(l) =  $\alpha$ -methylbenzene acetic acid(aq) + ammonia(aq), and (6) 3-indoleacetonitrile(aq) + 2H<sub>2</sub>O(l) = indole-3acetic acid(aq) + ammonia(aq). The equilibrium measurements showed that these reactions proceeded to completion. Thus, it was possible to set only lower limits for the values of the apparent equilibrium constants K'. However, it was possible to obtain precise values of the calorimetrically determined molar enthalpies of reaction  $\Delta_r H_m$ (cal). These values were then used in conjunction with an equilibrium model to calculate values of the standard molar enthalpies for chemical reference reactions that correspond to the above overall biochemical reactions.

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*Keywords:* Ammonia; Apparent equilibrium constant; Benzeneacetic acid; Benzoic acid; Benzonitrile; Benzylcyanide; Enthalpy; Entropy; Gibbs free energy; Indole-3-acetic acid; 3-Indoleacetonitrile;  $\alpha$ -Methylbenzeneacetic acid;  $\alpha$ -Methylbenzyl cyanide; 4-Phenylbutyonitrile; 4-Phenylbutyric acid; 3-Phenylpropanoic acid; 3-Phenylpropionitrile

# 1. Introduction

The nitrilase enzymes catalyze the direct hydrolysis of organic nitriles to the corresponding carboxylic acids, which are potentially useful as intermediates in the agricultural and pharmaceutical industries. The use of nitrilase enzymes has attracted substantial interest in the biotechnology community [1–4] because conventional chemical methods for nitrile hydrolysis entail the use of severe conditions such as the use of concentrated acid or base and high temperatures. Such harsh conditions are generally not useful when sensitive complex molecules, or chiral compounds are involved. Most importantly, the chemical methods do not permit asymmetric synthesis. In contrast, the nitrilase-catalyzed reactions proceed under mild conditions and produce a high yield of a stereospecific product. The importance of nitrilase enzymes has led recently to the development of a library consisting of over 200 new forms of this enzyme [3] (see Figure 1).

Having an active catalyst is central to making the desired bioprocess possible. However, a proper engineering of the process requires thermodynamic information in order to optimize the product yield and to obtain a correct heat balance in a bioreactor. Interestingly, there do not appear to be any thermodynamic results in the literature for the hydrolysis of nitriles. Accordingly, we have performed a thermodynamic investigation of several representative nitrilase-catalyzed reactions:

 $benzonitrile(aq) + 2H_2O(l) =$ benzoic acid(aq) + ammonia(aq), (1)

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FIGURE 1. The structures of the substances and the reactions studied herein.

$$\label{eq:benzylcyanide} \begin{split} \text{benzylcyanide}(aq) + 2H_2O(l) = \\ \\ \text{benzeneacetic acid}(aq) + ammonia(aq), \end{split} \tag{2}$$

3-phenylpropionitrile(aq) +  $2H_2O(l) =$ 

$$3-phenylpropanoic acid(aq) + ammonia(aq), \qquad (3)$$

4-phenylbutyonitrile(aq) +  $2H_2O(l) =$ 

$$4-phenylbutyric acid(aq) + ammonia(aq), \qquad (4)$$

 $\alpha$ -methylbenzyl cyanide(aq) + 2H<sub>2</sub>O(l) =

 $\alpha$ -methylbenzene acetic acid(aq) + ammonia(aq), (5)

$$\begin{array}{l} 3\text{-indoleacetonitrile}(aq)+2H_2O(l)=\\ & \text{indole-3-acetic acid}(aq)+ammonia(aq). \end{array} \tag{6}$$

## 2. Experimental

### 2.1. Materials

Pertinent information on the substances used in this study is given in table 1.<sup>1</sup> The purities of the nitriles were assessed by using a Hewlett Packard 5890 g.c. equipped with a flame ionization detector and a fused silica Phenomenex ZB-FFAP capillary column (30 m long, 0.53 mm i.d.). The injector and detector temperatures were (523 and 540) K, respectively. The head pressure

<sup>&</sup>lt;sup>1</sup> Certain commercial equipment, or instruments, or materials are identified in this paper to specify the experimental procedures adequately. Such identification is not intended to imply recommendation, or endorsement by the National Institute of Standards and Technology (NIST), nor is it intended to imply that the materials or equipment identified are necessarily the best available for the purpose.

TABLE 1

Principal substances used in this study with their Chemical Abstracts Service (CAS) registry numbers, empirical formulae, relative molecular masses  $M_r$ , mass fraction moisture contents w determined by Karl Fischer analysis, suppliers (A = Aldrich, B = BioCatalytics, M = Mallinckrodt, N = NIST, and S = Sigma), estimated mole fraction purities x, and method(s) used to determine x

| Substance                    | CAS No.   | Formula                                      | $M_{ m r}$ | W      | Supplier | x <sup>a</sup> | Method(s)         |
|------------------------------|-----------|--|------------|--------|----------|----------------|-------------------|
| Benzeneacetic acid           | 103-82-2  | C <sub>8</sub> H <sub>8</sub> O <sub>2</sub> | 136.18     |        | А        | 0.995          | g.c.              |
| Benzoic acid                 | 65-85-0   | $C_7H_6O_2$                                  | 122.12     |        | Ν        | >0.9999        | acidimetric assay |
| Benzonitrile                 | 100-47-0  | C <sub>7</sub> H <sub>5</sub> N              | 103.12     | 0.0014 | А        | 0.999          | g.c.              |
| Benzylcyanide                | 140-29-4  | C <sub>8</sub> H <sub>7</sub> N              | 117.15     | 0.0005 | А        | 0.999          | g.c.              |
| Indole-3-acetic acid         | 87-51-4   | $C_{10}H_9NO_2$                              | 175.18     |        | А        |                | -                 |
| 3-Indoleacetonitrile         | 771-51-7  | $C_{10}H_8N_2$                               | 156.18     | 0.0013 | А        | 0.996          | g.c.              |
| α-Methylbenzeneacetic acid   | 492-37-5  | $C_9H_{10}O_2$                               | 150.18     |        | А        | 0.997          | g.c.              |
| α-Methylbenzylcyanide        | 1823-91-2 | C <sub>9</sub> H <sub>9</sub> N              | 131.18     | 0.0010 | А        | 0.989          | g.c.              |
| Nitrilase <sup>b</sup>       |           |  |            |        | В        |                | c                 |
| 4-Phenylbutyric acid         | 1821-12-1 | $C_{10}H_{12}O_{2}$                          | 164.20     |        | А        | 0.999          | g.c.              |
| 4-Phenylbutyronitrile        | 2046-18-6 | $C_{10}H_{11}N$                              | 145.20     | 0.0011 | А        | 0.988          | g.c.              |
| 3-Phenylpropanoic acid       | 501-52-0  | $C_9H_{10}O_2$                               | 150.18     |        | А        | 0.999          | g.c.              |
| 3-Phenylpropionitrile        | 645-59-0  | C <sub>9</sub> H <sub>9</sub> N              | 131.18     | 0.0022 | А        | 0.996          | g.c.              |
| Phosphoric acid              | 7664-38-2 | H <sub>3</sub> PO <sub>4</sub>               | 97.995     |        | М        |                | C                 |
| Potassium phosphate, dibasic | 7758-11-4 | K <sub>2</sub> HPO <sub>4</sub>              | 174.16     |        | S        |                |                   |

Synonyms. 3-Phenylpropanoic acid, hydrocinnamic acid; 3-phenylpropionitrile, hydrocinnamonitrile; benzeneacetic acid, phenylacetic acid;  $\alpha$ -methylbenzeneacetic acid, (*R*)-2-phenylpropanoic acid; and nitrilase, nitrile aminohydrolase and E.C. No. 3.5.5.1.

<sup>*a*</sup> The estimated mole fraction purities are exclusive of the amounts of water in the samples.

<sup>b</sup> Lyophilized powders. Four different forms of nitrilases, designated by the vendor as NIT102, NIT106, NIT1001, and NIT1005, were used in this study. NIT102 was used for reaction (4), NIT106 for reactions (2), (5), and (6), NIT1001 for reaction (3), and NIT1005 for reaction (1).

of the helium carrier gas was 283 kPa. The initial column temperature of 333 K was held for 3 min and then raised to 510 K at a rate of 0.333 K  $\cdot$  s<sup>-1</sup> and held at 510 K for 15 min. Each of the nitriles was dissolved in methanol and then injected into the g.c. Our chromatographic column was not suitable for 3-indoleacetonitrile (indefinite retention of this substance in the column). Nevertheless, the vendor reported a mole fraction purity x = 0.996 for this substance based on their own g.c. method. Additional evidence for the purity of this substance comes from the fact that only a single peak was seen on its h.p.l.c. chromatogram (see Section 2.2). The mass fraction of water in each of the nitrilase samples was determined by means of Karl Fischer titration [5].

#### 2.2. Chromatography

A Hewlett–Packard Model 1100 h.p.l.c equipped with a UV detector was used for the separation of the nitriles and their respective reaction products. AZorbax ODS column (4.6 mm i.d., 250 mm long) and a Bio-Sil (Bio-Rad) C18 guard column were used for the separations. The mobile phases were: (A) methanol and (B) water. The volume fractions  $\phi$  of these mobile phases were:  $\phi(A) = 0.60$ ,  $\phi(B) = 0.40$  at time t = 0; and  $\phi(A) = 0.95$ ,  $\phi(B) = 0.05$  at t = 15.0 min. The wavelength  $\lambda$  of the detector was set at 215 nm for all reactions excepting reaction (6), where  $\lambda$  was set at 225 nm. In all cases, the flow rate was 0.0133 cm<sup>3</sup> · s<sup>-1</sup>. Typical retention times were: benzonitrile, 6.1 min; benzoic acid, 3.5 min; benzylcyanide, 5.8 min; benzeneacetic acid, 3.5 min; 3-phenylpropionitrile, 6.5 min; 3-phenylpropanoic acid, 3.6 min; 4-phenylbutyonitrile, 8.4 min; 4-phenylbutyric acid, 3.7 min;  $\alpha$ -methylbenzyl cyanide, 7.1 min;  $\alpha$ -methylbenzeneacetic acid, 3.6 min; 3-indoleacetonitrile, 5.8 min; indole-3-acetic acid, 3.5 min. Thus, for the six reactions studied herein, the substances of interest were well separated on the h.p.l.c. The response factor solutions for the substances of interest were determined by first preparing (gravimetrically) solutions that contained each of these substances in the same buffer solution used for either the calorimetric or extent of reaction measurements. Methanol, at the volume fraction 0.25, was also added to the buffer to insure complete dissolution of the nitriles. These solutions were then injected at least three times into the h.p.l.c. The response factor was then calculated as the ratio of the concentration of the substance {expressed as mol · (kg solution)<sup>-1</sup>} divided by the area on the chromatogram that corresponds to that substance. Limits of detection for each substance were also determined by injecting into the h.p.l.c. solutions that contained successively smaller concentrations of that substance.

#### 2.3. Extent of reaction measurements

Attempts were made to measure the apparent equilibrium constants [6] K' of reactions (1) to (6). These studies used the h.p.l.c method described in Section 2.2. For reactions (1) to (6), the solutions used for the forward direction of reaction consisted of {nitrile(m = 0.0048 to  $0.0094 \text{ mol} \cdot \text{kg}^{-1}$ } in phosphate buffer { $m(\text{K}_2\text{HPO}_4) = 0.092 \text{ mol} \cdot \text{kg}^{-1} + m(\text{H}_3\text{PO}_4) = 0.015 \text{ mol} \cdot \text{kg}^{-1} +$ 

NH<sub>4</sub>Cl( $m = 0.10 \text{ mol} \cdot \text{kg}^{-1}$ ) at pH = 7.5}. The reaction mixture used for the reverse direction of reaction consisted of {acid produced from corresponding nitrile (m = 0.0047 to 0.011 mol  $\cdot \text{kg}^{-1}$ ) in the aforementioned phosphate buffer}. The mass fraction of nitrilase in the respective reaction mixtures was  $\approx 0.0002$ . The forward and reverse reactions were allowed to proceed with gentle lateral shaking ( $\approx 30$  shakes  $\cdot \text{min}^{-1}$ ) at T = 298.15 K for 3 days.

## 2.4. Calorimetry

Descriptions of the microcalorimeters used in this study and their performance characteristics, the calibration and data-acquisition systems, and the computer programs used to treat the results have been given by Steckler *et al.* [7,8]. These calorimeters were calibrated electrically by using a high stability d.c. power supply, calibrated digital voltmeter, standard resistor, and time-interval counter. The electric potential differences U of the thermopiles in the microcalorimeters are measured with Agilent model 34420A Nanovolt Meters. The values of U are then recorded on a microcomputer and the areas of the thermograms are calculated by numerical integration.

The calorimetric sample vessels were fabricated from high-density polyethylene. Each vessel had two compartments that held, respectively,  $\approx 0.55$  and  $\approx 0.40$ cm<sup>3</sup> of solution. The substrate solutions were placed in the  $0.55 \text{ cm}^3$  compartment and the enzyme solutions were placed in the 0.40 cm<sup>3</sup> compartment. The substrate solutions consisted of the nitrile dissolved in a phosphate buffer. Prior to preparing these substrate solutions, we measured approximate values of the saturation molalities m(sat) of the nitriles in the phosphate buffer. For the calorimetric experiments, we wanted to be certain that all of the nitriles had dissolved in the buffer. Therefore, we limited the molalities of the nitriles in the substrate solutions to be no more 0.75m(sat) and also allowed  $\approx 24$  h for dissolution of these substances in the buffer. The enzyme solution consisted of nitrilase in the same phosphate buffer used for the substrate.

The vessels and their contents were allowed to thermally equilibrate in the microcalorimeters for  $\approx 60$  min before the enzyme and substrate solutions were mixed. After mixing, 15 to 30 min was allowed for reaction. Following reaction, the vessels were removed from the microcalorimeters and the h.p.l.c. was used for the analysis of the final solutions. In all cases, it was found that there was no detectable amount of nitrile in any of the final reaction mixtures. Based on the limits of detection of the nitriles, the mole fraction of unreacted nitrile, in all cases, was <0.001.

"Blank" enthalpy changes  $\Delta_{mix}H$  were determined in control experiments. The values for  $\Delta_{mix}H$  ranged from (-0.29 to 0.74) mJ for the mixing of the substrate solutions with the buffer, with an average value of 0.13 mJ;  $\Delta_{mix}H$  ranged from (-0.18 to 0.12) mJ for the mixing of the enzyme solutions with the buffer, with an average value of 0.03 mJ. We judge the total corrections applied for the blank enthalpy changes to be uncertain by  $\approx \pm 0.3$  mJ. Since the measured reaction enthalpy changes were in the range (-69 to -285) mJ, the uncertainties in the blank enthalpy changes lead to uncertainties of  $0.001\Delta_r H_m(cal)$  to  $0.004\Delta_r H_m(cal)$  in the final results. The quantity  $\Delta_r H_m(cal)$  is the calorimetrically determined molar enthalpy of reaction pertinent to the actual experimental conditions.

#### 2.5. Measurement of pH

Measurement of pH was done with an ThermoOrion Model 420 pH meter and a Radiometer combination glass micro-electrode at the temperature at which experiments were performed. The pH meter was calibrated with Radiometer standard buffers that bracketed the pHs of the solutions used in this study. The pHs of the reaction mixtures were calculated by using interpolation with the measured electric potential differences and the pHs of the standard buffers.

## 3. Results and discussion

#### 3.1. Thermodynamic formalism

For overall biochemical reactions, the measured quantities are apparent equilibrium constants K' and calorimetrically determined molar enthalpies of reaction  $\Delta_r H_m$ (cal) [6]. From these quantities, one can use an equilibrium model that considers the contributions from the various species and reactions in solution to calculate values of the equilibrium constant K and the standard molar enthalpy of reaction  $\Delta_r H_m^\circ$  for a selected reference reaction. Since water is a reactant in reactions (1) to (6), it is particularly important to define the equilibrium constants and standard states. In this regard, the apparent equilibrium constants for reactions (1) to (6), respectively, are

$$K' = m\{\text{benzoic acid}(aq)\} \cdot m\{\text{ammonia}(aq)\}/$$
$$m\{\text{benzonitrile}(aq)\}, \tag{7}$$

$$K' = m\{\text{benzeneacetic acid(aq)}\} \cdot m\{\text{ammonia(aq)}\}/$$
$$m\{\text{benzylcyanide(aq)}\}, \qquad (8)$$

$$K' = m\{3\text{-phenylpropanoic acid(aq)}\} \cdot \\ m\{\text{ammonia(aq)}\}/m\{3\text{-phenylpropionitrile(aq)}\},$$
(9)

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(15)

- $K' = m\{4-\text{phenylbutyric acid}(aq)\} \cdot m\{\text{ammonia}(aq)\}/$ m{4-phenylbutyonitrile(aq)}, (10)
- $K' = m\{\alpha \text{-methylbenzeneacetic acid}(aq)\} \cdot m\{\text{ammonia}(aq)\}/m\{\alpha \text{-methylbenzylcyanide}(aq)\},$ (11)

$$K' = m\{\text{indole-3-acetic acid}(aq)\} \cdot m\{\text{ammonia}(aq)\}/$$
  
m{3-indoleacetonitrile(aq)}. (12)

The molalities *m* in the above equations are the total molalities of the various ionic forms of the respective species. Thus, while the term benzoic acid is used in reaction (1) and in equation (7), it is understood that this term refers to an equilibrium mixture of two species, namely benzoate<sup>-</sup>(aq) and benzoic acid<sup>0</sup>(aq). Since the pK for benzoic acid<sup>0</sup>(aq) is 4.31, the neutral form will be predominant for pH < 4.31 and the benzoate ion will be predominant for pH > 4.31. The respective reference reactions corresponding to the overall biochemical reactions (1) to (6) are

$$benzonitrile^{0}(aq) + 2H_{2}O(l) =$$
  

$$benzoate^{-}(aq) + NH_{4}^{+}(aq), \qquad (13)$$

 $benzylcyanide^{0}(aq) + 2H_2O(l) =$ 

benzeneacetate<sup>-</sup>(aq) + 
$$NH_4^+(aq)$$
, (14)

3-phenylpropionitrile<sup>0</sup>(aq) + 
$$2H_2O(l) =$$
  
3-phenylpropanoate<sup>-</sup>(aq) +  $NH_4^+(aq)$ ,

4-phenylbutyonitrile<sup>0</sup>(aq) + 2H<sub>2</sub>O(l) =

 $4-phenylbutyrate^{-}(aq) + NH_{4}^{+}(aq), \qquad (16)$ 

- $\begin{aligned} &\alpha\text{-methylbenzylcyanide}^{0}(aq) + 2H_{2}O(l) = \\ &\alpha\text{-methylbenzeneacetate}^{-}(aq) + NH_{4}^{+}(aq), \end{aligned} \tag{17}$
- $\label{eq:2.1} \begin{array}{l} \mbox{3-indoleacetonitrile}^0(aq)+2H_2O(l)=\\ \mbox{indole-3-acetate}^-(aq)+NH_4^+(aq). \end{array} \tag{18}$

The respective equilibrium constants for reactions (13) to (18) are:

$$K = m\{\text{benzoate}^{-}(\text{aq})\} \cdot m\{\text{NH}_{4}^{+}(\text{aq})\} / [m\{\text{benzonitrile}(\text{aq})\} \cdot a_{w}^{2}], \qquad (19)$$

$$K = m\{\text{benzeneacetate}^{-}(\text{aq})\} \cdot m\{\text{NH}_{4}^{+}(\text{aq})\} / [m\{\text{benzylcyanide}(\text{aq})\} \cdot a_{w}^{2}], \qquad (20)$$

$$K = m\{3\text{-phenylpropanoate}^{-}(aq)\} \cdot m\{\mathrm{NH}_{4}^{+}(aq)\}/$$
$$[m\{3\text{-phenylpropionitrile}(aq)\} \cdot a_{\mathrm{w}}^{2}], \qquad (21)$$

 $K = m\{4\text{-phenylbutyrate}^{-}(aq)\} \cdot m\{NH_{4}^{+}(aq)\} / [m\{4\text{-phenylbutyonitrile}(aq)\} \cdot a_{w}^{2}], \qquad (22)$ 

$$K = m\{\alpha \text{-methylbenzeneacetate}^{-}(\mathrm{aq})\} \cdot m\{\mathrm{NH}_{4}^{+}(\mathrm{aq})\} / [m\{\alpha \text{-methylbenzylcyanide}(\mathrm{aq})\} \cdot a_{\mathrm{w}}^{2}], \qquad (23)$$

$$K = m\{\text{indole-3-acetate}^{-}(\text{aq})\} \cdot m\{\text{NH}_{4}^{+}(\text{aq})\} / [m\{3\text{-indoleacetonitrile}(\text{aq})\} \cdot a_{w}^{2}].$$
(24)

In this study, the standard state for the solute is the hypothetical ideal solution of unit molality  $(m^{\circ} = 1 \text{ mol} \cdot \text{kg}^{-1})$  and the standard state for the solvent is the pure solvent; the standard pressure  $p^{\circ} = 0.1$  MPa. The quantity  $a_{\text{w}}$  is the activity of water.

## 3.2. Results of experiments

The result of the extent of reaction measurements (Section 2.3) was that there were no measurable amounts of any of the nitriles in the reaction mixtures. Based upon the lower limits of the concentrations of these substances detectable with our h.p.l.c., it is possible to set lower limits for the values of the apparent equilibrium constants. Thus, for reactions (1) to (8) the values of K' (T = 298.15 K, pH 7.5) are: K'(1) > 650; K'(2) > 600; K'(3) > 540; K'(4) > 360; K'(5) > 310; and K'(6) > 540. Later in this paper (Section 3.4), we shall use a thermochemical cycle calculation to confirm our finding that at least one of these reactions proceeds to completion.

The results of the calorimetric measurements are given in table 2. A summary of the results follows:  $\Delta_{\rm r} H_{\rm m}({\rm cal}) = -(76.13 \pm 0.70) \, \text{kJ} \cdot {\rm mol}^{-1}$  for reaction (1);  $\Delta_{\rm r} H_{\rm m}({\rm cal}) = -(88.06 \pm 0.38) \text{ kJ} \cdot {\rm mol}^{-1}$  for reaction (2);  $\Delta_{\rm r} H_{\rm m}({\rm cal}) = -(90.73 \pm 0.43) \text{ kJ} \cdot {\rm mol}^{-1}$  for reaction (3);  $\Delta_{\rm r} H_{\rm m}({\rm cal}) = -(81.03 \pm 0.78) \text{ kJ} \cdot {\rm mol}^{-1}$  for reaction (4);  $\Delta_{\rm r} H_{\rm m}({\rm cal}) = -(82.59 \pm 0.52) \text{ kJ} \cdot {\rm mol}^{-1}$  for reaction (5); and  $\Delta_r H_m(\text{cal}) = -(97.61 \pm 0.49) \text{ kJ} \cdot \text{mol}^{-1}$  for reaction (6). The uncertainties given here are equal to two estimated standard deviations of the mean. We judge that reasonable estimates of possible systematic error in the of  $\Delta_r H_m(cal)$  are:  $0.002 \cdot \Delta_r H_m(cal)$ values to  $\pm 0.008 \cdot \Delta_r H_m$ (cal) due to impurities (including water) in the nitrile samples;  $\pm 0.0005 \cdot \Delta_r H_m$ (cal) due to possible errors in the extent of reaction; and  $\pm 0.003 \cdot \Delta_r H_m$ (cal) due to possible errors in the calorimetric measurements including the "blank" enthalpies. These estimates of possible systematic error are combined in quadrature together with the statistical uncertainty in the measured value of  $\Delta_r H_m$ (cal) expressed as one estimated standard deviation of the mean, to obtain combined standard uncertainties [9] for each result. These combined standard uncertainties are then multiplied by two to arrive at the final results from our experiments:  $\Delta_{\rm r} H_{\rm m}({\rm cal}) = -(76.1 \pm 0.9) \text{ kJ} \cdot {\rm mol}^{-1}$  for reaction (1);

| TABLE 2  |          |
|--|----------|
| Results of the calorimetric measurements for reactions (1) to (6) in phosphate buffer at $T =$ | 298.15 K |

| Experiment no. | pН            | $m(K_2HPO_4)$                                       | m(H <sub>3</sub> PO <sub>4</sub> )         | $10^3 \cdot m(\text{nitrile})$ | Im                  | $\Delta_{\rm r} H_{\rm m}({\rm cal})$ |
|----------------|---------------|---|--|--------------------------------|---------------------|---------------------------------------|
|                |               | $mol \cdot kg^{-1}$                                 | $mol \cdot kg^{-1}$                        | $mol \cdot kg^{-1}$            | $mol \cdot kg^{-1}$ | $kJ \cdot mol^{-1}$                   |
|                | F             | Reaction (1): benzonitrile                          | $(aq) + 2H_2O(1) = benz$                   | oic acid(aq) + ammonia(a       | a)                  |                                       |
| 1              | 7.51          | 0.1014  | 0.0117                                     | 3.957                          | 0.29                | -77.63                                |
| 2              | 7.51          | 0.1014  | 0.0117                                     | 4.019                          | 0.29                | -76.48                                |
| 3              | 7.51          | 0.1014  | 0.0117                                     | 3.974                          | 0.29                | -75.07                                |
| 4              | 7.51          | 0.1014  | 0.0117                                     | 3.898                          | 0.29                | -75.99                                |
| 5              | 7.51          | 0.1014  | 0.0117                                     | 4.003                          | 0.29                | -75.85                                |
| 6              | 7.51          | 0.1014  | 0.0117                                     | 4.305                          | 0.29                | -75.78                                |
|                |               | $\langle \Delta_{\rm r} H_{\rm m}({ m cs}) \rangle$ | $ a1\rangle = -(76.13 \pm 0.70)$           | $kJ \cdot mol^{-1}$            |                     |                                       |
|                | Reac          | tion (2): benzylcyanide(a                           | q) + 2H <sub>2</sub> O(1) = benzer         | neacetic acid(aq) + ammor      | nia(aq)             |                                       |
| 1              | 7.43          | 0.1014  | 0.00117                                    | 2.118                          | 0.29                | -88.59                                |
| 2              | 7.43          | 0.1014  | 0.00117                                    | 2.014                          | 0.29                | -88.09                                |
| 3              | 7.43          | 0.1014  | 0.00117                                    | 2.012                          | 0.29                | -88.57                                |
| 4              | 7.43          | 0.1014  | 0.00117                                    | 2.002                          | 0.29                | -87.40                                |
| 5              | 7.43          | 0.1014  | 0.00117                                    | 1.985                          | 0.29                | -87.74                                |
| 6              | 7.43          | 0.1014  | 0.00117                                    | 2.027                          | 0.29                | -87.91                                |
|                |               | $\langle \Delta_{\rm r} H_{\rm m}({ m c}) \rangle$  | $ al\rangle = -(88.06 \pm 0.38)$           | $kJ \cdot mol^{-1}$            |                     |                                       |
|                | Reaction (3   | 3): 3-phenylpropionitrile(a                         | $aq) + 2H_2O(1) = 3-phe$                   | enylpropanoic acid(aq) + a     | mmonia(aq)          |                                       |
| 1              | 7.45          | 0.1014  | 0.00117                                    | 2.571                          | 0.29                | -91.24                                |
| 2              | 7.45          | 0.1014  | 0.00117                                    | 2.626                          | 0.29                | -90.05                                |
| 3              | 7.45          | 0.1014  | 0.00117                                    | 2.558                          | 0.29                | -90.35                                |
| 4              | 7.45          | 0.1014  | 0.00117                                    | 2.530                          | 0.29                | -91.38                                |
| 5              | 7.45          | 0.1014  | 0.00117                                    | 2.571                          | 0.29                | -90.91                                |
| 6              | 7.45          | 0.1014  | 0.00117                                    | 2.558                          | 0.29                | -90.49                                |
|                |               | $\langle \Delta_{\rm r} H_{\rm m}({ m cc}) \rangle$ | $ al\rangle = -(90.73 \pm 0.43)$           | $kJ \cdot mol^{-1}$            |                     |                                       |
|                | Reaction      | (4): 4-phenylbutyonitrile                           | $(aq) + 2H_2O(l) = 4-ph$                   | enylbutyric acid(aq) + am      | monia(aq)           |                                       |
| 1              | 7.50          | 0.1014  | 0.00117                                    | 1.379                          | 0.29                | -81.08                                |
| 2              | 7.50          | 0.1014  | 0.00117                                    | 1.500                          | 0.29                | -79.53                                |
| 3              | 7.50          | 0.1014  | 0.00117                                    | 1.404                          | 0.29                | -82.21                                |
| 4              | 7.50          | 0.1014  | 0.00117                                    | 1.396                          | 0.29                | -80.48                                |
| 5              | 7.50          | 0.1014  | 0.00117                                    | 1.436                          | 0.29                | -81.13                                |
| 6              | 7.50          | 0.1014  | 0.00117                                    | 1.489                          | 0.29                | -81.78                                |
|                |               | $\langle \Delta_{\rm r} H_{ m m}({ m c})$           | $ al\rangle = -(81.03 \pm 0.78)$           | $kJ \cdot mol^{-1}$            |                     |                                       |
|                | Reaction (5): | α-methylbenzylcyanide(a                             | q) + 2H <sub>2</sub> O(1) = $\alpha$ -meth | hylbenzeneacetic acid(aq)      | + ammonia(aq)       |                                       |
| 1              | 7.53          | 0.1014  | 0.00117                                    | 3.458                          | 0.29                | -82.91                                |
| 2              | 7.53          | 0.1014  | 0.00117                                    | 3.466                          | 0.29                | -82.95                                |
| 3              | 7.53          | 0.1014  | 0.00117                                    | 3.461                          | 0.29                | -82.70                                |
| 4              | 7.53          | 0.1014  | 0.00117                                    | 3.468                          | 0.29                | -81.40                                |
| 5              | 7.53          | 0.1014  | 0.00117                                    | 3.414                          | 0.29                | -82.37                                |
| 6              | 7.53          | 0.1014  | 0.00117                                    | 3.509                          | 0.29                | -83.19                                |
|                |               | $\langle \Delta_{\rm r} H_{\rm m}({ m cs}) \rangle$ | $ al\rangle = -(82.59 \pm 0.52)$           | $kJ \cdot mol^{-1}$            |                     |                                       |
|                | Reaction      | n (6): 3-indoleacetonitrile                         | $(aq) + 2H_2O(1) = indc$                   | ble-3-acetic acid(aq) + amr    | nonia(aq)           |                                       |
| 1              | 7.55          | 0.1014  | 0.00117                                    | 0.8036                         | 0.29                | -97.43                                |
| 2              | 7.55          | 0.1014  | 0.00117                                    | 0.8228                         | 0.29                | -97.79                                |
| 3              | 7.55          | 0.1014  | 0.00117                                    | 0.8502                         | 0.29                | -97.71                                |
| 4              | 7.55          | 0.1014  | 0.00117                                    | 0.8064                         | 0.29                | -98.62                                |
| 5              | 7.55          | 0.1014  | 0.00117                                    | 0.8374                         | 0.29                | -97.27                                |
| 6              | 7.55          | 0.1014  | 0.00117                                    | 0.8429                         | 0.29                | -96.85                                |
|                |               | $\langle \Delta_{\rm r} H_{\rm m}({ m cc}) \rangle$ | $ al\rangle = -(97.61 \pm 0.49)$           | $kJ \cdot mol^{-1}$            |                     |                                       |

The molalities *m* in columns 3 to 5 are those obtained after mixing of the enzyme and substrate solutions and prior to any reaction. All molalities are equal to the sums of the molalities of the indicated substances in their various ionic forms. The approximate mass fractions *w* of the nitrilase were  $6 \cdot 10^{-4}$ ,  $4 \cdot 10^{-4}$ ,  $1 \cdot 10^{-3}$ ,  $2 \cdot 10^{-3}$ ,  $1 \cdot 10^{-3}$ , and  $4 \cdot 10^{-4}$  in reactions (1) to (8), respectively. The values of the ionic strength  $I_m$  are calculated. The uncertainties in the average values of  $\Delta_r H_m$ (cal) are equal to two estimated standard deviations of the mean.

$$\begin{split} \Delta_{\rm r} H_{\rm m}({\rm cal}) &= -(88.1 \pm 0.8) \ {\rm kJ} \cdot {\rm mol}^{-1} \ {\rm for} \ {\rm reaction} \ (2); \\ \Delta_{\rm r} H_{\rm m}({\rm cal}) &= -(90.7 \pm 0.8) \ {\rm kJ} \cdot {\rm mol}^{-1} \ {\rm for} \ {\rm reaction} \ (3); \\ \Delta_{\rm r} H_{\rm m}({\rm cal}) &= -(81.0 \pm 1.6) \ {\rm kJ} \cdot {\rm mol}^{-1} \ {\rm for} \ {\rm reaction} \ (4); \end{split}$$

 $\Delta_{\rm r} H_{\rm m}({\rm cal}) = -(82.6 \pm 1.4) \text{ kJ} \cdot {\rm mol}^{-1}$  for reaction (5); and  $\Delta_{\rm r} H_{\rm m}({\rm cal}) = -(97.6 \pm 1.1) \text{ kJ} \cdot {\rm mol}^{-1}$  for reaction (6). TABLE 3

The pKs and standard molar enthalpy changes  $\Delta_r H_m^\circ$  at T = 298.15 K and I = 0 for the aqueous ionization reactions of substances pertinent to this study and to the analysis of results from the literature. See Section 3 for the basis of these values

| Reaction  | pK        | $\Delta_{ m r} {H}^{\circ}_{ m m}$ |
|---|-----------|------------------------------------|
|   |           | $kJ \cdot mol^{-1}$                |
| $NH_4^+ = NH_3 + H^+$   | 9.245     | 51.95                              |
| benzeneacetic $acid^0 = benzeneacetate^{-1} + H^+$  | 4.310     | 2.0                                |
| benzoic $acid^0 = benzoate^{-1} + H^+$  | 4.202     | -0.46                              |
| indole-3-acetic acid = indole-3-acetate $^{-1}$ + H <sup>+</sup>  | $3.9^{a}$ | $\approx 1^a$                      |
| $\alpha$ -methylbenzeneacetic acid <sup>0</sup> = $\alpha$ -methylbenzeneacetate <sup>-1</sup> + H <sup>+</sup> | $4.3^{a}$ | $\approx 3^a$                      |
| 4-phenylbutyric acid = 4-phenylbutyrate <sup><math>-1</math></sup> + H <sup>+</sup>                             | 4.757     | $\approx 6^a$                      |
| 3-phenylpropanoic acid = 3-phenylpropanoate <sup><math>-</math></sup> + H <sup>+</sup>                          | 4.664     | 9.4                                |
| $H_2PO_4^- = HPO_4^{2-} + H^+$  | 7.198     | 3.6                                |

<sup>a</sup> Estimated value.

#### 3.3. Equilibrium modeling calculations

The pKs and standard molar enthalpy changes  $\Delta_r H_m^\circ$ for the  $H^+(aq)$  dissociation reactions of the reactants and of the buffer are needed to relate the experimental results for reactions (1) to (8) to thermodynamic quantities for their respective reference reactions (13) to (18). These pK and  $\Delta_r H_m^{\circ}$  values are given in table 3. The basis of the selected values is now discussed. The values for  $H_2PO_4^-(aq)$  and for  $NH_4^+(aq)$  are taken from a recent review [10]. The pKs and  $\Delta_r H_m^{\circ}$  values for benzeneacetic acid(aq), benzoic acid(aq), and 3-phenylpropanoic acid(aq) are from the database of Martell et al. [11]. The pK value for 4-phenylbutyric acid(aq) is also from this database [11]. In the absence of measured pK values for indole-3-acetic acid(aq) and for  $\alpha$ -methylbenzene acetic acid(aq), we have estimated the pK values of these substances to be, respectively,  $\approx 3.9$  and  $\approx 4.3$  based on their structural similarities to 2-indole carboxylic acid and benzeneacetic acid(aq). The pK value for 2-indole carboxylic acid was taken from Martell et al. [11]. We have used the known pK and  $\Delta_r H^\circ_m$  values of benzeneacetic acid(aq), benzoic acid(aq), and 3-phenylpropanoic acid(aq) to calculate an average value of the standard molar entropy change  $\Delta_r S_m^\circ = -72 \text{ J} \cdot \text{K}^{-1} \cdot$ mol<sup>-1</sup> for the ionization reactions of these three substances. This average value was then used with the pK values given in table 3 to estimate the values of  $\Delta_r H_m^\circ$ given in that table for the ionization reactions of indole-3-acetic acid(aq),  $\alpha$ -methylbenzene acetic acid(aq), and 4-phenylbutyric acid(aq). Examination of the structures of all six nitriles used in this study indicates that they have no ionizable hydrogens unless placed in very alkaline solutions.

The equilibrium model used for the calculation of the equilibrium constants K and standard molar enthalpies  $\Delta_r H_m^{\circ}$  for the reference reactions from the measured values of K' and  $\Delta_r H_m$ (cal) has been described [12,13]. The calculations include corrections for non-ideality and are made self-consistent with regard to the ionic strength. The non-ideality corrections

are based on the extended Debye-Hückel equation in which the "ion-size" parameter has been set at 1.6  $kg^{1/2} \cdot mol^{-1/2}$ . Use of the equilibrium model together with the values of  $\Delta_r H_m(cal)$  determined in this study and the pK and  $\Delta_r H^\circ_m$  values iven in table 3 leads to following values:  $\Delta_{\rm r} H_{\rm m}^{\circ}(13) = -(76.7 \pm 0.9)$ the  $kJ \cdot mol^{-1}; \ \Delta_r H_m^{\circ}(14) = -(89.0 \pm 0.8) \ kJ \cdot mol^{-1}; \Delta_r H_m^{\circ}$  $\begin{array}{l} (15) = -(91.3 \pm 0.8) \text{ kJ} \cdot \text{mol}^{-1}; \ \Delta_{r} H^{\circ}_{m}(16) = -(82.0 \pm 1.6) \text{ kJ} \cdot \text{mol}^{-1}; \ \Delta_{r} H^{\circ}_{m}(17) = -(83.3 \pm 1.4) \text{ kJ} \cdot \text{mol}^{-1}; \end{array}$ and  $\Delta_{\rm r} H^{\circ}_{\rm m}(18) = -(99.0 \pm 1.1) \text{ kJ} \cdot \text{mol}^{-1}$ . These values all pertain to T = 298.15 K and I = 0. The uncertainties given here are based on those assigned to the values of  $\Delta_r H_m$ (cal) (see Section 3.2). However, there is also a component of uncertainty due to possible errors in the parameters used in the equilibrium model. This latter component of uncertainty was estimated by perturbing pertinent quantities in the equilibrium model with an assumed possible error. Thus, the pK values for the acids were perturbed by  $\pm 0.2$  and the values of  $\Delta_{\rm r} H_{\rm m}^{\circ}$  for the acid dissociation reactions were also perturbed by  $\pm 3.0 \text{ kJ} \cdot \text{mol}^{-1}$ . Finally, the "ion-size" parameter was perturbed by  $\pm 0.3 \text{ kg}^{1/2} \cdot \text{mol}^{-1/2}$ . The combined effects of these perturbations on the calculated values of  $\Delta_r H_m^{\circ}$  were all <0.17 kJ  $\cdot$  mol<sup>-1</sup>. Since the uncertainties in the values of  $\Delta_r H_m(cal)$  were substantially larger than 0.17 kJ · mol<sup>-1</sup>, the uncertainties in the calculated values given immediately above have not been changed. These results are given in table 4.

#### 3.4. Thermochemical cycle calculations

There do no appear to be any values of *K* or  $\Delta_r H_m^{\circ}$  in the literature for the hydrolysis of the nitriles studied herein or for any other nitriles. Nevertheless, we are able to perform a thermochemical cycle calculation that leads to values of  $\Delta_r G_m^{\circ}$ , *K*,  $\Delta_r H_m^{\circ}$ , and  $\Delta_r S_m^{\circ}$  for reaction (13) at T = 298.15 K, I = 0, and p = 0.1 MPa. Several of the values of the thermochemical quantities used in this calculation are summarized in table 5. The calculation also used the CODATA [14] values for the standard molar enthalpies of formation  $\Delta_f H_m^{\circ}$  of CO<sub>2</sub>(g), H<sub>2</sub>O(l), and

| Values of the standard molar enthalpy changes $\Delta_r H_m^\circ$ for the several reference reactions considered herein  |                                  |  |  |
|---|----------------------------------|--|--|
| Reaction  | $\Delta_{ m r} H_{ m m}^{\circ}$ |  |  |
|   | $kJ \cdot mol^{-1}$              |  |  |
| (13) benzonitrile <sup>0</sup> (aq) + 2 H <sub>2</sub> O(l) = benzoate <sup>-</sup> (aq) + NH <sub>4</sub> <sup>+</sup> (aq)  | $-(76.7 \pm 0.9)$                |  |  |
| (14) benzylcyanide <sup>0</sup> (aq) + 2 H <sub>2</sub> O(l) = benzeneacetate <sup>-</sup> (aq) + $NH_4^+$ (aq)   | $-(89.0 \pm 0.8)$                |  |  |
| (15) 3-phenylpropionitrile <sup>0</sup> (aq) + 2 $H_2O(l) = 3$ -phenylpropanoate <sup>-</sup> (aq) + NH <sub>4</sub> <sup>+</sup> (aq)                              | $-(91.3 \pm 0.8)$                |  |  |
| (16) 4-phenylbutyonitrile <sup>0</sup> (aq) + 2 $H_2O(l) = 4$ -phenylbutyrate <sup>-</sup> (aq) + $NH_4^+(aq)$  | $-(82.0 \pm 1.6)$                |  |  |
| (17) $\alpha$ -methylbenzylcyanide <sup>0</sup> (aq) + 2 H <sub>2</sub> O(l) = $\alpha$ -methylbenzeneacetate <sup>-</sup> (aq) + NH <sub>4</sub> <sup>+</sup> (aq) | $-(83.3 \pm 1.4)$                |  |  |
| (18) 3-indoleacetonitrile <sup>0</sup> (aq) + 2 H <sub>2</sub> O(l) = indole-3-acetate <sup>-</sup> (aq) + NH <sub>4</sub> <sup>+</sup> (aq)                        | $-(99.0 \pm 1.1)$                |  |  |

The standard state is the hypothetical ideal solution at unit molality. The basis of the uncertainties is discussed in Section 3.3.

TABLE 5

TABLE 4

Values of the thermodynamic quantities at T = 298.15 K and pressure p = 0.1 MPa for reactions and for substances pertinent to the thermochemical pathway calculations done in this study (see Section 3.4)

| Entry no. | Substance or reaction   | $\Delta_{ m r} G_{ m m}^{\circ}$ | $\Delta_{ m r} {H}^{\circ}_{ m m}$ | $S^\circ_{ m m}$                                       |
|-----------|---|----------------------------------|------------------------------------|--|
|           |   | $kJ \cdot mol^{-1}$              | $kJ \cdot mol^{-1}$                | $\mathbf{J}\cdot\mathbf{K}^{-1}\cdot\mathrm{mol}^{-1}$ |
| 1         | $C_7H_5N(l) + 8.25 O_2(g) = 7 CO_2(g) + 2.5 H_2O(l) + 0.5 N_2(g)$ |                                  | $-3632.34^{a}$                     |  |
| 2         | $C_7H_6O_2(cr) + 7.5O_2(g) = 7CO_2(g) + 3 H_2O(l)$                |                                  | $-3226.86^{b}$                     |  |
| 3         | $C_7H_5N(l)$  |                                  |                                    | 209.1 <sup>c</sup>                                     |
| 4         | $C_7H_6O_2(cr)$   |                                  |                                    | 167.73 <sup>d</sup>                                    |
| 5         | $C_7H_5N(l) = C_7H_5N(aq)$  | 9.3 <sup>e</sup>                 | $18.2^{e}$                         |  |
| 6         | $C_7H_6O_2(cr) = C_7H_6O_2(aq)$                                   | 8.85 <sup>f</sup>                | $22.5^{f}$                         |  |
| 7         | $C_7H_6O_2(aq) = C_7H_5O_2(aq) + H^+(aq)$                         | 23.99 <sup>g</sup>               | $-0.46^{g}$                        |  |

The thermodynamic quantities are the standard molar enthalpy of reaction  $\Delta_r H_m^\circ$ , the standard molar Gibbs free energy of reaction  $\Delta_r G_m^\circ$ , and the standard molar entropy  $S_m^\circ$ . The empirical formulas of the various substances are: benzonitrile,  $C_7H_5N$ ; and benzoic acid,  $C_7H_6O_2$ . The standard state for aqueous solutes is the hypothetical ideal solution at unit molality.

<sup>*a*</sup> Based on the value of the standard molar enthalpy of combustion  $\Delta_c H_m$  of  $C_7 H_5 N(l)$  determined by Evans and Skinner [15].

<sup>b</sup> Based on the value of  $\Delta_f H_m^\circ$  for  $C_7 H_6 O_2(cr)$  given by Domalski and Hearing [16] which, in turn, is based on the value of the standard molar enthalpy of combustion of  $C_7 H_6 O_2(cr)$  determined by Churney and Armstrong [17].

<sup>c</sup> Based on the value of  $S_{\rm m}^{\circ}$  reported by Lebedev *et al.* [18].

<sup>*d*</sup> Based on the value of  $S_{m}^{\circ}$  reported by Arvidsson *et al.* [19].

<sup>e</sup> Calculated from the solubility measurements of Pryanikova and Markina [20].

<sup>f</sup> Calculated from the solubility measurements of Ward and Cooper [21].

<sup>g</sup> Based on the values of pK and  $\Delta_r H_m^{\circ}$  selected by Martell *et al.* [11].

 $NH_4^+(aq)$ , as well as the CODATA values for the standard molar entropies  $S_m^{\circ}$  of C(cr), H<sub>2</sub>(g), H<sub>2</sub>O(l),  $NH_4^+(aq)$ . This  $O_2(g),$ and thermochemical pathway leads to the following values for reaction (13):  $\Delta_{\rm r} G_{\rm m}^{\circ} = -65.5 \text{ kJ} \cdot \text{mol}^{-1}$ ,  $K = 3 \cdot 10^{11}$ ,  $\Delta_{\rm r} H_{\rm m}^{\circ} = -106.1 \text{ kJ} \cdot \text{mol}^{-1}$ , and  $\Delta_{\rm r} S_{\rm m}^{\circ} = -136 \text{ J} \cdot \text{K}^{-1} \cdot \text{mol}^{-1}$ . The experimental result for  $\Delta_{\rm r} H_{\rm m}^{\circ}$  was  $-(76.7 \pm 0.9)$  $kJ \cdot mol^{-1}$ . The difference of 29.4  $kJ \cdot mol^{-1}$  in the two values is larger than one would like to see. Nevertheless, having some confidence in our experimental results, we believe that this difference is attributable to some errors in the values used in the thermochemical cycle calculation. Likely candidates for these errors are the values of the standard molar enthalpies of solution  $\Delta_r H^\circ_{\mathfrak{m}}$  for benzoic acid(cr) and for benzonitrile(l) which were calculated from the temperature dependence of experimentally determined solubilities. It is also possible that the value of the standard molar enthalpy of combustion  $\Delta_{\rm c} H_{\rm m}^{\circ}$  of benzonitrile(l) is in error. In any case, the thermochemical cycle calculation confirms our experimental finding that the equilibrium constant for the hydrolysis

of benzonitrile(aq) is very large and that the reaction, for all practical purposes, leads to a complete hydrolysis of this nitrile to the acid. A similar conclusion is reasonable for the other hydrolysis reactions studied hererin.

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