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A thermodynamic investigation of some reactions involving prephenic acid

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Calorimetric enthalpies of reaction have been measured for the following enzyme-catalysed reactions at the temperature 298.15 K:

$$\text{prephenate(aq)} = \text{phenylpyruvate(aq)} + \text{carbon dioxide(aq)},$$

$$\text{prephenate(aq)} + \text{NAD}_{\text{ox}}\text{(aq)} + \text{H}_2\text{O(l)} = \text{4-hydroxyphenylpyruvate(aq)} + \text{NAD}_{\text{red}}\text{(aq)} + \text{carbon dioxide(aq)}.$$

Here, NAD_{ox} and NAD_{red} are, respectively, the oxidized and reduced forms of β-nicotinamide adenine dinucleotide. The enzymes that catalyse these respective reactions, prephenate dehydratase and prephenate dehydrogenase, were prepared by expression of the appropriate plasmids using the techniques of molecular biology. The calorimetric measurements together with the equilibrium modeling calculations lead to a standard molar enthalpy change $\Delta_r H^o_m = -(126 \pm 5)\ \text{kJ}\cdot\text{mol}^{-1}$ for the reference reaction:

$$\text{prephenate}^{2-}\text{(aq)} = \text{phenylpyruvate}^{-}\text{(aq)} + \text{HCO}_3^-\text{(aq)}.$$

Similarly, $\Delta_r H^o_m = -(74 \pm 3)\ \text{kJ}\cdot\text{mol}^{-1}$ for the reference reaction:

$$\text{prephenate}^{2-}\text{(aq)} + \text{NAD}_{\text{ox}}^-\text{(aq)} + \text{H}_2\text{O(l)} = \text{4-hydroxyphenylpyruvate}^{-}\text{(aq)} + \text{NAD}_{\text{red}}^{2-}\text{(aq)} + \text{HCO}_3^-\text{(aq)} + \text{H}^+\text{(aq)}.$$

Both results pertain to $T = 298.15$ K and ionic strength $I = 0$. Benson estimates for the entropies lead to approximate values of the equilibrium constants $K \approx 1\cdot10^{26}$ and $K \approx 1\cdot10^{12}$, respectively, for the above two reference reactions. © 1999 Academic Press

KEYWORDS: enthalpy; 4-hydroxyphenylpyruvic acid; β-nicotinamide adenine dinucleotide; phenylpyruvic acid; prephenate dehydratase; prephenate dehydrogenase; prephenic acid

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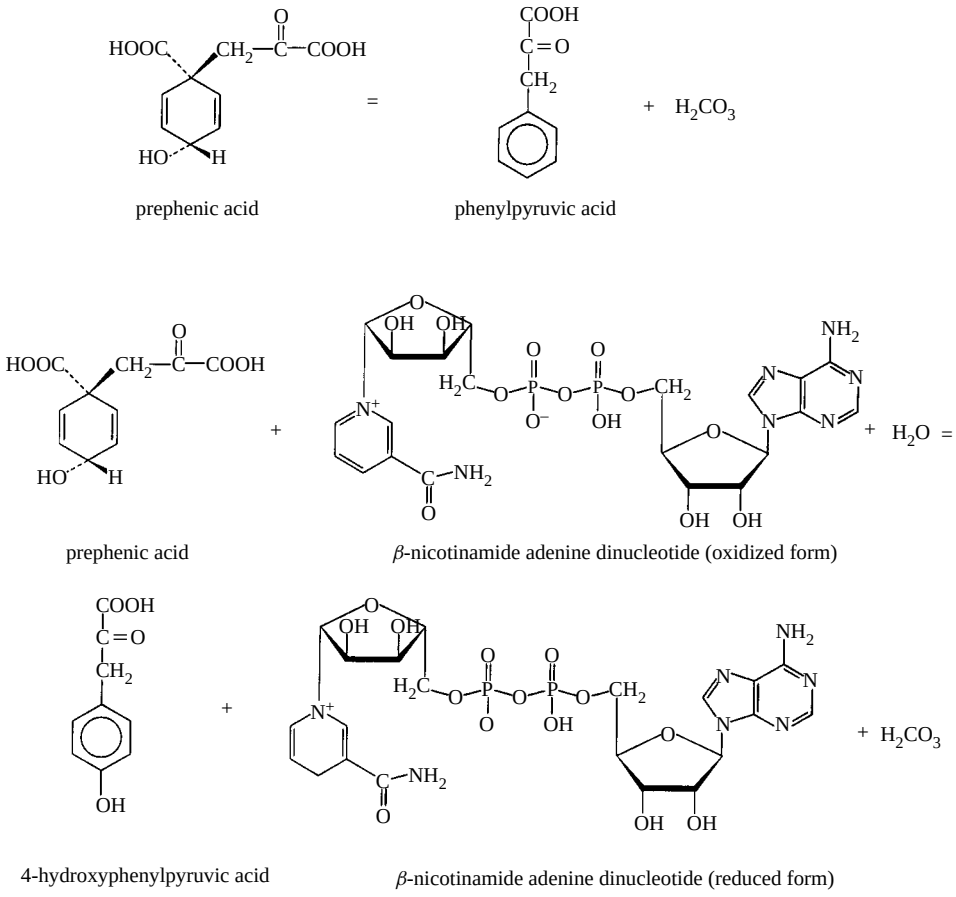

FIGURE 1. Structures of the substances in reactions (1) and (2). The neutral forms of the substances are shown.

1. Introduction

The enzymes prephenate dehydratase (EC 4.2.1.51) and prephenate dehydrogenase (EC 1.3.1.12) catalyse, respectively, the reactions:

$$\text{prephenate(aq)} = \text{phenylpyruvate(aq)} + \text{carbon dioxide(aq)}, \tag{1}$$

$$\text{prephenate(aq)} + \text{NAD}_{\text{ox}}\text{(aq)} + \text{H}_2\text{O(l)} = \text{4-hydroxyphenylpyruvate(aq)} + \text{NAD}_{\text{red}}\text{(aq)} + \text{carbon dioxide(aq)}. \tag{2}$$

Here, NAD_{ox} and NAD_{red} are, respectively, the oxidized and reduced forms of β-nicotinamide adenine dinucleotide. The structures of these substances are shown in figure 1.

These two reactions, which are the first steps in two branches of the chorismate metabolic pathway,[1] are followed *in vivo* by transamination reactions in which the phenylpyruvate and

4-hydroxyphenylpyruvate are converted, respectively, to L-phenylalanine and L-tyrosine. In this same metabolic pathway, prephenate is formed from chorismate by an enzyme-catalysed (chorismate mutase, EC 5.4.99.5) Claisen rearrangement. The transamination reactions and the conversion of chorismate to prephenate have been the subject of recent thermodynamic studies.[2, 3] Accordingly, the aim in the present study was to perform calorimetric and equilibrium measurements on reactions (1) and (2) and thus obtain a complete picture of the thermodynamics of a major portion of the chorismate metabolic pathway. This necessitated the preparation of prephenate dehydratase and prephenate dehydrogenase by starting with the expression of the appropriate plasmids and by using the techniques of molecular biology. In addition to its inherent biochemical interest, this pathway is also of importance because of its potential use for the large-scale production of aromatic amino acids and other valuable products.[4, 5]

2. Experimental

Relevant information on the substances used in this study is given in table 1.† In the absence of a sample of prephenic acid having sufficient purity, it was decided to prepare this substance *in situ* by using a well-characterized sample of chorismic acid[2] and the enzyme chorismate mutase (EC 5.4.99.5). This conversion of chorismate to prephenate was found to be essentially complete under the conditions used for the calorimetric experiments (see below). Specifically, the mole fraction of chorismate remaining was $<8\cdot10^{-4}$ following a reaction time of 1 h. Previous kinetic results[6, 7] showed that the half life for the decomposition of prephenate via reaction (1) is 130 h at the temperature 298.15 K and pH = 7.0. Thus, for the calorimetric experiments, where the prephenate was in solution for ≈1 h prior to initiation of the reaction(s) of interest, the mole fraction of prephenate converted to (phenylpyruvate + carbon dioxide + H_2O) was ≈0.0053.

Prephenate dehydrogenase was produced by expression of the plasmid pJX3 of Xia *et al.*[8] This plasmid is a recombinant of pUC19 with a 1600 base DNA insert from *Erwinia herbicola*. This insert contains a defective pheA gene and a tyrA gene that is missing the coding for 37 amino acids at its amine terminus (replaced by 18 amino acids from the polycloning site of the lacZ α-peptide of pUC19). The resulting DNA encodes for a monofunctional prephenate dehydrogenase with no chorismate mutase activity. The protein was expressed in *E. coli* KA12 cells that are deficient in pheA and tyrA resulting in no endogenous chorismate mutase, or prephenate dehydratase, or prephenate dehydrogenase activity.[9] The cells were grown in a Terrific Broth medium batch culture[10] at T = 310 K for ≈15 h. The cells were harvested by centrifugation at the acceleration $a \approx 3000\cdot g_n$ (g_n = 9.80665 m·s^{-2}), washed in the buffer {tris (hydroxymethyl) aminomethane (Tris) (concentration c = 0.01 mol·dm^{-3}) + ethylenediaminetetraacetic acid (EDTA) (c = 0.0001 mol·dm^{-3}) + dithiothreitol (DTT) (c = 0.001 mol·dm^{-3}), adjusted with HCl to pH = 7.4}. These cells were then re-harvested by centrifugation

†Certain commercial equipment, 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, mole fraction purity x as stated by supplier (M = Mallinckrodt, S = Sigma), and method used to determine x. The mole fraction purities are exclusive of the amount of water, or, for NAD_{ox} and NAD_{red}, the amounts of acetone and ethanol in the samples

Substance	CAS registry number	Formula	M_r	w	Supplier	x	Method [a]
Chorismate mutase [a]			$4.35 \cdot 10^4$				
Chorismic acid	617–12–9	$C_{10}H_{10}O_6$	226.19	0.102	S	0.951	h.p.l.c.
4-hydroxyphenylpyruvic acid	156–39–8	$C_9H_8O_4$	180.16	0.1102	S	0.991	h.p.l.c.
NAD_{ox}	53–84–9	$C_{21}H_{27}N_7O_{14}P_2$	663.43	0.0636	S	0.98 [b]	h.p.l.c. and enzymatic assay
NAD_{red}	606–68–8	$C_{21}H_{27}N_7O_{14}P_2Na_2$	709.41	0.0521	S	0.98 [c]	h.p.l.c. and enzymatic assay
Phenylpyruvic acid, sodium salt	114–76–1	$C_9H_7O_3Na$	186.14	0.107	S	0.98	t.l.c.
Phosphoric acid	7664–38–2	H_3PO_4	98.00		M	> 0.995	(acid + base) titration
Potassium phosphate, dibasic	7758–11–4	K_2HPO_4	174.18		S	> 0.995	(acid + base) titration
Prephenic acid [d]	126–49–8	$C_{10}H_{10}O_6$	226.19				
Prephenate dehydratase			$7.1 \cdot 10^4$ [e]				
Prephenate dehydrogenase			$7.8 \cdot 10^4$ [e]				
Sodium phosphate, dibasic	7558–79–4	Na_2HPO_4	141.96		S	> 0.99	

[a] The chorismate mutase, a homotrimer of subunits having $M_r = 1.45 \cdot 10^4$, was kindly provided by Dr Prasad Reddy. The purification and assay of this enzyme have been described previously.(9)

[b] The NAD_{ox} contained a mass fraction of acetone equal to 0.008.

[c] The NAD_{red} contained a mass fraction of ethanol equal to 0.034.

[d] Prephenic acid was prepared *in situ* (see Experimental section). The amount of prephenate formed in solution was calculated from the amount of chorismic acid used in its preparation.

[e] Prephenate dehydratase and prephenate dehydrogenase are both homodimers having respective subunit M_r values equal to $3.05 \cdot 10^4$ and $3.90 \cdot 10^4$.

at the same acceleration to produce a cell pellet. This was followed by re-suspension (mass concentration γ of the cell pellet in the buffer $= 0.25\ \mathrm{kg \cdot dm^{-3}}$) in a second buffer {3-(*N*-morpholino) propanesulfonic acid (Mops) ($c = 0.02\ \mathrm{mol \cdot dm^{-3}}$ + sodium acetate ($c = 0.0083\ \mathrm{mol \cdot dm^{-3}}$) + EDTA ($c = 0.001\ \mathrm{mol \cdot dm^{-3}}$) + DTT ($c = 0.001\ \mathrm{mol \cdot dm^{-3}}$), adjusted with a Tris base to pH = 7.4}. All of the aforementioned procedures were carried out at $T = 277$ K. The resulting cell mass (15 g) was kept frozen at $T = 193$ K and thawed at $T \approx 296$ K immediately prior to the purification step. The lysis of the thawed cells was accomplished by adjusting the pH to 7.8 with KOH ($c = 5.0\ \mathrm{mol \cdot dm^{-3}}$) and then adding EDTA ($c = 5\ \mathrm{mol \cdot dm^{-3}}$), DTT ($c = 0.001\ \mathrm{mol \cdot dm^{-3}}$), and lysozyme ($\gamma = 0.2\ \mathrm{g \cdot dm^{-3}}$). After incubation in the refrigerator ($T = 277$ K) for 1 h to 2 h, deoxyribonuclease ($\gamma = 1\ \mathrm{g \cdot dm^{-3}}$), and $MgSO_4$ ($c = 0.01\ \mathrm{mol \cdot dm^{-3}}$) were added and incubation was continued for one additional hour. The lysed cells were centrifuged at $a = 2.9 \cdot 10^4 \cdot g_n$ for 1 h and the supernatant was saved for purification of prephenate dehydrogenase.

The cell lysate was loaded onto a diethylaminoethane (DEAE) Fast-Flow Sepharose column (Pharmacia Biotech, Piscataway, NJ) which was equilibrated with buffer A {KH_2PO_4 ($c = 0.02\ \mathrm{mol \cdot dm^{-3}}$) + DTT ($c = 0.001\ \mathrm{mol \cdot dm^{-3}}$) adjusted to pH=7.5 with KOH(aq)}. The column was washed with the aforementioned buffer followed by a gradient of KCl ($c = 0$ to $c = 0.4\ \mathrm{mol \cdot dm^{-3}}$) in buffer A. Active fractions were pooled and concentrated by using an Amicon cell with a YM10 membrane. The sample was diluted with buffer A and re-concentrated to reduce the KCl concentration 13-fold. The protein was then loaded onto a hydroxyapatite column and eluted with a gradient of buffer A ($c = 0.02\ \mathrm{mol \cdot dm^{-3}}$ to $c = 0.30\ \mathrm{mol \cdot dm^{-3}}$). Active fractions were again pooled and $(NH_4)_2SO_4$ was added until the concentration of $(NH_4)_2SO_4$ reached $1.0\ \mathrm{mol \cdot dm^{-3}}$. The pH was then adjusted to 7.5 with either KOH(aq) or HCl(aq). The pooled active fractions were subjected to a hydrophobic interaction chromatography step (Phenyl-6 Fast-Flow Sepharose column, Pharmacia Biotech, Piscataway, NJ). The column was washed with a gradient of $(NH_4)_2SO_4$ ($c = 0.7\ \mathrm{mol \cdot dm^{-3}}$ to $c = 0$) in buffer A. Prephenate dehydrogenase did not elute until the column was washed with water. The mass fraction $w \approx 0.9$ of prephenate dehydrogenase in the resultant protein was determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis. The mass concentration γ of the protein in solution was $2.8\ \mathrm{g \cdot dm^{-3}}$. The activity of the prephenate dehydrogenase was $3.6 \cdot 10^{-4}\ \mathrm{mol \cdot s^{-1} \cdot g^{-1}}$. This activity was measured by following the reduction of NAD_{ox} to NAD_{red} via reaction (2) in {K_2HPO_4 ($c = 0.05\ \mathrm{mol \cdot dm^{-3}}$) +$NAD_{ox}$ ($c = 5 \cdot 10^{-4}\ \mathrm{mol \cdot dm^{-3}}$) + prephenate ($c = 4 \cdot 10^{-4}\ \mathrm{mol \cdot dm^{-3}}$), pH = 7.5} at $T = 296$ K. This followed the procedure of Byng *et al.*[11] except that the absorbance of NAD_{red} at the wavelength $\lambda = 340$ nm was monitored instead of fluorescence.

Prephenate dehydratase was prepared by expression of the plasmid pJZ1g[12] containing the monofunctional pheC gene of *Pseudomonas aeruginosa* in *E. coli* KA12 cells.[6] The culture and harvest of the cells was as described above for the preparation of the prephenate dehydrogenase with the exception that isopropylthiogalactoside ($c = 0.001\ \mathrm{mol \cdot dm^{-3}}$) was added to the medium once the cells had reached the logarithmic phase of growth. Cell lysis was accomplished with the use of a bead beater. A two-step chromatographic procedure was used for the purification of prephenate dehydratase. First, the protein was loaded onto a Q-Sepharose anion exchange column (Pharmacia Biotech, Piscataway, NJ) equilibrated

with buffer B {K_2HPO_4 ($c = 0.02$ mol·dm^{-3}) + DTT ($c = 0.001$ mol·dm^{-3}), pH = 8.5} and eluted using a KCl gradient ($c = 0$ to $c = 0.30$ mol·dm^{-3}) in buffer B. Active fractions were pooled and diluted with buffer C { K_2HPO_4 ($c = 0.02$ mol·dm^{-3}) +DTT ($c = 0.001$ mol·dm^{-3}), pH = 7.2} to reduce the KCl concentration and then loaded onto a hydroxyapatite column that had been equilibrated with buffer C. The prephenate dehydratase was eluted with a gradient of buffer C {$c = (0.02$ mol·dm^{-3} to 0.30 mol· dm^{-3})}. Prephenate dehydratase activity was assayed by carrying out reaction 1 in {Tris ($c = 0.025$ mol·dm^{-3}) + prephenic acid ($c = 5.10^{-4}$ mol·dm^{-3}), pH = 8.2} at $T = 297$ K. After incubation at $T = 297$ K, aliquots were removed at appropriate time intervals and analysed by h.p.l.c. Here, a Hewlett-Packard 1050 h.p.l.c. equipped with a u.v. detector set at $\lambda = 210$ nm and a Hewlett-Packard Hypersil C-18 column (4 mm i.d., 250 mm long) were used. The separation was isocratic (flow rate $q_V = 0.0133$ cm^3·s^{-1}) with the mobile phase {acetonitrile (volume fraction $\phi = 0.20$) +K_2HPO_4 ($c = 0.01$ mol·dm^{-3}, pH = 7.5) ($\phi = 0.80$)}. The retention time for phenylpyruvate was 4.2 min and the retention time of prephenate was 2.5 min (the void volume of the column). The activity of the enzyme was $4.2 \cdot 10^{-4}$ mol·s^{-1}·g^{-1} with $\gamma = 6$ g·dm^{-3} and a mass fraction purity > 0.8 as judged by SDS-PAGE analysis.

A Hewlett-Packard h.p.l.c. equipped with a u.v. detector set at $\lambda = 215$ nm and a Hewlett-Packard Hypersil C-18 column (4 mm i.d., 250 mm long) were used for the analysis of chorismate, prephenate, phenylpyruvate, 4-hydroxyphenylpyruvate, NAD_{ox}, and NAD_{red}. The column was thermostated at $T = 313$ K for the study of reaction (1) and was kept at ambient temperature (≈295 K) for reaction (2). The mobile phase for reaction (1) consisted of (A) {tetrabutyl ammonium hydroxide ($c = 0.01$ mol·dm^{-3}) + $NH_4H_2PO_4$ ($c = 0.025$ mol·dm^{-3})} adjusted to pH = 7.0 with H_3PO_4 and (B) acetonitrile; the flow rate q_V was 0.0133 cm^3·s^{-1}. The following gradient of these two mobile phases was formed: ϕ(A) = 0.85 and ϕ(B) = 0.15 at time $t = 0$; ϕ(A) = 0.80 and ϕ(B) = 0.20 at $t = 10$ min; and ϕ(A) = 0.50 and ϕ(B) = 0.50 at $t = 20$ min. With this procedure, typical retention times were: chorismate, 8.8 min; prephenate, 9.8 min; and phenylpyruvate, 18.3 min. For reaction (2), the mobile phase consisted of {K_2HPO_4 ($c = 0.03$ mol·dm^{-3}) + methanol ($\phi = 0.04$)} at pH = 6.2. This was run isocratically ($q_V = 0.0133$ cm^3·s^{-1}). Under these conditions, typical retention times were: prephenate 3.1 min; chorismate, 3.5 min; NAD_{ox}, 8.6 min; 4-hydroxyphenylpyruvate, 12.1 min; and NAD_{red}, 13.6 min.

Attempts were made to determine apparent equilibrium constants for reactions (1) and (2) by approaching equilibrium from both directions of the reaction. The solution used for the forward direction of reaction (1) contained prephenate (molality $m = 0.00264$ mol ·kg^{-1}) in a K_2HPO_4 buffer ($m = 0.10$ mol·kg^{-1}, pH = 7.19). The solution used for the reverse direction of reaction contained phenylpyruvate ($m = 0.00259$ mol·kg^{-1}) and sodium carbonate ($m = 0.0106$ mol·kg^{-1}) in the same phosphate buffer. The enzyme prephenate dehydratase was then added to these solutions so that the mass fraction of the enzyme in these solutions was ≈$1.5 \cdot 10^{-4}$. Following equilibration for 19 h, the molalities of prephenate and phenylpyruvate were measured by using the chromatographic procedure described above. The pHs of the reaction mixtures for the forward and reverse direction of the reaction were 7.14 and 7.47, respectively. A similar procedure was used for reaction (2). Here the solution used for the forward direction of the reaction contained

prephenate ($m = 0.00227\ \text{mol}\cdot\text{kg}^{-1}$) and NAD_{ox} ($m = 0.00392\ \text{mol}\cdot\text{kg}^{-1}$) in a Na_2HPO_4 buffer ($m = 0.1\ \text{mol}\cdot\text{kg}^{-1}$, pH = 7.50). The solution used for the reverse direction of the reaction contained 4-hydroxyphenylpyruvate ($m = 0.00291\ \text{mol}\cdot\text{kg}^{-1}$), sodium carbonate ($m = 0.00974\ \text{mol}\cdot\text{kg}^{-1}$), and NAD_{red} ($m = 0.00286\ \text{mol}\cdot\text{kg}^{-1}$) in the same phosphate buffer. The prephenate dehydrogenase was then added to these solutions so that the mass fraction of the enzyme in these solutions was $\approx 1.3\cdot 10^{-4}$. These solutions were also placed in 20 cm^3 Teflon capped glass bottles and gently shaken at about 50 r.p.m. in a water-bath thermostated at $T = 298.15$ K. Following equilibration for 21 h, the molalities of prephenate, 4-hydroxyphenylpyruvate, NAD_{ox}, and NAD_{red} were determined chromatographically. The pHs of the reaction mixtures for the forward and reverse directions of the reaction were 7.25 and 7.84, respectively. The limit of detectability of prephenate was $\approx 4\cdot 10^{-6}\ \text{mol}\cdot\text{kg}^{-1}$ under the chromatographic conditions employed in this study.

Three heat-conduction microcalorimeters were used for the enthalpy of reaction measurements. They were calibrated electrically with a high stability d.c. power supply, calibrated digital voltmeter, standard resistor, and time-interval counter. Descriptions of the microcalorimeters and their performance characteristics, the data-acquisition system, and the computer programs used to treat the results have been given by Steckler *et al*.[13, 14] The data-acquisition system has recently undergone significant modernization. The voltages of the thermopiles of the microcalorimeters are now measured with Hewlett-Packard model 34420A Nanovolt Meters. These voltages are then recorded on a microcomputer with a data-acquisition program written in Hewlett-Packard HP-VEE. The integration of the areas of the thermograms is done by using a code written in C++.

The calorimetric sample vessels were fabricated from high density polyethylene. Each vessel had two compartments that held, respectively, ≈ 0.55 cm^3 and ≈ 0.40 cm^3 of solution. The substrate solutions were placed in the 0.55 cm^3 compartment and the enzyme solutions were placed in the 0.40 cm^3 compartment. The substrate solutions for reactions (1) and (2) initially contained chorismate and chorismate mutase. As pointed out above, the chorismate mutase-catalysed conversion of chorismate to prephenate was rapid and essentially complete. In order to carry out reaction (2) NAD_{ox} was added to the buffer used for the preparation of the substrate and enzyme solutions. Prephenate was the limiting reactant in reaction (2). The enzyme solutions were prepared by addition of the prephenate dehydratase and prephenate dehydrogenase to the respective stock buffer solutions that were used for the preparation of the substrate solutions. The aim of this procedure was to minimize the blank enthalpies that are a consequence of the mixing of the enzyme and substrate solutions.

The vessels and their contents were allowed to equilibrate in the microcalorimeters for ≈ 60 min before the enzyme and substrate solutions were mixed. After this mixing, approximately 30 min were allowed for reactions (1) and (2). Following the reaction, the vessels were removed from the microcalorimeters and their contents were promptly analysed to determine the amount of prephenate in solution. The mole fraction of unreacted prephenate was ≈ 0.001 for reaction (1) and < 0.001 for reaction (2). The amount of substance n that had undergone reaction was based on the amount of prephenate in solution at the initiation of the calorimetric experiment. This amount was calculated from the amount of chorismic acid used in the *in situ* preparation of the prephenate. In performing this calculation, corrections were applied for the impurities in the chorismic acid. Thus, the correction to

the amount of prephenate in solution due to the impurities (including water) in the chorismic acid was $0.151 \cdot n$ (see table 1). A small correction ($0.0053 \cdot n$) was also made for the amount of prephenate that had undergone reaction (1) spontaneously (see above) and prior to the mixing of the enzyme and substrate solutions. Additionally, an even smaller correction ($\leq 0.001 \cdot n$) was made for the amount of unreacted prephenate. The "blank" enthalpy changes for mixing of the substrate solutions with the buffer ranged from -0.33 mJ to 1.87 mJ. For the mixing of the enzyme solutions with the respective buffers, these enthalpies ranged from -0.72 mJ to 1.62 mJ. These "blank" enthalpies of mixing were applied as corrections to the measured calorimetric enthalpies which were ≈ -500 mJ for reaction (1) and ≈ -325 mJ for reaction (2).

Measurement of pH was done with an Orion Model 811 pH meter and a Radiometer combination glass micro-electrode. The pH meter was calibrated with Radiometer standard buffers and with a standard phosphate buffer (pH = 7.42 at $T = 298.15$ K). This latter buffer was prepared from KH_2PO_4 and Na_2HPO_4, standard reference materials 186-Id and 186-IId, respectively, from the National Institute of Standards and Technology.

3. Results and discussion

The apparent equilibrium constants[15] for reactions (1) and (2), respectively, are:

$$K' = \{m(\text{phenylpyruvate}) \cdot m(\text{carbon dioxide})\}/\{m(\text{prephenate}) \cdot m^{\circ}\}, \tag{3}$$

$$K' = \{m(\text{4-hydroxyphenylpyruvate}) \cdot m(\text{NAD}_{\text{red}}) \cdot m(\text{carbon dioxide})\}/ \{m(\text{prephenate}) \cdot m(\text{NAD}_{\text{ox}}) \cdot m^{\circ}\}. \tag{4}$$

The molalities m in the above equation are the total molalities of the various charged and uncharged species that are formed from the dissociation of the various substances in solution. In particular, in reactions (1) and (2), and equations (3) and (4), "carbon dioxide" represents the total molalities of the various carbon dioxide related species, namely CO_3^{2-}(aq), HCO_3^- (aq), CO_2 (aq), and H_2CO_3 (aq). To maintain this distinction throughout this paper, "carbon dioxide" will refer to the sum of all of these species and "CO_2(aq)" will refer to the equilibrium mixture of the species $\{CO_2(aq) + H_2CO_3(aq)\}$. Specifically, the thermodynamic properties of H_2CO_3(aq) are taken to be equal to the thermodynamic properties of $\{CO_2(aq) + H_2O(l)\}$.[16] The standard molality ($m^{\circ} = 1\ \text{mol} \cdot \text{kg}^{-1}$) has been introduced in equations (2) and (3) to keep the apparent equilibrium constants dimensionless.

In discussing the thermodynamics of these reactions, it is useful to introduce reference reactions that pertain to specific ionic forms:

$$\text{prephenate}^{2-}(aq) = \text{phenylpyruvate}^{-}(aq) + HCO_3^-(aq), \tag{5}$$

$$\text{prephenate}^{2-}(aq) + NAD_{ox}^-(aq) + H_2O(l) = \text{4-hydroxyphenylpyruvate}^{-}(aq) + NAD_{red}^{2-}(aq) + HCO_3^-(aq) + H^+(aq). \tag{6}$$

The equilibrium constants for reactions (5) and (6), respectively, are:

$$K = \{m(\text{phenylpyruvate}^{-}) \cdot m(HCO_3^-)\}/\{m(\text{prephenate}^{2-}) \cdot m^{\circ}\}, \tag{7}$$

$$K = \{m(\text{4-hydroxyphenylpyruvate}^-)\cdot m(\text{NAD}^{2-}_{\text{red}})\cdot m(\text{HCO}_3^-)\cdot m(\text{H}^+)\}/$$
$$\{m(\text{prephenate}^{2-})\cdot m(\text{NAD}^-_{\text{ox}})\cdot (m^{\text{o}})^2\}. \quad (8)$$

The standard state used in this study is the hypothetical ideal solution of unit molality ($m^{\text{o}} = 1\ \text{mol}\cdot\text{kg}^{-1}$). It will be shown below that the species in reactions (5) and (6) are the predominant species at the pH values at which the calorimetric and equilibrium measurements were performed.

The result from the equilibrium measurements was that there was no measurable amount of prephenate in any of the reaction mixtures. Thus, it was not possible to measure apparent equilibrium constants for reactions (1) and (2). Based upon the lower limit of the amount of prephenate detectable with our chromatographic procedures, we can state that $K'(T = 298.15\ \text{K}, \text{pH} \approx 7.3) > 2$ for reaction (1), and $K'(T = 298.15\ \text{K}, \text{pH} \approx 7.6) > 9$ for reaction (2). Later in this paper it will be possible to estimate the order of magnitude of these equilibrium constants.

The results of the calorimetric measurements are given in table 2. Since carbon dioxide is a product of reactions (1) and (2), and since each microcalorimeter reaction vessel has a vapor space above the solution in it, one must consider the following equilibrium in the treatment of the results:

$$\text{CO}_2(\text{aq}) = \text{CO}_2(\text{g}). \quad (9)$$

Specifically, the aim in the calorimetric experiments was to determine values of the calorimetrically determined molar enthalpies[15] $\Delta_{\text{r}}H_{\text{m}}$ (cal) for reactions (1) and (2). However, since there is an enthalpy change ΔH associated with the formation of $\text{CO}_2(\text{g})$ via reaction (9), it is necessary to make an appropriate correction to the measured ΔH for any $\text{CO}_2(\text{g})$ formed. The amount of $\text{CO}_2(\text{g})$ formed in each experiment is calculated by using the value of the Henry's law constant:

$$K_{\text{H}} = p\{\text{CO}_2(\text{g})\cdot m^{\text{o}}\}/[m\{\text{CO}_2(\text{aq})\}\cdot p^{\text{o}}]. \quad (10)$$

Here, $p^{\text{o}} = 0.1$ MPa. The values $K_{\text{H}} = 29.7$ and the standard molar enthalpy change $\Delta_{\text{r}}H^{\text{o}}_{\text{m}} = 19.75\ \text{kJ}\cdot\text{mol}^{-1}$ for reaction (9) at $T = 298.15$ K and $p = 0.1$ MPa were calculated from the standard thermodynamic properties given in the CODATA tables.[17] Note that the CODATA tables[17] refer to $\text{CO}_2(\text{aq})$ as "$\text{CO}_2(\text{aq})$, undissoc". However, it is evident from the paper of Berg and Vanderzee[18] (on which the CODATA table entries for "$\text{CO}_2(\text{aq})$, undissoc" are based) that "$\text{CO}_2(\text{aq})$, undissoc" is the mixture of the species $\{\text{CO}_2(\text{aq}) + \text{H}_2\text{CO}_3(\text{aq})\}$. The importance of keeping this distinction clear has been emphasized by Alberty[19,20] who has also calculated standard molar formation properties for the aqueous species CO_2 and H_2CO_3, as well as standard molar transformed formation properties of aqueous carbon dioxide (the mixture of all carbon dioxide related species).

By solving all of the pertinent chemical equilibrium equations (see below), it is possible to calculate the amount of $\text{CO}_2(\text{g})$ formed during an experiment. By using the known value of $\Delta_{\text{r}}H^{\text{o}}_{\text{m}}$ for reaction (9), one can then calculate a correction to the measured molar enthalpy of reaction $\Delta_{\text{r}}H^{\text{o}}_{\text{m}}$ (meas) which then leads to the desired value(s) of $\Delta_{\text{r}}H_{\text{m}}$ (cal). Thus, the following average values of $\Delta_{\text{r}}H_{\text{m}}$ (cal) are obtained: $\langle\Delta_{\text{r}}H_{\text{m}}\ (\text{cal})\rangle = -(127.2 \pm 0.5)\ \text{kJ}\cdot\text{mol}^{-1}$ for reaction (1), and $\langle\Delta_{\text{r}}H_{\text{m}}\ (\text{cal})\rangle = -(79.2 \pm 0.7)\ \text{kJ}\cdot\text{mol}^{-1}$ for reaction (2). The uncertainties given here are equal to two estimated standard deviations of the mean.

TABLE 2. Results of the calorimetric measurements for biochemical reactions (1) and (2) in a phosphate buffer at $T = 298.15$ K. The molalities m 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. Abbreviations used in this table are: Preph, prephenate; NAD_{ox}, β-nicotinamide adenine dinucleotide (oxidized form); and NAD_{red}, β-nicotinamide adenine dinucleotide (reduced form). $\Delta_r H_m$ (meas) is the measured molar enthalpy of reaction which includes the change in enthalpy for reaction (9), $CO_2(aq) = CO_2(g)$. Correction for the enthalpy change for reaction (9) leads to the values of $\Delta_r H_m$ (cal), the calorimetrically determined enthalpies of reactions (1) and (2), given in column 7. The values of the ionic strength I_m are calculated. Prephenate dehydratase and prephenate dehydrogenase were used to catalyse reactions (1) and (2), respectively. The respective mass fractions w of these enzymes in solution were $2.5 \cdot 10^{-4}$ and $1.2 \cdot 10^{-4}$. The uncertainties in $\Delta_r H_m$ (cal) are equal to two estimated standard deviations of the mean

Reaction (1): prephenate(aq) = phenylpyruvate(aq) + carbon dioxide(aq)						
pH	$\frac{m(K_2HPO_4)}{mol \cdot kg^{-1}}$	$\frac{m(H_3PO_4)}{mol \cdot kg^{-1}}$	$\frac{10^3 \cdot m(Preph)}{mol \cdot kg^{-1}}$	$\frac{I_m}{mol \cdot kg^{-1}}$	$\frac{\Delta_r H_m(meas)}{kJ \cdot mol^{-1}}$	$\frac{\Delta_r H_m(cal)}{kJ \cdot mol^{-1}}$
7.17	0.09309	0.01959	4.834	0.35	−124.1	−127.9
7.17	0.09312	0.01960	4.888	0.35	−123.3	−127.0
7.17	0.09312	0.01960	4.888	0.35	−123.2	−127.3
7.17	0.09303	0.01958	4.899	0.35	−123.4	−127.3
7.17	0.09294	0.01956	4.771	0.35	−122.6	−126.3
7.17	0.09295	0.01956	4.785	0.35	−122.7	−126.4
$\langle \Delta_r H_m \text{ (cal)} \rangle = -(127.0 \pm 0.5) \text{ kJ} \cdot \text{mol}^{-1}$						

TABLE 2—*continued*

Reaction (2): prephenate(aq) + NAD_{ox}(aq) + H_2O(l) = 4-hydroxyphenylpyruvate(aq) + NAD_{red}(aq) + carbon dioxide(aq)							
pH	$\frac{m(Na_2HPO_4)}{mol \cdot kg^{-1}}$	$\frac{m(H_3PO_4)}{mol \cdot kg^{-1}}$	$\frac{10^3 \cdot m(Preph)}{mol \cdot kg^{-1}}$	$\frac{10^3 \cdot m(NAD_{ox})}{mol \cdot kg^{-1}}$	$\frac{I_m}{kJ \cdot mol^{-1}}$	$\frac{\Delta_r H_m \text{ (meas)}}{kJ \cdot mol^{-1}}$	$\frac{\Delta_r H_m \text{ (cal)}}{kJ \cdot mol^{-1}}$
6.98	0.09301	0.00851	5.284	10.25	0.32	−74.2	−79.5
6.98	0.09299	0.00851	5.248	10.25	0.32	−74.3	−79.4
6.98	0.09298	0.00850	5.231	10.25	0.32	−74.8	−80.0
6.98	0.09299	0.00851	5.243	10.25	0.32	−73.2	−77.8
6.98	0.09311	0.00852	5.404	10.26	0.32	−73.8	−78.5
6.98	0.09303	0.00851	5.300	10.25	0.32	−73.8	−78.7
$\langle \Delta_r H_m \text{ (cal)} \rangle = -(79.0 \pm 0.7)\ kJ \cdot mol^{-1}$							

The uncertainties in the values of $\langle \Delta_r H_m \text{ (cal)} \rangle$ represent only the random errors inherent in the measurements and do not reflect the possible systematic errors which are now considered. We judge that reasonable estimates of the standard uncertainties[21] due to possible systematic errors in the values of $\Delta_r H_m$ (cal) are: $0.018 \cdot \Delta_r H_m$ (cal) due to impurities in the chorismate (this includes the uncertainty in its moisture content); $0.001 \cdot \Delta_r H_m$ (cal) for the amount of prephenate that had undergone reaction (1) spontaneously and prior to the mixing of the enzyme and substrate solutions; $0.003 \cdot \Delta_r H_m$ (cal) due to uncertainties in the calorimetric measurements; and $0.003 \cdot \Delta_r H_m$ (cal) and $0.006 \cdot \Delta_r H_m$ (cal) for reactions (1) and (2), respectively, due to uncertainties in the CO_2 correction. The standard uncertainties in the CO_2 corrections are based on the assumption that these corrections are uncertain by no more than ± 10 per cent. These estimates of the possible systematic error are combined in quadrature together with the statistical uncertainties in the measured values of $\Delta_r H_m$ (cal), expressed as one estimated standard deviation of the mean, to obtain combined standard uncertainties.[21] These combined standard uncertainties are then multiplied by two to arrive at the final set of results for $\Delta_r H_m$ (cal): $\Delta_r H_m \text{ (cal)} = -(127.2 \pm 4.7)\ \text{kJ} \cdot \text{mol}^{-1}$ for reaction (1) at $T = 298.15$ K, pH = 7.17, and ionic strength $I_m = 0.35\ \text{mol} \cdot \text{kg}^{-1}$; $\Delta_r H_m \text{ (cal)} = -(79.2 \pm 3.1)\ \text{kJ} \cdot \text{mol}^{-1}$ for reaction (2) at $T = 298.15$ K, pH = 6.98, and $I_m = 0.32\ \text{mol} \cdot \text{kg}^{-1}$.

The pK values and standard molar enthalpies for the proton dissociation reactions of the reactants and of the other solutes in solution are needed to relate the experimental results for reactions (1) and (2) to thermodynamic quantities for the respective reference reactions (5) and (6). These pK values and standard molar enthalpies $\Delta_r H_m^o$ are given in table 3. The pK and $\Delta_r H_m^o$ values for the ionization of phenylpyruvic acid are from Martell and Smith.[22] Moore and Underwood[23] performed a potentiometric titration of NAD_{ox} and obtained p$K = (3.88 \pm 0.02)$ for NAD_{ox}^0 at $T = 298.15$ K and $I = 0$. We estimate pK $\approx$3.9 for NAD_{red}^- (aq) at $T = 298.15$ K and $I = 0$ on the basis of the structural similarity of NAD_{ox} and NAD_{red}. The value for the pK of prephenate$^-$ is a previous estimate from Kast *et al.*[2] In the absence of values from the literature, we estimated the pK values of 4-hydroxyphenylpyruvic acid(aq) from the known[22] pK values of phenylpyruvic acid(aq) and L-tyrosine(aq). The thermodynamic quantities for the ionizations of H_2CO_3, HCO_3^-, and $H_2PO_4^-$ were calculated from the standard molar thermodynamic properties given in the CODATA tables.[17] We note that the values of the thermodynamic quantities for the ionizations of H_2CO_3 and HCO_3^- used in this study (see table 3) are essentially the same as those that are calculated from the values of the standard molar thermodynamic properties given in table 7 in the paper by Berg and Vanderzee.[18]

It is evident from the pK values given in table 3 that the species used in the reference reactions (5) and (6) are also the predominant species within the respective pseudoisomer[15] groups. Additionally, the pK values of all of the reactants, with the exception of H_2CO_3, are well removed (*i.e.* $|\text{p}K - \text{pH}| \geq 2.5$) from the pH values at which the measurements were performed. Thus, the most essential pK needed for the equilibrium calculations is that of H_2CO_3. Fortunately, the value of this pK is well known.[18]

The equilibrium model used for the calculation of standard molar enthalpies $\Delta_r H_m^o$ for the reference reactions from the measured values of $\Delta_r H_m$ (cal) has been described previously.[24] This model has been modified recently so that it now utilizes the Mathemat-

TABLE 3. The pK values and standard molar enthalpy changes $\Delta_r H^o_m$ for the aqueous proton dissociation reactions of the substances pertinent to this study at $T = 298.15$ K and $I = 0$. See Results and discussion for the basis of these values

Reaction	pK	$\Delta_r H^o_m$ / kJ·mol^{-1}
4-hydroxyphenylpyruvic acid = 4-hydroxyphenylpyruvate$^-$ + H$^+$	2.5 [a]	
4-hydroxyphenylpyruvate$^-$ = 4-hydroxyphenylpyruvate^{2-} + H$^+$	10.5 [a]	
$NAD^0_{ox} = NAD^-_{ox} + H^+$	3.88	
$NAD^-_{red} = NAD^{2-}_{red} + H^+$	3.9 [a]	
phenylpyruvic acid = phenylpyruvate$^-$ + H$^+$	2.54	
prephenate$^-$ = prephenate^{2-} + H$^+$	4.5 [a]	
$H_2CO_3 = HCO^-_3 + H^+$	6.353	9.16
$HCO^-_3 = CO^{2-}_3 + H^+$	10.327	14.70
$H_2PO^-_4 = HPO^{2-}_4 + H^+$	7.212	3.6

[a] Estimated values.

ica[25] computer code of Alberty and Krambeck[26] to solve the simultaneous non-linear equations that describe the various equilibria. This Mathematica code has been extended so as to include corrections for non-ideality and so that the calculations are made self-consistent[24] with regard to the ionic strength. The non-ideality corrections are based on the extended Debye–Hückel equation[24] in which the "ion-size" parameter has been set at 1.6 kg$^{1/2}$·mol$^{-1/2}$. As pointed out above, it is necessary to consider the formation of CO_2(g) in the microcalorimeter vessel. Accordingly, reaction (9) is also incorporated into these equilibrium calculations. Thus, by applying this model and by using both the experimental values of $\Delta_r H_m$ (cal) and the thermodynamic quantities given in table 3, we obtain $\Delta_r H^o_m = -(125.6 \pm 4.7)$ kJ·mol^{-1} for reaction (5) and $\Delta_r H^o_m = -(73.9 \pm 3.1)$ kJ·mol^{-1} for reaction (6). Both values pertain to $T = 298.15$ K and $I = 0$.

The equilibrium model is also used to calculate the change in binding of the hydrogen ion $\Delta_r N(H^+)$. Thus, $\Delta_r N(H^+) = 0.0964$ for reaction (1) at $T = 298.15$ K, pH = 7.17, and $I_m = 0.35$ mol·kg^{-1}, and $\Delta_r N(H^+) = -0.857$ for reaction (2) at $T = 298.15$ K, pH = 6.98, and $I_m = 0.32$ mol·kg^{-1}. These values of $\Delta_r N(H^+)$ were used in the buffer protonation corrections[27] used to calculate the values of $\Delta_r H^o_m$ for the reference reactions (5) and (6) from the experimentally determined values of $\Delta_r H_m$ (cal) for the overall biochemical reactions (1) and (2).

The uncertainties in the values of $\Delta_r H^o_m$ for the reference reactions (5) and (6) have two components: the experimental uncertainties in the measured values of $\Delta_r H_m$ (cal) and possible errors in the quantities used in the equilibrium model. This latter component of uncertainty was examined by perturbing each of the pertinent quantities in the model by an assumed possible error. Specifically, the pK of H_2CO_3 was perturbed by ± 0.01, $\Delta_r H^o_m$ for

the ionization of H_2CO_3 by ± 0.1 kJ·mol^{-1}, the pK of $H_2PO_4^-$ by ± 0.01, $\Delta_r H_m^o$ for the ionization of $H_2PO_4^-$ by ± 0.2 kJ·mol^{-1}, and the "ion-size" parameter used in the activity coefficient model by ± 0.3 kg$^{1/2}$·mol$^{-1/2}$. The combined effect of these perturbations in the calculated values of $\Delta_r H_m^o$ was relatively small (≤ 0.3 kJ·mol^{-1}) in comparison with the experimental uncertainties in $\Delta_r H_m$ (cal). Therefore, the final uncertainties in the calculated values of $\Delta_r H_m^o$ for reactions (5) and (6) are the same as the uncertainties in the values of $\Delta_r H_m$ (cal). The final set of rounded results for $T = 298.15$ K and $I = 0$ are: $\Delta_r H_m^o = -(126 \pm 5)$ kJ·mol^{-1} for reaction (5), and $\Delta_r H_m^o = -(74 \pm 3)$ kJ·mol^{-1} for reaction (6).

This study appears to be the first in which values of calorimetric enthalpies have been determined for reactions (1) and (2). Additionally, there do not appear to be any thermodynamic cycles that lead to values of these enthalpies, or any other thermodynamic quantities for these reactions. Nevertheless, some insight into the thermodynamics of these reactions can be obtained by calculating the difference between the standard molar enthalpies of formation $\Delta_f H_m^o$ of phenylpyruvate$^-$ (aq) and 4-hydroxyphenylpyruvate$^-$ (aq). First, from the review of Miller and Smith-Magowan,[28] we have $\Delta_r G_m^o = 20.2$ kJ·mol^{-1} and $\Delta_r H_m^o = -30.7$ kJ·mol^{-1} at $T = 298.15$ K and $I_m = 0.1$ mol·kg^{-1} for the reaction:

$$NAD_{ox}^-(aq) + H_2(g) = NAD_{red}^{2-}(aq) + H^+(aq). \tag{11}$$

Adjustment of these quantities to $I = 0$ with the extended Debye–Hückel theory (see above) yields $\Delta_r G_m^o = 22.65$ kJ·mol^{-1} and $\Delta_r H_m^o = -31.94$ kJ·mol^{-1} for reaction (11). Thus, we have the difference $[\Delta_f H_m^o\{NAD_{red}^{2-}(aq)\} - \Delta_r H_m^o\{NAD_{ox}^-(aq)\}] = -31.94$ kJ·mol^{-1}, and, with the standard molar entropy of $H_2(g)$,[17] we also have the difference in the standard partial molar entropies $[S_{2,m}^o\{NAD_{red}^{2-}(aq)\} - S_{2,m}^o\{NAD_{ox}^-(aq)\}] = -52.4$ J·K^{-1}·mol^{-1}. These differences will be used in the calculations that follow. From the CODATA tables ,[17] we calculate $\Delta_r H_m^o = -285.83$ kJ·mol^{-1} ($T = 298.15$ K, $p^o = 0.1$ MPa) for the reaction:

$$H_2(g) + (1/2)O_2(g) = H_2O(l). \tag{12}$$

The appropriate summation of reactions (5), (6), (11), and (12) gives the reaction:

$$\text{phenylpyruvate}^-(aq) + (1/2)O_2(g) = \text{4-hydroxyphenylpyruvate}^-(aq). \tag{13}$$

Then, $\Delta_r H_m^o(13) = -\Delta_r H_m^o(5) + \Delta_r H_m^o(6) - \Delta_r H_m^o(11) + \Delta_r H_m^o(12) = -(202 \pm 6)$ kJ·mol^{-1}. Note, however, that $\Delta_r H_m^o(13)$ is also equal to $\Delta_f H_m^o$ [{4-hydroxyphenylpyruvate$^-$(aq)} $-\Delta_f H_m^o$\{phenylpyruvate$^-$(aq)\}]. This difference of $-(202 \pm 6)$ in the $\Delta_f H_m^o$ values of these two substances corresponds to the addition of a hydroxyl group to position 4 of the aromatic ring. Some evidence of the reasonableness of this difference can be obtained by a comparison with the difference in the $\Delta_f H_m^o$ values of the structurally similar pair {phenol (C_6H_6O) + benzene (C_6H_6)}.

To obtain the difference in the $\Delta_f H_m^o$ values of this pair of substances, we use values of $\Delta_r H_m^o$ for the following reactions:

$$6C(s) + 3H_2(g) = C_6H_6(l), \tag{14}$$

$$6C(s) + 3H_2(g) + (1/2)O_2(g) = C_6H_6O(s), \tag{15}$$

$$C_6H_6(l) = C_6H_6(aq), \tag{16}$$

$$C_6H_6O(s) = C_6H_6O(aq). \tag{17}$$

For reactions (14) and (15) we have, respectively, $\Delta_r H^o_m = -165.1\ kJ\cdot mol^{-1}$ and $\Delta_r H^o_m = 49.0\ kJ\cdot mol^{-1}$ from Pedley *et al.*[29] We also have $\Delta_r H^o_m = 2.1\ kJ\cdot mol^{-1}$ for reaction (16) from May *et al.*,[30] and $\Delta_r H^o_m = 12.93\ kJ\cdot mol^{-1}$ for reaction (17) from Nichols and Wadsö.[31] All of these results pertain to $T = 298.15$ K and $p = 0.1$ MPa. Then,

$$\Delta_f H^o_m\{\text{phenol(aq)}\} - \Delta_f H^o_m\{\text{benzene(aq)}\} = -\Delta_r H^o_m(14) + \Delta_r H^o_m(15) - \Delta_r H^o_m(16) + \Delta_r H^o_m(17) = -203.2 kJ\cdot mol^{-1}. \tag{18}$$

However, we had already calculated $\Delta_f H^o_m$ [{4-hydroxyphenylpyruvate$^-$(aq)} − $\Delta_f H^o_m$ {phenylpyruvate$^-$(aq)}] = $-(202 \pm 6)\ kJ\cdot mol^{-1}$. Thus, it is seen that there is an excellent correlation in the difference in the $\Delta_f H^o_m$ values of these structurally similar pairs of substances.

As pointed out earlier, it was not possible to determine equilibrium constants for reactions (5) and (6). However, by estimating values of $\Delta_r S^o_m$ for these reactions, and by combining these estimates with the measured values of $\Delta_r H^o_m$, we can obtain values of $\Delta_r G^o_m$ and then approximate values of the equilibrium constants. First, estimates of S^o_m for prephenic, phenylpyruvic, and 4-hydroxyphenylpyruvic acids in both the gas and liquid phases were accomplished by using the Benson method[32,33] with the group values tabulated by Domalski and Hearing.[34] There were too many missing group values to allow for meaningful estimates for the solid phase. These Benson estimates were then combined with the values of S^o_m for HCO_3^-(aq) and H_2O(l)[17] and with the difference $[S^o_{2,m}\{NAD^{2-}_{red}(aq)\} - S^o_{2,m}\{NAD^-_{ox}(aq)\}]$ (see above) to obtain values of $\Delta_r S^o_m$ for the reference reactions (5) and (6). It is important to note that these calculations completely neglect the hydration of the prephenic, phenylpyruvic, and 4-hydroxyphenylpyruvic acids as well as the ionization of these species. Additionally, in the absence of Benson group values for C–$(H)_2$(CO)(C_B), CO–(CO)(C), CO–(CO)(O), C–$(C_d)_2$(CO)(H), and C–(C_d)(CO)$(H)_2$, we used the existing values for the respective groups C–$(H)_2$(CO)(C), CO–(C)(O), C–$(C)_2$(CO)(H), and C–(C)(CO)$(H)_2$.

Thus, based upon these Benson estimates and the aforementioned auxiliary thermodynamic quantities, we have $\Delta_r S^o_m \approx 85\ J\cdot K^{-1}\cdot mol^{-1}$ for reaction (5) and $\Delta_r S^o_m \approx -20\ J\cdot K^{-1}\cdot mol^{-1}$ for reaction (6). The value of $\Delta_r S^o_m$ for reaction (5) obtained by using the gas phase Benson estimate differed by $40\ J\cdot K^{-1}\cdot mol^{-1}$ from the value obtained by using the Benson estimate for the liquid phase; the corresponding difference for reaction (6) was $16\ J\cdot K^{-1}\cdot mol^{-1}$. Thus, these quantities, which pertain to $T = 298.15$ and $p = 0.1$ MPa, must be considered very approximate. In any case, we use these estimated values of $\Delta_r S^o_m$ together with our experimentally determined values of $\Delta_r H^o_m$ (see above) to calculate $\Delta_r G^o_m \approx -151\ kJ\cdot mol^{-1}$ for reaction (5) and $\Delta_r G^o_m \approx -68\ kJ\cdot mol^{-1}$ for reaction (6).

Thus, $K \approx 1\cdot 10^{26}$ for reaction (5) and $K \approx 1\cdot 10^{12}$ for reaction (6). These values are consistent with our experimental findings and show that, at equilibrium, both reactions are expected to proceed to completion.

Using the analogous procedure to that just used to obtain the estimated values of $\Delta_r S^o_m$, we calculate $\Delta_r H^o_m \approx -134$ kJ·mol^{-1} for reaction (5) and $\Delta_r H^o_m \approx -73$ kJ·mol^{-1} for reaction (6). However, unlike the situation for the entropies, it was not necessary to estimate any group values. The value of $\Delta_r H^o_m$ for reaction (5) obtained by using the gas phase Benson estimate differed by 38 kJ·mol^{-1} from the value obtained by using the Benson estimate for the liquid phase; the corresponding difference for reaction (6) was 50 kJ·mol^{-1}. Interestingly, these estimated average values of $\Delta_r H^o_m$, albeit very uncertain, are close to the experimental results $\Delta_r H^o_m = -(126 \pm 5)$ kJ·mol^{-1} for reaction (5) and $\Delta_r H^o_m = -(74 \pm 3)$ kJ·mol^{-1} for reaction (6). This suggests a need and an opportunity to further develop the Benson method to include reactions in solution.

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