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A thermodynamic study of the reactions: {2-dehydro-3-deoxy-D-*arabino*-heptanoate 7-phosphate(aq) = 3-dehydroquinate(aq) + phosphate(aq)} and {3-dehydroquinate(aq) = 3-dehydroshikimate(aq) + H_2O(l)}

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Microcalorimetry and high-performance liquid chromatography (h.p.l.c.) have been used to conduct a thermodynamic investigation of reactions catalyzed by 3-dehydroquinate synthase and by 3-dehydroquinate dehydratase. These are the second and third reactions in the metabolic pathway leading to the formation of chorismate. The two reactions are: {DAHP(aq) = 3-dehydroquinate(aq) + phosphate(aq)} and {3-dehydroquinate(aq) = 3-dehydroshikimate(aq) + H_2O(l)}. The h.p.l.c. measurements showed that the first reaction proceeded to completion and that the value of the apparent equilibrium constant for the second reaction was $K' = (4.6 \pm 1.5)$ (Hepes buffer, temperature $T = 298.15$ K, pH = 7.50, and ionic strength $I_m = 0.065\,\text{mol}\cdot\text{kg}^{-1}$). Calorimetric measurements led to a molar enthalpy of reaction $\Delta_r H_m(\text{cal}) = -(50.9 \pm 1.1)\ \text{kJ}\cdot\text{mol}^{-1}$ (Hepes buffer, $T = 298.15$ K, pH = 7.46, $I_m = 0.070\,\text{mol}\cdot\text{kg}^{-1}$) for the first reaction and to $\Delta_r H_m(\text{cal}) = (2.3 \pm 2.3)\ \text{kJ}\cdot\text{mol}^{-1}$ (Hepes buffer, $T = 298.15$ K, pH = 7.42, $I_m = 0.069\,\text{mol}\cdot\text{kg}^{-1}$) for the second reaction. These results were analyzed in terms of a chemical equilibrium model that accounts for the multiplicity of ionic states of the reactants and products. These calculations gave thermodynamic quantities at $T = 298.15$ K and $I_m = 0$ for chemical reference reactions involving specific ionic forms. For the reaction $DAHP^{3-}(aq) = 3\text{-dehydroquinate}^{-}(aq) + HPO_4^{2-}(aq)$, the standard molar enthalpy of reaction $\Delta_r H_m^\circ = -(51.1 \pm 4.5)\ \text{kJ}\cdot\text{mol}^{-1}$. For the reaction 3-de-hydroquinate(aq) = 3-dehydroshikimate(aq) + H_2O(l), the equilibrium constant

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$K = (4.6 \pm 1.5)$ and $\Delta_r H^\circ_m = (2.3 \pm 2.3)\,\mathrm{kJ \cdot mol^{-1}}$. A Benson type approach was used to estimate the standard molar entropy change $\Delta_r S^\circ_m$ for the first reference reaction and led to the value $K \approx 2 \cdot 10^{14}$ for this reaction. Values of the apparent equilibrium constants and the standard transformed Gibbs free energy changes $\Delta_r G^\circ_m$ under approximately physiological conditions are given for the biochemical reactions. Published by Elsevier Science Ltd.

KEYWORDS: apparent equilibrium constant; DAHP synthase; 2-dehydro-3-deoxy-D-*arabino*-heptanoate 7-phosphate; 3-dehydroquinate synthase; dehydratase; DHQ; enthalpy; entropy; Gibbs free energy

1. Introduction

In a previous study,[1] thermodynamic results were obtained for the first reaction in the chorismate metabolic pathway[2]

$$\begin{aligned}&\text{phospho}\textit{enol}\text{pyruvate(aq)} + \text{D-erythrose 4-phosphate(aq)} + \mathrm{H_2O(l)}\\ &\quad = \text{2-dehydro-3-deoxy-D-}\textit{arabino}\text{-heptanoate 7-phosphate(aq)} + \text{phosphate(aq)}.\end{aligned} \tag{1}$$

The substance 2-dehydro-3-deoxy-D-*arabino*-heptanoate 7-phosphate is commonly known as DAHP and this abbreviation† will be used subsequently. In aqueous solution, DAHP exists as an equilibrium mixture of a linear form and a pyranose form (see figure 1). The pyranose is the more stable of these two forms[1] and is known to be the predominant (mole fraction $x > 0.99$) form of DAHP[3] in aqueous solution. The next two steps in the chorismate pathway are

$$\text{DAHP(aq)} = \text{3-dehydroquinate(aq)} + \text{phosphate(aq)}, \tag{2}$$

$$\text{3-dehydroquinate(aq)} = \text{3-dehydroshikimate(aq)} + \mathrm{H_2O(l)}. \tag{3}$$

These reactions are catalyzed, respectively, by 3-dehydroquinate synthase (EC 4.6.1.3) and by 3-dehydroquinate dehydratase (EC 4.2.1.10). The catalytic activity of 3-dehydroquinate synthase depends on the presence of the cofactors NAD_{ox} and Zn^{2+}.[3,4] The crystal structure of *Aspergillus nidulans* 3-dehydroquinate synthase is known[5] as is the structure of type I 3-dehydroquinate dehydratase from *Salmonella typhi* and type II 3-dehydroquinate dehydratase from *Mycobacterium tuberculosis*.[6] The two types of 3-dehydroquinate dehydratase have distinct structures but catalyze the same overall reaction.[6] Additionally, there is a substantial body of information on the molecular biology involving the enzymes that catalyze these two reactions.[2,7–13] However, the only thermodynamic results in the literature appear to be an early report by Mitsuhashi and

†Chemical abbreviations used in this paper are: Bis–Tris propane, 1,3-bis[tris(hydroxymethyl)methylamino]propane; DAHP, 2-dehydro-3-deoxy-D-*arabino*-heptanoate 7-phosphate; DTT, DL-dithiothreitol; E4P, D-erythrose 4-phosphate; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; and NAD_{ox}, oxidized form of β-nicotinamide-adenine dinucleotide.

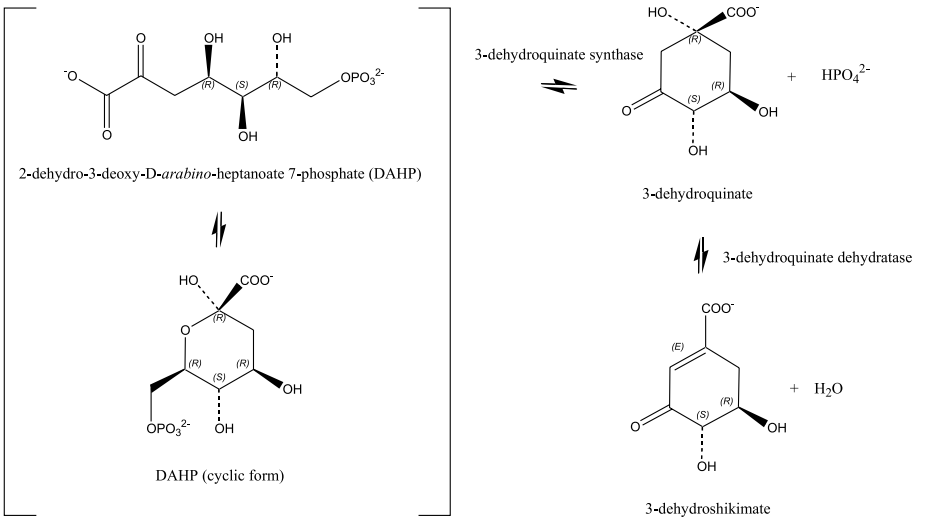

FIGURE 1. Structures of the substances in reactions (2) and (3). The predominant form of the substances at pH = 7.5 are shown.

Davis[14] of the apparent equilibrium constant for reaction (3). There does not appear to be any thermodynamic information available on reaction (2).

The experimental approach taken in this study was to prepare DAHP *in situ* by using reaction (1). This was done by starting with a well characterized sample of phospho*enol*pyruvate and an excess of D-erythrose 4-phosphate. This reaction is catalyzed by DAHP synthase and is known to proceed quantitatively to completion.[1] This was again confirmed in the present study. The *in situ* prepared solution of DAHP served as the starting point for the study of reactions (2) and (3). Thus, in the calorimetric study of reaction (2), 3-dehydroquinate synthase was added to the DAHP solution and the extent of reaction was determined by using h.p.l.c. The combined reaction $\{\alpha_2\cdot$reaction (2) + $\alpha_3\cdot$reaction (3)$\}$

$$\alpha_2\{\text{DAHP(aq)} = \text{3-dehydroquinate(aq)} + \text{phosphate(aq)} + \text{H}_2\text{O(l)}\} + \alpha_3\{\text{3-dehydroquinate(aq)} = \text{3-dehydroshikimate(aq)} + \text{H}_2\text{O(l)}\} \quad (4)$$

was studied calorimetrically by adding the two enzymes (3-dehydroquinate synthase + 3-dehydroquinate dehydratase) to the DAHP solution. Here α_2 and α_3 are, respectively, the degrees or fractions of reactions (2) and (3) that occur under actual experimental conditions. H.p.l.c. was again used to measure the extents of reactions (2) and (3). The molar enthalpy change for reaction (3) was then calculated from the measured enthalpy change for reaction (4), the measured extents of reactions (2) and (3), and the already measured molar enthalpy change for reaction (2). The results have been used to calculate values of standard thermodynamic quantities that pertain to reference reactions that involve specific ionic species.

2. Experimental

CHEMICALS

Pertinent information on the substances used in this study is given in table 1.[‡] The sample of phospho*enol*pyruvate was carefully characterized by using two different h.p.l.c methods. It is the same sample as used previously.[1] The mass fractions of water in the samples of phospho*enol*pyruvate, D-erythrose 4-phosphate, and β-nicotinamide-adenine-dinucleotide (oxidized) were determined by means of Karl Fischer titration.[1]

DAHP was prepared *in situ* by addition of DAHP synthase (mass fraction $w = 3.6 \cdot 10^{-5}$) to a solution containing phospho*enol*pyruvate and excess D-erythrose 4-phosphate. The buffer was Hepes {concentration $c = 0.098\,\mathrm{mol \cdot dm^{-3}}$, adjusted to pH = 7.65 with NaOH(aq)}; $MnCl_2$ ($c = 0.00098\,\mathrm{mol \cdot dm^{-3}}$) was present as a cofactor. This reaction was carried out at the temperature $T = 298.15\,\mathrm{K}$ with gentle lateral shaking of the reaction mixture for $\approx 60\,\mathrm{min}$. The h.p.l.c. analysis (Dionex DX 500 Ion Chromatograph, see below) showed that, based on the limit of detection of PEP (molality $m \approx 5 \cdot 10^{-7}\,\mathrm{mol \cdot kg^{-1}}$), the mass fraction of phospho*enol*pyruvate left in the reaction mixture was < 0.0001. The final DAHP solution was divided into several small tubes and stored at $T \approx 253\,\mathrm{K}$. Thus, the molality of the *in situ* prepared DAHP was calculated from the stoichiometry of reaction (1) and is based on the well-known purity of the sample of phospho*enol*pyruvate, the fact that the reaction proceeds to completion, and the measured stability of DAHP in solution (see below). A sample of 3-dehydroquinate in H_2O(l) was prepared by using previously described procedures.[15] Both the DAHP and 3-dehydroquinate were stored at $T \approx 193\,\mathrm{K}$.

ENZYMES

The DAHP synthase was provided by Professor Ronald Bauerle (University of Virginia) and its preparation has been described earlier.[1] This enzyme was present at a mass fraction $w = 0.006$ in a solution containing {1,3-bis[tris(hydroxymethyl)methylamino]propane (Bis–Tris propane) ($c = 0.020\,\mathrm{mol \cdot dm^{-3}}$) + phospho*enol*pyruvate ($c = 1.50 \cdot 10^{-4}\,\mathrm{mol \cdot dm^{-3}}$), pH = 6.8}.

The 3-dehydroquinate synthase and 3-dehydroquinate dehydratase were purified following previously described methods.[5, 11,16] The 3-dehydroquinate synthase was stored in the buffer [($KH_2PO_4 + K_2HPO_4$) {c(total phosphate)$\approx 0.20\,\mathrm{mol \cdot dm^{-3}}$} + DTT ($c = 0.001\,\mathrm{mol \cdot dm^{-3}}$) + KCl ($c = 0.1\,\mathrm{mol \cdot dm^{-3}}$), pH = 6.6]. The 3-dehydroquinate dehydratase was stored in the buffer [($KH_2PO_4 + K_2HPO_4$) {c(total phosphate) ≈ 0.025 $\mathrm{mol \cdot dm^{-3}}$} + DTT ($c = 0.0005\,\mathrm{mol \cdot dm^{-3}}$), pH = 7.2]. The mass fractions of 3-dehydroquinate synthase and 3-dehydroquinate in their respective solutions were 0.0011 and

[‡]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, 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 (Fi = Fisher, Fl = Fluka, and S = Sigma), and method used to determine x

Substance	CAS No.	Formula	M_r	w	Supplier	x	Method
2-Dehydro-3-deoxy-D-*arabino*-heptanoate 7-phosphate[a]	2627-73-8	$C_7H_{13}O_{10}P$	288.15		[b]		
2-Dehydro-3-deoxyphospho-heptanoate aldolase[c]			$1.520 \cdot 10^5$		[d]		
3-Dehydroquinate	10534-44-8	$C_7H_{10}O_6$	190.15		[b,d]		
3-Dehydroquinate dehydratase[e]			$2.7732 \cdot 10^4$		[d]		
3-Dehydroquinate synthase[f]			$4.2949 \cdot 10^4$		[d]		
3-Dehydroshikimate	2922-42-1	$C_7H_8O_5$	172.14		[b]		
D-Erythrose 4-phosphate	103302-15-4	$C_4H_8O_7PNa$	222.07	0.037	S	≈ 0.90	t.l.c.; enzymatic assay
N-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid[g]	7365-45-9	$C_8H_{18}N_2O_4S$	238.31		S		
Manganese chloride	7773-01-5	$MnCl_2$	125.84		Fl	> 0.99	Argentometric
β-Nicotinamide-adenine dinucleotide (oxidized)[h]	53-84-9	$C_{21}H_{27}N_7O_{14}P_2$	663.43	0.0636	S		
Phospho*enol*pyruvate, potassium salt	4265-07-0	$C_3H_4O_6PK$	206.13	0.0140	S	> 0.99	h.p.l.c.; enzymatic assay
Sodium hydroxide	1310-73-2	NaOH	39.997		Fi	> 0.99	Acid titration
Zinc sulfate heptahydrate	7446-20-0	$ZnSO_4 \cdot 7H_2O$	287.56		S		

The mole fraction purities are exclusive of the amounts of water in the samples.
[a] Referred to as DAHP in the text.
[b] Prepared *in situ* (see Experimental).
[c] EC 4.1.2.15; referred to as DAHP synthase in text.
[d] Prepared for this study (see Experimental).
[e] EC 4.2.1.10.
[f] EC 4.6.1.3.
[g] Referred to as Hepes in text.
[h] Referred to as NAD_{ox} in text.

0.0014. Glycerol was present in both preparations (volume fraction $\phi = 0.50$). The 3-dehydroquinate synthase and 3-dehydroquinate dehyratase were stored, respectively, at $T \approx 253\,\mathrm{K}$ and $T \approx 193\,\mathrm{K}$. Prior to use the 3-dehydroquinate synthase underwent three exchanges ($\phi = 10$) with Hepes buffer $\{c = 0.098\,\mathrm{mol \cdot dm^{-3}}$, adjusted to pH = 7.65 with NaOH(aq)$\}$ by using a Millipore Ultrafree Centrifugal Filter {volume $V = 15\,\mathrm{cm^3}$, relative molecular mass cut-off = 5000, centrifugation at $1400 \cdot g_n$ ($g_n = 9.80665\,\mathrm{m \cdot s^{-2}}$) and at $T = 277\,\mathrm{K}$}.

CHROMATOGRAPHY

Quantitative analysis of phospho*enol*pyruvate, NAD_{ox}, D-erythrose 4-phosphate, DAHP, and 3-dehydroquinate was done by using a Dionex DX 500 Ion Chromatograph with an ED50 conductivity detector (cell set at $T = 308\,\mathrm{K}$; temperature compensation $= 1.7\,\mathrm{K^{-1}}$; self-regenerating suppressor current $i = 300\,\mathrm{mA}$) and a Dionex AS11 anion exchange separation column (4 mm i.d., 250 mm long) with an AG11 guard column (4 mm i.d., 50 mm long). Both columns were thermostatted at $T = 308\,\mathrm{K}$. The mobile phases were (I) water and (II) aqueous KOH generated by a Dionex EG-40 eluant generator and an EGC-KOH cartridge. The following gradient of these two mobile phases was formed: volume fraction $\phi(\mathrm{I}) = 0.90$ and $\phi(\mathrm{II}) = 0.10$ at time $t = 0$; $\phi(\mathrm{I}) = 0.90$ and $\phi(\mathrm{II}) = 0.10$ at $t = 1\,\mathrm{min}$; $\phi(\mathrm{I}) = 0.80$ and $\phi(\mathrm{II}) = 0.20$ at $t = 10\,\mathrm{min}$; $\phi(\mathrm{I}) = 0.20$ and $\phi(\mathrm{II}) = 0.80$ at $t = 17\,\mathrm{min}$; $\phi(\mathrm{I}) = 0.20$ and $\phi(\mathrm{II}) = 0.80$ at $t = 19\,\mathrm{min}$; $\phi(\mathrm{I}) = 0$ and $\phi(\mathrm{II}) = 1.00$ at $t = 20\,\mathrm{min}$. The flow rate was $0.01667\,\mathrm{cm^3 \cdot s^{-1}}$. Approximate retention times were 1.9 min for 3-dehydroquinate, 2.3 min for chloride, 3.5 min for NAD_{ox}, 4.7 min for sulfate, 4.8 min for D-erythrose 4-phosphate, 8.8 min for phosphate, 11.1 min for DAHP, and 14.0 min for phospho*enol*pyruvate.

Since 3-dehydroshikimate could not be detected on the Dionex DX 500 Ion Chromatograph, another chromatographic method was used for the analysis of this substance. The chromatograph used was an Hewlett–Packard model 1100 h.p.l.c. equipped with a u.v. detector set at the wavelength $\lambda = 240\,\mathrm{nm}$ and a Zorbax Extend-C18 (4.6 mm i.d., 250 mm long) thermostatted at $T = 308\,\mathrm{K}$. The mobile phases were (III) KH_2PO_4 ($c = 0.05\,\mathrm{mol \cdot dm^{-3}}$, pH = 4.9) and (IV) methanol. The following gradient of these two mobile phases was formed: $\phi(\mathrm{III}) = 0.95$ and $\phi(\mathrm{IV}) = 0.05$ at $t = 0$; $\phi(\mathrm{III}) = 0.90$ and $\phi(\mathrm{IV}) = 0.10$ at $t = 10\,\mathrm{min}$. The flow rate was $0.0133\,\mathrm{cm^3 \cdot s^{-1}}$. The retention time of 3-dehydroshikimate was 3.4 min.

Interferences with both the 3-dehydroquinate and 3-dehydroshikimate chromatographic peaks were observed upon injection of the *in situ* prepared DAHP solution into the respective chromatographs. These interferences necessitated appropriate corrections which were made by subtracting the values of the interference peak areas A_{int} from the respective total peak areas A_{total} for 3-dehydroquinate and 3-dehydroshikimate. The values of A_{int} were found, in the worst case, to be reproducible to within $\pm 0.07 \cdot A_{int}$ (two estimated standard deviations of the mean). Since, the corrections for these interferences were $\approx 0.14 \cdot A_{total}$ and $\approx 0.40 \cdot A_{total}$ for 3-dehydroquinate and 3-dehydroshikimate, respectively, this correction represents the greatest source of uncertainty in both the calorimetric and equilibrium measurements performed on reaction (3).

The chromatographic response factor of DAHP is based on its concentration which is known from its *in situ* preparation. Since the conversion of DAHP to 3-dehydroquinate via reaction (2) was found to be complete, the response factor of 3-dehydroquinate is based on the stoichiometry of this conversion and the known initial concentration of DAHP in the reaction mixture. Since there was also a spontaneous conversion of 3-dehydroquinate to 3-dehydroshikimate (see below), the peak areas corresponding to 3-dehydroquinate were extrapolated to $t = 0$ to obtain the value of the peak area corresponding to 3-dehydroquinate prior to the formation of any 3-dehydroshikimate. The response factor of 3-dehydroshikimate was obtained by following its formation with the Hewlett–Packard 1100 h.p.l.c. and, at the same time, the loss of 3-dehydroquinate was monitored by using the Dionex chromatograph. The amount of 3-dehydroshikimate formed, and subsequently its response factor, was obtained by using the chromatographic results together with the stoichiometry of reaction (3). Because of the aforementioned interferences as well as the other factors inherent in the chromatography, we judge the chromatographic analyses for the molalities m of 3-dehydroquinate and of 3-dehydroshikimate to be reliable to $\approx \pm 0.10 \cdot m$.

DEGRADATION STUDIES

The possibility of spontaneous degradation of several key substances is of consequence to this study. Therefore, studies of the instabilities of the pertinent substances were performed by monitoring the change with time of the chromatographic peak areas corresponding to these substances. All studies were performed at $T = 298.15\,\mathrm{K}$. The instability of PEP had been studied previously[1] using the buffer {tris(hydroxymethyl)aminomethane ($m = 0.0982\,\mathrm{mol \cdot kg^{-1}}$) adjusted with HCl(aq) to pH = 8.21} and the mole fraction change with time $\dot{x}$ was found to be $\approx 0.0005\,\mathrm{h^{-1}}$. Similar studies on the other substances gave the following results: DAHP with the buffer {tris(hydroxymethyl)aminomethane ($m = 0.105\,\mathrm{mol \cdot kg^{-1}}$) adjusted with HCl(aq) to pH = 8.20}, $\dot{x} \approx 0.0007$; 3-dehydroquinate with the buffer {Hepes ($m = 0.098\,\mathrm{mol \cdot kg^{-1}}$) adjusted to pH = 7.65 with NaOH(aq)}, $\dot{x} = 0.028\,\mathrm{h^{-1}}$; and 3-dehydroshikimate with the buffer {Hepes ($m = 0.098\,\mathrm{mol \cdot kg^{-1}}$) adjusted to pH = 7.65 with NaOH(aq)}, $\dot{x} \approx 0.0005\,\mathrm{h^{-1}}$. These instability studies were performed for periods of time ranging from 3 h to 6 h. The 3-dehydroquinate used was the sample synthesized for this study. The 3-dehydroshikimate was prepared *in situ* by adding 3-dehydroquinate dehydratase to the synthesized sample of 3-dehydroquinate. After 45 min of reaction, the 3-dehydroquinate dehydratase was removed by centrifugation (Millipore Ultrafree Centrifugal Filter, $V = 4\,\mathrm{cm^3}$, relative molecular mass cut-off = 5000, centrifugation at $2860 \cdot g_n$ and at $T = 277\,\mathrm{K}$) and the degradation of the 3-dehydroshikimate that had been formed was studied. It is noted that changes in mole fraction $\dot{x} < \approx 0.007\,\mathrm{h^{-1}}$ are at or near the lower limit of detection of the chromatographic measurements for these substances and, for our purpose, can be taken equal to zero. Thus, 3-dehydroquinate is the only substance for which instability is an issue. The product of its spontaneous degradation is believed to be 3-dehydroshikimate based upon the fact that the retention time of the degradation product formed was identical to the retention time of the 3-dehydroshikimate that was formed following the addition of 3-dehydroquinate dehydratase to 3-dehydroquinate.

EQUILIBRIUM MEASUREMENTS

An attempt to measure the apparent equilibrium constant of reaction (2) at $T = 298.15\,\mathrm{K}$ was made by using the first of the chromatographic methods described above (Dionex DX 500 Ion Chromatograph). The reaction mixture used for the forward direction of reaction consisted of {DAHP ($m = 0.0028\,\mathrm{mol \cdot kg^{-1}}$) in Hepes buffer ($m = 0.098\,\mathrm{mol \cdot kg^{-1}}$)} at pH = 7.46. The reaction mixture used for the reverse direction of reaction consisted of [3-dehydroquinate ($m = 0.0054\,\mathrm{mol \cdot kg^{-1}}$) + ($K_2HPO_4 + H_3PO_4$) {$m$(total phosphate) = $0.0084\,\mathrm{mol \cdot kg^{-1}}$} + Bis–Tris propane ($m = 0.052\,\mathrm{mol \cdot kg^{-1}}$)] at pH = 7.76. The mass fraction of 3-dehydroquinate synthase in the respective reaction mixtures was ≈ 0.0003. The forward and reverse reactions were allowed to proceed at $T = 298.15\,\mathrm{K}$ for 1 h and 24 h, respectively.

The position of equilibrium of reaction (3) was studied by starting with a solution of *in situ* prepared DAHP and by adding to it 3-dehydroquinate synthase and the cofactors NAD_{ox} and $ZnSO_4$. Following ≈ 60 min of reaction at $T = 298.15\,\mathrm{K}$, three injections of the reaction mixture were made into the Dionex chromatograph. These measurements showed the complete conversion of DAHP to 3-dehydroquinate and also allowed for the determination of the chromatographic peak area corresponding to 3-dehydroquinate. The enzyme 3-dehydroquinate dehydratase was then added to this reaction mixture and reaction (3) was allowed to proceed for ≈ 50 min. The molalities of 3-dehydroquinate and of 3-dehydroshikimate were measured by using the chromatographic methods described above. The results permitted the calculation the apparent reaction quotient Q' for reaction (3). In the absence of a sample of 3-dehydroshikimate to carry out reaction (3) from the "reverse" direction of reaction, we adopted an alternative tactic of testing whether or not equilibrium had been achieved. This was done by dividing the final reaction mixture into two portions and adding additional 3-dehydroquinate dehydratase to one portion and additional DAHP (*in situ* prepared) to the other portion. Approximately 60 min of equilibration time was allowed for both the "enzyme addition" and for the "substrate addition" experiments. All equilibrations were carried out using gentle lateral shaking (≈ 60 shakes $\mathrm{min^{-1}}$) in a thermostat set at $T = 298.15\,\mathrm{K}$. This alternative tactic looks to see if the value of Q' changes due to these additions. The absence of change (within the experimental uncertainties) in the value of Q', while not as conclusive as results obtained from both directions of reaction, is still good evidence that equilibrium has been achieved.

MICROCALORIMETRY

Descriptions of the microcalorimeters used in this study and their performance characteristics, the data acquisition system, and the computer programs used to treat the results have been given by Steckler *et al.*[17,18] 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 Hewlett–Packard 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 highdensity polyethylene. Each vessel had two compartments that held, respectively, $\approx 0.55\,cm^3$ and $\approx 0.40\,cm^3$ 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. For reaction (2), the substrate solution was the *in situ* prepared DAHP and the enzyme solution was DHQ synthase that had been subject to a buffer exchange with the same Hepes buffer used in the preparation of DAHP. For reaction (4), the substrate solution again was *in situ* prepared DAHP and the enzyme solution consisted of the two enzymes DHQ synthase and DHQ dehydratase. Catalytic concentrations of $ZnSO_4$ and NAD_{ox} were present as cofactors in both reactions.

The vessels and their contents were allowed to thermally equilibrate in the microcalorimeters for ≈ 60 min before the enzyme and substrate solutions were mixed. After mixing, ≈ 62 min was allowed for reaction (2) and ≈ 26 min for reaction (4) (note: the mass fraction of 3-dehydroquinate dehydratase used to carry out reaction (4) was twice that used for reaction (2)). Following the respective reactions, the vessels were removed from the microcalorimeters and the chromatographs were then used for the analysis of the reaction mixtures. In the study of reaction (2), it was found that the mole fraction of 3-dehydroquinate converted to 3-dehydroshikimate was 0.0063. Consequently, the correction to the measured enthalpy change ΔH for this small amount of reaction was negligible ($< 0.0005 \cdot \Delta H$). In all cases, it was found that the mole fraction of unreacted DAHP following either reactions (2) or reaction (4) was < 0.001.

"Blank" enthalpy changes $\Delta_{mix}H$ were determined in control experiments. Thus, for mixing of the substrate solution {very similar substrate solutions were used for both reactions (2) and (4)} with the buffer, $\Delta_{mix}H$ was $-(7.8 \pm 1.2)$ mJ. For the mixing of the enzyme solutions with the buffer, $\Delta_{mix}H$ was (0.2 ± 0.7) mJ for reaction (2) and $-(11.3 \pm 0.2)$ mJ for reaction (4). These values of $\Delta_{mix}H$ were applied as corrections to the measured enthalpies ΔH which were in the range $-(99$ to $133)$ mJ for reaction (2) and $(-118$ to $124)$ mJ for reaction (4). We judge the total correction applied for the blank enthalpy changes to be uncertain by $\approx \pm 1.5$ mJ. This leads to uncertainties of $\approx 0.013 \cdot \Delta_r H_m(\text{cal})$ in the final results, where $\Delta_r H_m(\text{cal})$ is the calorimetrically determined molar enthalpy of reaction pertinent to the actual experimental conditions.

Measurement of pH was done with an Orion Model 811 pH meter and a Radiometer combination glass microelectrode. The pH meter was calibrated with Radiometer standard buffers (pH = 7.00 and 9.18) that bracketed the pHs of the solutions used in this study. The electric potential differences U were recorded and pH values were calculated by interpolation.

3. Results and discussion

THERMODYNAMIC FORMALISM

The apparent equilibrium constants for reactions (2) and (3), respectively, are

$$K' = m(\text{3-dehydroquinate}) \cdot m(\text{phosphate}) / \{m(\text{DAHP}) \cdot m^\circ\}, \qquad (5)$$

$$K' = m(\text{3-dehydroshikimate})/m(\text{3-dehydroquinate}). \quad (6)$$

The molalities m in the above equations are the total molalities of the various charged and uncharged species that are formed from the dissociation of the biochemical substances in solution. By convention,[19] the water has been omitted in the expressions for the apparent equilibrium constants for reaction (3). Following our usual practice, we have selected chemical reference reactions that involve the predominant species present under the experimental conditions used in our study. Thus, the selected reference reactions are

$$\text{DAHP}^{3-}(\text{aq}) = \text{3-dehydroquinate}^{-}(\text{aq}) + \text{HPO}_4^{2-}(\text{aq}), \quad (7)$$

$$\text{3-dehydroquinate}^{-}(\text{aq}) = \text{3-dehydroshikimate}^{-}(\text{aq}) + \text{H}_2\text{O}(\text{l}). \quad (8)$$

The respective equilibrium constants for these reference reactions are

$$K = m(\text{3-dehydroquinate}^{-}) \cdot m(\text{HPO}_4^{2-})/m(\text{DAHP}^{3-}), \quad (9)$$

$$K = m(\text{3-dehydroshikimate}^{-})/m(\text{3-dehydroquinate}^{-}). \quad (10)$$

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 of the solvent is the pure solvent; the standard pressure $p^\circ = 0.1\,\text{MPa}$.

RESULTS OF EXPERIMENTS

The result of the equilibrium measurement performed on reaction (2) from the forward direction of reaction was that there was no measurable amount of DAHP left in the reaction mixture. Similarly, DAHP could not be detected in the experiment performed starting from the reverse direction of reaction. Using the limit of detection of DAHP of $\approx 3 \cdot 10^{-6}\,\text{mol} \cdot \text{kg}^{-1}$, we obtain a lower limit for the value of the apparent equilibrium constant, $K' > 15$ at $T = 298.15\,\text{K}$ and pH = 7.46 to 7.76 . The results of the equilibrium measurements performed on reaction (3) are given in table 2. It should be noted that, because a sample of 3-dehydroshikimate was not available, three different types of experiments were carried out to test if equilibrium had been achieved (see Experimental). It is seen that the value of the reaction quotient Q' obtained on adding additional 3-dehydroquinate dehydratase led to a value of Q' slightly higher but still in agreement with the value of Q' obtained on the original reaction mixture. However, the value of Q' obtained by addition of DAHP to the reaction mixture led to a slightly lower value of Q', i.e., the difference between the Q' values is slightly larger than the combined uncertainties. While it is possible that the 3-dehydroquinate dehydratase may have lost some of its activity, one cannot completely eliminate the possibility that a true equilibrium may not have been achieved. This fact will be reflected in the final uncertainty that is assigned to the value of K'.

The results of the calorimetric measurements are given in tables 3 and 4. Since, in the measurements performed on reaction (2), a small fraction ($x = 0.0063$) of the 3-dehydroquinate was converted to 3-dehydroshikimate, it was necessary to make a correction

TABLE 2. Results of equilibrium measurements for reaction (3), 3-dehydroquinate(aq) = 3-dehydroshikimate(aq) + H_2O(l), at $T = 298.15$ K, pH = 7.50, and a calculated ionic strength $I_m = 0.065\,\text{mol} \cdot \text{kg}^{-1}$

Type of experiment	m(Hepes) / mol · kg^{-1}	m(NaOH) / mol · kg^{-1}	m(glycerol) / mol · kg^{-1}	$10^3 \cdot m$(phosphate) / mol · kg^{-1}	$10^3 \cdot m(\text{NAD}_{ox})$ / mol · kg^{-1}	$10^3 \cdot m(\text{ZnSO}_4)$ / mol · kg^{-1}	$10^3 \cdot m$(DHQ) / mol · kg^{-1}	$10^3 \cdot m$(DHS) / mol · kg^{-1}	Q'
F	0.0980	0.0603	0.304	2.30	0.192	0.328	0.168	0.803	4.8 ± 0.5
EA	0.0903	0.0556	0.727	5.12	0.177	0.302	0.142	0.749	5.3 ± 0.8
SA	0.0981	0.0604	0.289	2.30	0.182	0.311	0.246	0.904	3.7 ± 0.4
$K' = \langle Q' \rangle = 4.6 \pm 1.0$									

The molalities m in columns 2 to 9 are those measured after equilibration times of 50 to 60 min. All molalities are equal to the sums of the molalities of the indicated substances in their various ionic forms. The three different types of equilibrium measurements described in the text (see Experimental) are: F, the measurement from the forward direction of reaction; EA, the experiment involving the addition of 3-dehydroquinate dehydratase to the reaction mixture; and SA, the experiment involving the addition of DAHP to the reaction mixture. Abbreviations used in this table are: Hepes, N-(2-hydroxyethyl)piperazine-N'-2-ethanesulfonic acid; NAD_{ox}, oxidized form of β-nicotinamide-adenine dinucleotide; DHQ, 3-dehydroquinate; and DHS, 3-dehydroshikimate. The mass fractions w of DAHP synthase, 3-dehydroquinate synthase, and 3-dehydroquinate dehydratase in the reaction mixtures were $\approx 1 \cdot 10^{-5}$, $\approx 2 \cdot 10^{-4}$, and $\approx 1 \cdot 10^{-4}$, respectively. Additional substances were also present in the reaction mixtures at the indicated molalities: Bis–Tris propane ($m \approx 3 \cdot 10^{-5}\,\text{mol} \cdot \text{kg}^{-1}$); DL-dithiothreitol ($m \approx 5 \cdot 10^{-5}\,\text{mol} \cdot \text{kg}^{-1}$); D-erythrose 4-phosphate ($m \approx 2 \cdot 10^{-3}\,\text{mol} \cdot \text{kg}^{-1}$); and $MnCl_2$ ($m \approx 2 \cdot 10^{-4}\,\text{mol} \cdot \text{kg}^{-1}$). The values of the reaction quotient Q' for reaction (3) are given in column 10. The value of the apparent equilibrium constant K' for reaction (3) is the average of the values of Q'. The uncertainties are equal to two estimated standard deviations of the mean.

TABLE 3. Results of the calorimetric measurements for reaction (2), DAHP(aq) = 3-dehydroquinate(aq) + phosphate(aq), at $T = 298.15\,\mathrm{K}$. pH = 7.46, pMn = $-\lg[m(\mathrm{Mn}^{2+})] = 3.30$, and ionic strength $I_\mathrm{m} = 0.070\,\mathrm{mol \cdot kg^{-1}}$

Experiment	m(Hepes)	m(NaOH)	$10^3 \cdot m$(E4P)	$10^3 \cdot m(\mathrm{NAD_{ox}})$	$10^3 \cdot m(\mathrm{ZnSO_4})$	$10^3 \cdot m$(DAHP)	$\Delta_\mathrm{r}H_\mathrm{m}$(cal)
	$\mathrm{mol \cdot kg^{-1}}$	$\mathrm{mol \cdot kg^{-1}}$	$\mathrm{mol \cdot kg^{-1}}$	$\mathrm{mol \cdot kg^{-1}}$	$\mathrm{mol \cdot kg^{-1}}$	$\mathrm{mol \cdot kg^{-1}}$	$\mathrm{kJ \cdot mol^{-1}}$
1	0.0998	0.0614	3.76	0.015	0.018	3.128	−50.6
2	0.0998	0.0614	3.41	0.017	0.021	2.840	−51.2
3	0.0998	0.0614	3.84	0.013	0.016	3.193	−50.7
4	0.0998	0.0614	3.38	0.016	0.020	2.812	−50.5
5	0.0998	0.0614	3.69	0.018	0.022	3.071	−50.8
6	0.0998	0.0614	3.49	0.014	0.017	2.901	−50.7
7	0.0999	0.0614	3.21	0.016	0.019	2.673	−51.9

$\langle \Delta_\mathrm{r}H_\mathrm{m}(\mathrm{cal}) \rangle = -(50.9 \pm 0.4)\ \mathrm{kJ \cdot mol^{-1}}$

The values of pMn and I_m are calculated. The molalities m in columns 2 to 7 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: Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; E4P, D-erythrose 4-phosphate; $\mathrm{NAD_{ox}}$, oxidized form of β-nicotinamide-adenine dinucleotide; and DAHP, 2-dehydro-3-deoxy-D-*arabino*heptanoate 7-phosphate. The solution also contained some Bis–Tris propane ($m \approx 8 \cdot 10^{-5}\,\mathrm{mol \cdot kg^{-1}}$) and $\mathrm{MnCl_2}$ ($m \approx 6 \cdot 10^{-4}\,\mathrm{mol \cdot kg^{-1}}$). The mass fractions w of DAHP synthase and 3-dehydroquinate synthase were $\approx 2 \cdot 10^{-5}$ and $\approx 4 \cdot 10^{-4}$, respectively. $\Delta_\mathrm{r}H_\mathrm{m}$(cal) is the calorimetrically determined molar enthalpy of reaction (2). The uncertainty in the average value of $\Delta_\mathrm{r}H_\mathrm{m}$(cal) is equal to two estimated standard deviations of the mean.

TABLE 4. Results of the calorimetric measurements for reaction (4), α_2 {DAHP(aq) = 3-dehydroquinate(aq) + phosphate(aq) + H_2O(l)} + α_3 {3-dehydroquinate(aq) = 3-dehydroshikimate(aq) + H_2O(l)}, at $T = 298.15$ K, pH = 7.42, and ionic strength $I_m = 0.069\,mol \cdot kg^{-1}$

Experiment	m(Hepes)	m(NaOH)	m(glycerol)	$10^3 \cdot m$(E4P)	$10^3 \cdot m(NAD_{ox})$	$10^3 \cdot m(ZnSO_4)$	$10^3 \cdot m$(DAHP)	$\Delta_r H$	$\Delta_r H_m$(cal)
	$mol \cdot kg^{-1}$	$mol \cdot kg^{-1}$	$mol \cdot kg^{-1}$	$mol \cdot kg^{-1}$	$mol \cdot kg^{-1}$	$mol \cdot kg^{-1}$	$mol \cdot kg^{-1}$	J	$kJ \cdot mol^{-1}$
1	0.0978	0.0602	0.277	3.40	0.168	0.206	2.828	−0.118	0.3
2	0.0979	0.0602	0.255	3.59	0.155	0.190	2.986	−0.117	3.0
3	0.0979	0.0602	0.261	3.53	0.159	0.194	2.942	−0.122	2.1
4	0.0978	0.0602	0.270	3.46	0.164	0.201	2.879	−0.121	2.2
5	0.0980	0.0603	0.243	3.70	0.147	0.181	3.080	−0.124	3.7
6	0.0978	0.0602	0.274	3.42	0.167	0.204	2.847	−0.119	2.2
$\langle \Delta_r H_m(cal) \rangle = (2.3 \pm 0.9)\ kJ \cdot mol^{-1}$									

The molalities in columns 2 to 8 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: Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; E4P, D-erythrose 4-phosphate; NAD_{ox}, oxidized form of β-nicotinamide-adenine dinucleotide; and DAHP, 2-dehydro-3-deoxy-D-*arabino*heptanoate 7-phosphate. The solution also contained some Bis–Tris propane ($m \approx 0.000072\,mol \cdot kg^{-1}$), $MnCl_2$ ($m \approx 0.00058\,mol \cdot kg^{-1}$), and KH_2PO_4 ($m \approx 0.0011\,mol \cdot kg^{-1}$). The mass fractions w of DAHP synthase, 3-dehydroquinate synthase, and 3-dehydroquinate dehydratase were ≈ 0.00002, ≈ 0.0003, and ≈ 0.00006, respectively. The quantities $\Delta_r H$ and $\Delta_r H_m$(cal) are, respectively, the measured enthalpy of reaction and the calorimetrically determined molar enthalpy of reaction (3) {see Section 3 for the basis of the calculation of $\Delta_r H_m$(cal)}. The extents of reactions (2) and (3) are, respectively, $\xi_2 = \alpha_2 \cdot m$(DAHP) and $\xi_3 = \alpha_3 \cdot m$(DAHP), where m(DAHP) is the initial molality of DAHP (column 8). The fractions α of reactions (2) and (3) were found by using h.p.l.c. to be $\alpha_2 = 1.000$ and $\alpha_3 = 0.734$. The uncertainty in the average value of $\Delta_r H_m$(cal) is equal to two estimated standard deviations of the mean.

for the enthalpy change brought about by this reaction. This adjustment to the measured enthalpy change ΔH was made by using a preliminary value of $\Delta_r H_m(\text{cal})(3)$ obtained from the calorimetric study of reaction (4). The quantity $\Delta_r H_m(\text{cal})(3)$ is the calorimetrically determined molar enthalpy change for reaction (3); it includes the enthalpy of protonation of the buffer.[20] The aforementioned adjustment for conversion of 3-dehydroquinate to 3-dehydroshikimate was found to be negligible ($< 0.0005 \cdot \Delta H$) and subsequent refinements of the value of $\Delta_r H_m(\text{cal})(3)$ did not significantly change the value of $\Delta_r H_m(\text{cal})(2)$. For the experiments performed on reaction (4), the measured enthalpy change is given by

$$\Delta H = \xi_2 \cdot \Delta_r H_m(\text{cal})(2) + \xi_3 \Delta_r H_m(\text{cal})(3). \tag{11}$$

Here, ξ_2 and ξ_3 are the respective extents of reactions (2) and (3). The quantities $\Delta_r H_m(\text{cal})(2)$ and $\Delta_r H_m(\text{cal})(3)$ are the respective calorimetric molar enthalpies of reaction for these two reactions under the actual conditions of reaction. Since ξ_2, ξ_3, and ΔH were measured and since $\Delta_r H_m(\text{cal})(2)$ was already known, $\Delta_r H_m(\text{cal})(3)$ could then be calculated. In summary, the experimentally determined values are: $\Delta_r H_m(\text{cal})(2) = -(50.9 \pm 0.4)\ \text{kJ} \cdot \text{mol}^{-1}$ (Hepes buffer, $T = 298.15\,\text{K}$, $\text{pH} = 7.46$, $I_m = 0.070\,\text{mol} \cdot \text{kg}^{-1}$); $K' = (4.6 \pm 1.0)$ for reaction (3) (Hepes buffer, $T = 298.15\,\text{K}$, $\text{pH} = 7.50$, $I_m = 0.065\,\text{mol} \cdot \text{kg}^{-1}$); and $\Delta_r H_m(\text{cal})(3) = (2.3 \pm 0.9)\ \text{kJ} \cdot \text{mol}^{-1}$ (Hepes buffer, $T = 298.15\,\text{K}$, $\text{pH} = 7.42$, $I_m = 0.069\,\text{mol} \cdot \text{kg}^{-1}$). The value of $\Delta_r H_m(\text{cal})(3)$ given here was calculated {equation (11)} by using the value $\Delta_r H_m(\text{cal})(2) = -50.8\,\text{kJ} \cdot \text{mol}^{-1}$ which pertains to the same conditions of measurement used for the study of reaction (4). This value is based on an adjustment of the experimentally determined value of $\Delta_r H_m(\text{cal})(2)$ to the aforementioned conditions of reaction and was done with our equilibrium model (see below).

The uncertainties in the measured values of $\Delta_r H_m(\text{cal})$ and K' represent only the random errors inherent in the measurements and do not reflect possible systematic errors which are now considered. We judge that reasonable estimates of the standard uncertainties[21] in the measured values of $\Delta_r H_m(\text{cal})$ for reaction (2) are: $0.006 \cdot \Delta_r H_m(\text{cal})$ due to possible impurities in the sample of phosphoenolpyruvate; $0.008 \cdot \Delta_r H_m(\text{cal})$ due to possible errors in the calorimetric measurements; $0.002 \cdot \Delta_r H_m(\text{cal})$ due to possible errors in the extent of reaction; and $< 0.001 \cdot \Delta_r H_m(\text{cal})$ due to a possible error in the correction for reaction (3). For the results leading to $\Delta_r H_m(\text{cal})$ for reaction (3) the estimates of possible systematic error are: $0.18 \cdot \Delta_r H_m(\text{cal})$ due to possible impurities in the sample of phospho*enol*pyruvate; $0.27 \cdot \Delta_r H_m(\text{cal})$ due to possible errors in the calorimetric measurements; and $0.34 \cdot \Delta_r H_m(\text{cal})$ due to possible errors in the measured extents of reaction and in the enthalpy correction for reaction (2). These rather large possible fractional errors reflect the fact that the absolute value of $\Delta_r H_m(\text{cal})$ for reaction (3) is small and therefore any uncertainties represent a substantial fraction of its value. The estimates of possible systematic error in the value of K' for reaction (3) are: $0.05 \cdot K'$ due to possible chromatographic error; and $0.10 \cdot K'$ due to the possibility that equilibrium may not have been achieved. These estimates of possible systematic error are combined in quadrature together with the statistical uncertainties in the measured values of $\Delta_r H_m(\text{cal})$, expressed as one estimated standard deviations of the mean, to obtain

combined standard uncertainties.[21] These combined standard uncertainties are then multiplied by two to arrive at the final results all at $T = 298.15\,\mathrm{K}$: $\Delta_r H_m(\mathrm{cal}) = -(50.9 \pm 1.1)\,\mathrm{kJ \cdot mol^{-1}}$ for reaction (2) (Hepes buffer, pH = 7.46, $I_m = 0.070\,\mathrm{mol \cdot kg^{-1}}$); $\Delta_r H_m(\mathrm{cal}) = (2.3 \pm 2.3)\ \mathrm{kJ \cdot mol^{-1}}$ for reaction (3) (Hepes buffer, pH = 7.42, $I_m = 0.069\,\mathrm{mol \cdot kg^{-1}}$); and $K' = (4.6 \pm 1.5)$ for reaction (3) (Hepes buffer, pH = 7.50, $I_m = 0.065\,\mathrm{mol \cdot kg^{-1}}$).

EQUILIBRIUM MODEL

The pKs and standard molar enthalpies for the H^+ dissociation reactions of the reactants and products are needed to relate the experimental results for reactions (2) and (3) to thermodynamic quantities for the reference reactions (8) and (9). The values selected for this purpose are given in table 5. The pKs and standard molar enthalpies $\Delta_r H_m^\circ$ for $DAHP^{2-}(aq)$, $DAHP \cdot Mn^-(aq)$, D-erythrose 4-phosphate$^-$(aq), D-erythrose 4-phosphate·Mn(aq), and $MnHPO_4(aq)$ are previously used estimates[1] that are based on structurally similar substances. Similarly, in the absence of experimental results for 3-dehydroquinate(aq) and 3-dehydroshikimate(aq) we have estimated their pK and $\Delta_r H_m^\circ$ values by using critically selected values[22] of these quantities for the structurally similar substances (*S*)-malic acid and fumaric acid, respectively. The values of pK and $\Delta_r H_m^\circ$ for $H_2PO_4^-(aq)$ and Hepes$^\pm$(aq) are from a recent review.[23]

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 $\Delta_r H_m(\mathrm{cal})$ has been described.[24,25] 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''

TABLE 5. The pKs and standard molar enthalpy changes $\Delta_r H^\circ_m$ at $T = 298.15\,\mathrm{K}$ and $I = 0$ for the aqueous ionization reactions of substances pertinent to this study and to the analysis of results from the literature

Reaction	pK	$\Delta_r H_m^\circ/(\mathrm{kJ \cdot mol^{-1}})$
$DAHP^{2-} = DAHP^{3-} + H^+$	6.8[a]	−17[a]
$DAHP \cdot Mn^- = DAHP^{3-} + Mn^{2+}$	1.3[a]	−21[a]
3-Dehydroquinate = 3-dehydroquinate$^-$ + H^+	5.1[a]	−1[a]
3-Dehydroshikimate = 3-dehydroshikimate$^-$ + H^+	4.5[a]	−3[a]
D-Erythrose 4-phosphate$^-$ = D-erythrose 4-phosphate^{2-} + H^+	6.7[a]	−11[a]
D-Erythrose 4-phosphate · Mn = D-erythrose 4-phosphate + Mn^{2+}	1.3[a]	−20[a]
$H_2PO_4^- = HPO_4^{2-} + H^+$	7.198	3.6
$MnHPO_4 = HPO_4^{2-} + Mn^{2+}$	2.7	−12
Hepes$^\pm$ = H^+ + Hepes$^-$	7.564	20.4

Abbreviations used in this table are: DAHP, 2-dehydro-3-deoxy-D-*arabino*-heptanoate 7-phosphate; and Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid. See Results and discussion for the basis of these values.

[a] Estimated.

parameter has been set at $1.6\,kg^{1/2} \cdot mol^{-1/2}$. By using the equilibrium model with the experimental results for K' and $\Delta_r H_m(cal)$ and with the thermodynamic quantities given in table 5, we calculate: $\Delta_r H_m^\circ = -(51.1 \pm 1.1)\ kJ \cdot mol^{-1}$ for the reference reaction (7); $K = (4.6 \pm 1.5)$ and $\Delta_r H_m^\circ = (2.3 \pm 2.3)\,kJ \cdot mol^{-1}$ for the reference reaction (8). The equilibrium model is also used to calculate the changes in binding $\Delta_r N(H^+)$ of the hydrogen ion. Thus, for reaction (2) at $T = 298.15\,K$, pH = 7.46, and $I_m = 0.070\,mol \cdot kg^{-1}$, $\Delta_r N(H^+) = 0.150$. For reaction (3) at $T = 298.15\,K$, pH = 7.42, and $I_m = 0.069\ mol \cdot kg^{-1}$, $\Delta_r N(H^+) = -0.0029$. It should be noted that since the values of $\Delta_r N(H^+)$ are small, the buffer protonation corrections[20] are also small.

The uncertainties in the calculated values of K and $\Delta_r H_m^\circ$ are based on the respective uncertainties in the experimentally determined values of K' and $\Delta_r H_m(cal)$. However, there is also a component of uncertainty due to uncertainties in the parameters used in the equilibrium model. This latter component of uncertainty was examined by perturbing each of the pertinent quantities in the model with an assumed possible error. Thus, the pK values were perturbed as follows: ± 0.5 for $DAHP^{2-}(aq)$, $DAHP \cdot Mn^-(aq)$, 3-dehydroquinate(aq), 3-dehydroshikimate(aq), D-erythrose 4-phosphate$^-$(aq), and D-erythrose 4-phosphate·Mn(aq); ± 0.2 for $MnHPO_4(aq)$; and ± 0.003 for $HPO_4^{2-}(aq)$ and Hepes(aq). The values of $\Delta_r H_m^\circ/(kJ \cdot mol^{-1})$ for the dissociation reactions were also perturbed as follows: ± 5 for $DAHP^{2-}(aq)$, $DAHP \cdot Mn^-(aq)$, 3-dehydroquinate(aq), 3-dehydroshikimate(aq), D-erythrose 4-phosphate(aq), and D-erythrose 4-phosphate $\cdot$ Mn(aq); ± 2 for $MnHPO_4(aq)$; and ± 0.5 for Hepes(aq) and $HPO_4^{2-}(aq)$. The "ion-size" parameter used in the activity coefficient model was also perturbed by $\pm 0.3\,kg^{1/2}\,mol^{-1/2}$. The final uncertainties in the values of the thermodynamic quantities for the reference reactions were obtained by combining, in quadrature, the experimental uncertainties with the estimated uncertainties attributable to the model. Thus, the results with expanded uncertainties are: $\Delta_r H_m^\circ = -(51.1 \pm 4.5)\ kJ \cdot mol^{-1}$ for reaction (7); and $K = (4.6 \pm 1.5)$ and $\Delta_r H_m^\circ = (2.3 \pm 2.3)\,kJ \cdot mol^{-1}$ for reaction (8). The only difference is in the uncertainty in the value of $\Delta_r H_m^\circ$ for reaction (7) which has been very significantly increased because of the uncertainty in the estimated pK value of DAHP. Should this pK value become known, the present results could be recalculated to yield a more reliable value of $\Delta_r H_m^\circ$ for reaction (7). These summary values are given in table 6 together with a value of $\Delta_r H_m^\circ$ for the reaction

$$DAHP^{3-}(aq) = 3\text{-dehydroshikimate}^-(aq) + HPO_4^{2-}(aq) + H_2O(l) \qquad (12)$$

which is equal to reaction (7) + reaction (8). Table 6 also contains values of the standard molar entropy changes $\Delta_r S_m^\circ$ and standard molar Gibbs free energy changes $\Delta_r G_m^\circ$ for these three reactions. Several of these values are based on an estimated value of $\Delta_r S_m^\circ$ for reaction (7). The basis of this estimate is given below.

COMPARISON WITH PREVIOUS RESULTS

There do not appear to be any thermodynamic results in the literature for reaction (2). For reaction (3), Mitsuhashi and Davis[14] report the value $K' = (15 \pm 1)$ at $T = 302.15\,K$ and pH = 7.4. However, examination of their paper indicates two possible difficulties.

TABLE 6. Equilibrium constants K, standard molar Gibbs free energy changes $\Delta_r G^\circ_m$, standard molar enthalpy changes $\Delta_r H^\circ_m$, and standard molar entropy changes $\Delta_r S^\circ_m$ at $T = 298.15\,\mathrm{K}$ and $I = 0$ for the reference reactions in aqueous solution that are pertinent to this study; see Section 3 for the basis of these values

Reaction		K	$\Delta_r G^\circ_m/(\mathrm{kJ \cdot mol^{-1}})$	$\Delta_r H^\circ_m/(\mathrm{kJ \cdot mol^{-1}})$	$\Delta_r S^\circ_m/(\mathrm{J \cdot K^{-1} \cdot mol^{-1}})$
$DAHP^{3-} = 3\text{-dehydroquinate}^- + HPO_4^{2-}$	(7)	$\approx 2 \cdot 10^{14}$[a]	≈ -81[a]	$-(51.1 \pm 4.5)$	≈ 101[a]
$3\text{-dehydroquinate}^- = 3\text{-dehydroshikimate}^- + H_2O$	(8)	4.6 ± 1.5	$-(3.8 \pm 0.8)$	2.3 ± 2.3	20 ± 8
$DAHP^{3-} = 3\text{-dehydroshikimate}^- + HPO_4^{2-} + H_2O$	(12)	$\approx 8 \cdot 10^{14}$[a]	≈ -85[a]	$-(48.8 \pm 5.1)$	≈ 121[a]

The standard state for the solutes is the hypothetical ideal solution of unit molality.

[a] Based on an estimated value of $\Delta_r S^\circ_m = 101\,\mathrm{J \cdot K^{-1} \cdot mol^{-1}}$ for reaction (7).

First, they[14] did not measure the concentration of 3-dehydroquinate. Therefore their results are based on the stoichiometry of reaction (3) and their analysis of the change in concentration of 3-dehydroshikimate. More significantly, their[14] figure 4 shows the change in absorbance (called optical density in their paper) of 3-dehydroshikimate at $\lambda = 234\,\text{nm}$ as a function of time beginning with the addition of 3-dehydroquinate dehydratase to the solution containing 3-dehydroshikimate. In this figure, one can see that the measured absorbance drops to approximately one half of its original value. If it is assumed that the concentration of 3-dehydroshikimate is proportional to the measured absorbance, one arrives at the value $K' = 1.0_2$ which is significantly different than the reported value $K' = 15$.

STRUCTURAL CONSIDERATIONS

Reaction (3) involves the conversion of a 2-hydroxy acid to the corresponding 2,3-unsaturated acid. Structurally similar changes occur in the following reactions:

$$(S)\text{-malate}^{2-}(\text{aq}) = \text{fumarate}^{2-}(\text{aq}) + \text{H}_2\text{O}(\text{l}), \tag{13}$$

$$\text{D-isocitrate}^{3-}(\text{aq}) = \text{cis-aconitate}^{3-}(\text{aq}) + \text{H}_2\text{O}(\text{l}). \tag{14}$$

For reaction (13) we have $K = 0.238$ based on the results of Gajewski *et al.*[26] and $K = 0.50$ for reaction (14) is calculated from Wilhoit's[27] table of formation properties. These values are comparable in magnitude with the value $K = (4.6 \pm 1.5)$ obtained in the present study and with the value $K = 1.0$ which we calculated from Mitsuhashi and Davis'[14] figure 4.

It is also interesting to compare the results obtained herein with values estimated by means of the Benson group-contribution method. To do this, we have used the thermochemical scheme shown in figure 2 and that involves two reactions:

$$\text{DAHP}^{3-}(\text{aq}) + \text{H}_2\text{O}(\text{l}) = \text{THTPCA}^{-}(\text{aq}) + \text{HPO}_4^{2-}(\text{aq}), \tag{15}$$

$$\text{THTPCA}^{-}(\text{aq}) = \text{3-dehydroquinate}^{-}(\text{aq}) + \text{H}_2\text{O}(\text{l}). \tag{16}$$

Here, THTPCA is an abbreviation for 2,4,5-trihydroxy-6-hydroxymethyl-tetrahydropyran-2-carboxylic acid. This substance is introduced solely as a means to performing the Benson calculation. Reaction (7) is equal to reaction (15) + reaction (16). We have estimated values of $\Delta_r H^\circ_m$ and $\Delta_r S^\circ_m$ for reaction (15) by using the values of these quantities obtained by Tewari *et al.*[28] for the structurally similar reaction

$$\text{D-glucose 6-phosphate}^{2-}(\text{aq}) + \text{H}_2\text{O}(\text{l}) = \text{D-glucose}(\text{aq}) + \text{HPO}_4^{2-}(\text{aq}). \tag{17}$$

The Benson method was used with the parameters tabulated by Domalski and Hearing[29] to estimate values of the standard molar enthalpy of formation $\Delta_f H^\circ_m$ and the standard molar entropy S°_m for THTPCA(l) and for 3-dehydroquinate(l). It was then assumed that the enthalpies of solution and ionization of THTPCA(l) and of 3-dehydroquinate(l) cancelled in the calculation of $\Delta_r H^\circ_m$ and $\Delta_r S^\circ_m$ for reaction (16). The needed values of

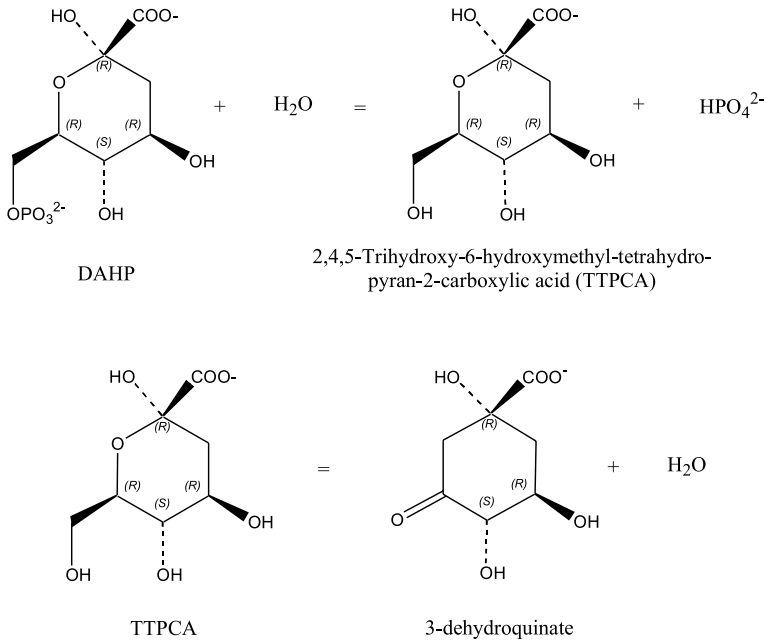

FIGURE 2. Structures of the substances used for the estimation of $\Delta_r H_m^\circ$ and $\Delta_r S_m^\circ$ for reaction (7) by using the Benson group-contribution method.

$\Delta_f H_m^\circ$ and S_m° for $H_2O(l)$ were taken from Cox *et al.*[30] Thus, the above estimation scheme leads to $\Delta_r H_m^\circ \approx -85\,\mathrm{kJ \cdot mol^{-1}}$ and $\Delta_r S_m^\circ \approx -101\,\mathrm{J \cdot K^{-1} \cdot mol^{-1}}$ for reaction (7). The difference between the predicted value and the experimental value $\Delta_r H_m^\circ = -(51.1 \pm 4.5)\ \mathrm{kJ \cdot mol^{-1}}$ is larger than one would hope for. However, it must be appreciated that some major assumptions were made in this calculation. Nevertheless, we use the estimated value of $\Delta_r S_m^\circ$ for reaction (7) together with the experimental value of $\Delta_r H_m^\circ$ to estimate a value of $\Delta_r G_m^\circ$ and then K for reaction (7). The approximate value $K \approx 2 \cdot 10^{12}$ is consistent with our experimental result that K' for reaction (2) is greater than 15.

APPARENT EQUILIBRIUM CONSTANTS UNDER APPROXIMATELY PHYSIOLOGICAL CONDITIONS

It is desirable to have values of the apparent equilibrium constants for the reactions considered herein under approximately physiological conditions which are taken to be[31] $T = 311.15\,\mathrm{K}$, pH = 7.0, and $I_m = 0.25\,\mathrm{mol \cdot kg^{-1}}$. By using the equilibrium model and with the values given in table 6 we calculate values of $K' \approx 3 \cdot 10^{13}$ and $K' = 4.8$ for reactions (2) and (3), respectively. For the biochemical reaction

$$\mathrm{DAHP(aq) = 3\text{-dehydroshikimate(aq) + phosphate(aq) + H_2O(l)}}, \qquad (18)$$

$K' \approx 1 \cdot 10^{14}$. The corresponding values of the standard molar transformed Gibbs free energy changes are $\Delta_r G'^{\circ}_m \approx (-80.3, -4.1, \text{ and } -84.4)\ \text{kJ} \cdot \text{mol}^{-1}$ for the overall biochemical reactions (2), (3), and (18), respectively.

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