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Thermodynamics of the conversion of penicillin G to phenylacetic acid and 6-aminopenicillanic acid

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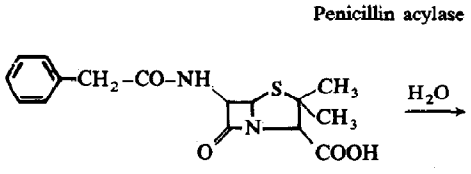
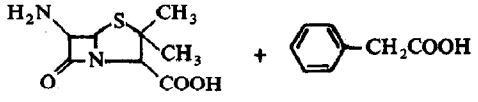
The thermodynamics of the enzymatic conversion (penicillin acylase) of aqueous penicillin G to phenylacetic acid and 6-aminopenicillanic acid have been studied using both high-pressure liquid-chromatography and microcalorimetry. The reaction was carried out in aqueous phosphate buffer over the pH range 6.0–7.6, at ionic strengths from 0.10 to 0.40 mol kg^{-1}, and at temperatures from 292 to 322 K. The data have been analyzed using a chemical equilibrium model with an extended Debye-Hückel expression for the activity coefficients. For the reference reaction, penicillin $G^-(aq) + H_2O(l)$ = phenylacetic $acid^-(aq) + 6$-aminopenicillanic $acid^-(aq) + H^+(aq)$, the following parameters have been obtained: $K = (7.35 \pm 1.5) \times 10^{-8}$ mol kg^{-1}, $\Delta G^0 = 40.7 \pm 0.5$ kJ mol^{-1}, $\Delta H^0 = 29.7 \pm 0.6$ kJ mol^{-1}, and $\Delta C_p^0 = -240 \pm 50$ J mol^{-1} K^{-1} at 298.15 K and at the thermochemical standard state. The extent of reaction for the overall conversion is highly dependent upon the pH.

1. Introduction

Many penicillin antibiotics used in medicine are produced semi-synthetically by chemical modification of the basic penicillin structure. The starting point for chemical modification is frequently 6-aminopenicillanic acid [1]. It can be produced by the hydrolysis of penicillin G using the enzyme penicillin acylase (EC 3.5.1.11):



penicillin G
(PGH_2)



6-aminopenicillanic acid
($APAH_2$)

phenylacetic acid
(ϕAH)

In 1984 nearly 2500 tons of 6-aminopenicillanic acid were produced using this reaction [2]. When manufacturing large quantities of materials the efficiency of product yield becomes a matter of

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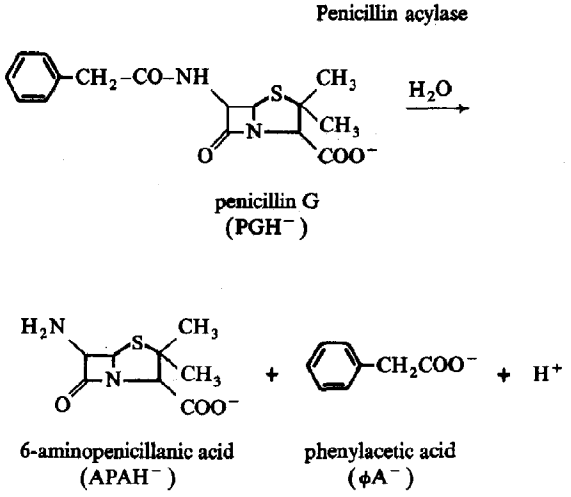
Abbreviations: APA^{2-}, $(6\text{-aminopenicillanic acid})^{2-}$; PG^{2-}, $(\text{penicillin G})^{2-}$; ϕA^-, $(\text{phenylacetic acid})^-$. Protonated forms are indicated by the addition of an appropriate number of hydrogen ions to these species. In penicillin G the first ionization is from the carboxyl group and the second from the amine group. In 6-aminopenicillanic acid, the first ionization of the neutral molecule is from the carboxyl group and the subsequent one from the amine group. The $APAH_3^+$ ion is formed by protonation of the neutral molecule at the amine group.

practical and economic importance. Chemical thermodynamics provides the formalisms and methods needed to obtain an understanding of the conditions under which the product yield can be optimized. The literature, however, contains only one equilibrium investigation [3] of this reaction, and there are no calorimetric data. Therefore, we have undertaken a systematic investigation of the thermodynamics of this process. A principal objective was the determination of the Gibbs energy (ΔG^0), enthalpy (ΔH^0), and heat capacity (ΔC_p^0) changes at 298.15 K for the following reference reaction:



Since the thermodynamic parameters for the reference reaction refer to the thermochemical standard state (hypothetical ideal solution of unit molality), they are independent of pH. The thermodynamics of the overall conversion, however, will be shown to have a high dependence on pH.

In this investigation we have used high-pressure liquid chromatography (HPLC) to determine equilibrium constants as a function of temperature (292–322 K), pH (6–7.5) and ionic strength (0.1–0.4 mol kg^{-1}). Microcalorimetry was used to measure enthalpies of reaction as a function of temperature and pH. The calorimetry provides an inherently more accurate method for the determination of the enthalpy and heat capacity changes than can be obtained from a calculation using the temperature dependency of equilibrium constants. Also, a test of the accuracy of the two sets of measurements is the agreement of the enthalpy change determined using calorimetry with a value of the enthalpy determined from the temperature dependency of the equilibrium data. The experimental data have been treated in terms of a chemical equilibrium model which considers both the multiplicity of the species in solution and ionic strength effects. It provides a coherent treatment of the thermodynamics of the overall process in terms of these effects.

2. Experimental

Phenylacetic acid and the sodium salt of penicillin G were purchased from Sigma *. 6-Aminopenicillanic acid and immobilized penicillin acylase, used for the equilibrium measurements, were supplied by Beecham Laboratories, Piscataway, NJ. The moisture contents of phenylacetic acid, penicillin G and 6-aminopenicillanic acid were determined by Karl Fischer analysis to be less than 0.1 mass%. The soluble penicillin acylase, used for the calorimetric measurements, was supplied by Boehringer Mannheim and Dr. Richard Virden, University of Newcastle upon Tyne, U.K. It was dialyzed against phosphate buffer prior to use. KH_2PO_4 and K_2HPO_4, used in the preparation of the buffer and the mobile phase, were obtained from Fisher Chemical Co.

Equilibrium measurements for the conversion of penicillin G to 6-aminopenicellanic acid and phenylacetic acid were carried out starting either with penicillin G (for forward reactions) or with 6-aminopenicillanic acid and phenylacetic acid (for reverse reactions) in phosphate buffer. The substrate solutions were prepared by dissolving known amounts of substrate(s) in phosphate buffer. The immobilized penicillin acylase was added to each solution and the solutions were then equilibrated in a thermostatted water bath controlled to within $\pm$ 0.05 K. The solutions were continuously stirred

* Certain commercial materials and products are identified in this paper to specify adequately the experimental procedures. Such identification does not imply recommendation or endorsement by the National Bureau of Standards.

with a Teflon-coated magnet prior to injection into the HPLC. Generally reactions were carried out for approx. 24 h.

Quantitative analysis of the equilibrium mixtures was performed by using a Zorbax ODS, 4.6 × 250 mm column and a Hewlett-Packard model 1090 liquid chromatograph equipped with a diode array detector. The mobile phase consisted of 29% methanol and 71% 0.02 M KH_2PO_4 at pH 4.7. The mobile phase flow rate was 0.7 ml min^{-1}. The detector was set at 220 nm. Under these conditions the retention times were 3.4, 8.3 and 19.8 min, respectively, for APA, φA and PG. A typical chromatogram is shown in fig. 1. The areas under these peaks correspond to the total amounts of 6-aminopenicillanic acid, phenylacetic acid and penicillin G in solution; i.e., they include all ionic forms.

The microcalorimetric techniques have been previously described [4]. Heat measurements were performed by mixing two separate solutions, designated as enzyme and substrate solutions, respectively, in the microcalorimeters. The enzyme solution contained the soluble penicillin acylase and the substrate solution contained penicillin G. The penicillin acylase had been dialyzed against the phosphate buffer used to contain both it and the substrate solution. Since this reaction proceeds substantially to the right under the experimental conditions used in this investigation, the reverse reaction was not carried out in the microcalorimeter.

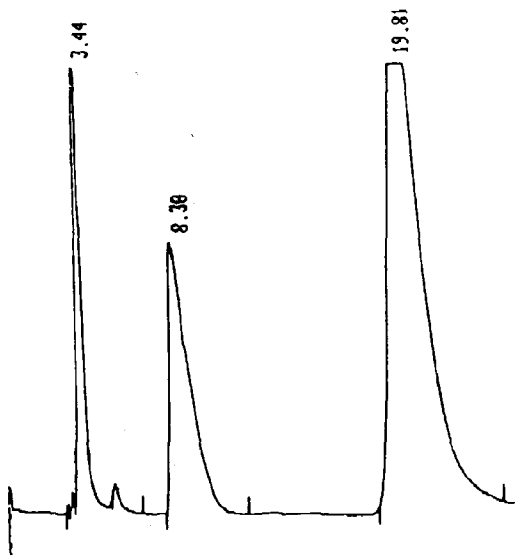


Fig. 1. Chromatogram of mixture of 6-aminopenicillanic acid, phenylacetic acid and penicillin G. Retention times of these compounds: 3.44, 8.30 and 19.81 min, respectively.

Approximate values of ionization constants of penicillin G and 6-aminopenicillanic acid were determined using acid-base titrations with a combination pH electrode as the end-point indicator.

3. Results and discussion

For the conversion of penicillin G to phenylacetic acid and 6-aminopenicillanic acid the reference reaction can be represented as:

$$PGH^- + H_2O = \phi A^- + APAH^- + H^+ \qquad (A)$$

The overall conversion is represented as

$$\sum PG + H_2O = \sum \phi A + \sum APA + n_H H^+ \qquad (A')$$

where Σ represents the totality of the different ionic states of a given substance in solution and n_H is the number of protons produced or absorbed as a part of the reaction. The equilibrium constants (K) and enthalpy changes (ΔH) for the reference reaction and for other related equilibria are given in table 1. Application of equilibrium thermodynamics to this system leads to the following:

$$f_{PG} = [PGH^-]/[\sum PG] = \{[H^+]/K_{1PG} + 1 + K_{2PG}/[H^+]\}^{-1} \qquad (1)$$

$$f_{\phi A} = [\phi A^-]/[\sum \phi A] = \{[H^+]/K_{\phi A} + 1\}^{-1} \qquad (2)$$

$$f_{APA} = [APAH^-]/[\sum APA] = \{[H^+]^2/(K_{1APA}K_{2APA}) + [H^+]/K_{2APA} + 1 + K_{3APA}/[H^+]\}^{-1} \qquad (3)$$

$$K_{A'} = [\sum APA][\sum \phi A]/[\sum PG] = K_A f_{PG}/([H^+] f_{APA} f_{\phi A}) \qquad (4)$$

Table 1

Thermodynamic parameters at 298.15 K relevant to the conversion of penicillin G to phenylacetic acid and 6-aminopenicillanic acid in aqueous solution

The standard state is the hypothetical ideal solution of unit molality. The values of K_A, ΔH^0_A, $\Delta C^0_{p,A}$, and pK_{2APA} were obtained from the regression analysis.

Process	pK or equilibrium constant	ΔH^0 / kJ mol^{-1}	ΔC^0_p / J mol^{-1} K^{-1}	References
$PGH^- + H_2O = \phi A^- + APAH^- + H^+$	$K_A = (7.35 \pm 1.5) \times 10^{-8}$	29.7 ± 0.6	-240 ± 50	this work; regressed from experimental data
$PGH_2 = PGH^- + H^+$	$pK_{1PG} \approx 2.6$	–	–	estimated
$PGH^- = PG^{2-} + H^+$	$pK_{2PG} \approx 9.0$	–	–	this work, pH titration
$\phi AH = \phi A^- + H^+$	$pK_{\phi A} = 4.3$	-3.7	-105	Smolyakov and Primanchuk [5]; also see references cited in ref. 6
$APAH_3^+ = APAH_2 + H^+$	$pK_{1APA} = 2.6$	–	–	Berezin et al. [7]
$APAH_2 = APAH^- + H^+$	$pK_{2APA} \approx 5.4$	≈ 31	≈ -200	ΔH^0 and ΔC^0_p were estimated; pK_{2APA} is based upon a regression analysis
$APAH^- = APA^{2-} + H^+$	$pK_{3APA} \approx 9.9$	–	–	this work, pH titration
$H_2PO_4^- = HPO_4^{2-} + H^+$	$pK_P = 7.20$	4.14	-149	the values of pK and ΔH^0 are from Larson and Hepler [8]; ΔC^0_p is calculated from data in the compilation of Abraham and Marcus [9]

$$n_H = 1 + f_{PG}\{[H^+]/K_{1PG} - K_{2PG}/[H^+]\} - f_{APA}\{2[H^+]^2/(K_{1APA}K_{2APA}) + [H^+]/K_{2APA} - K_{3APA}/[H^+]\} - f_{\phi A}\{[H^+]/K_{\phi A}\} \quad (5)$$

$$\Delta H_{A'} = \Delta H_A + f_{PG}\{[H^+]\Delta H_{1PG} - K_{2PG}\Delta H_{PG}/[H^+]\} - f_{APA}\{([H^+]^2/(K_{1APA}K_{2APA}))(\Delta H_{2APA} + \Delta H_{1APA}) + [H^+]\Delta H_{2APA} - K_{2APA}\Delta H_{3APA}/[H^+]\} - f_{\phi A}\{[H^+]\Delta H_{\phi A}/K_{\phi A}\} \quad (6)$$

In the above equations, square brackets represent concentrations in solution expressed either as mol (kg solution)$^{-1}$ or as mol (kg solvent)$^{-1}$ and the f terms are the fractions of a given substance existing in a specified ionic state. $K_{A'}$ is the equilibrium constant which is obtained from the chromatographic experiments where total concentrations of reactant and products are determined. The calorimetrically determined enthalpy also includes a contribution due to the heat of buffer protonation which is equal to $-n_H\Delta H_{buffer}$, where the latter quantity is the enthalpy of ionization of the buffer used in the system.

The temperature dependence of the Gibbs energies (ΔG^0) or equilibrium constants and the enthalpies of the reaction can be calculated from:

$$\Delta G^0_T = -RT \ln K = \Delta H^0_\theta + \Delta C^0_p(T - \theta) + T(\Delta G^0_\theta - \Delta H^0_\theta)/\theta - T\Delta C^0_p \ln(T/\theta) \quad (7)$$

$$\Delta H^0_T = \Delta H^0_\theta + \Delta C^0_p(T - \theta) \quad (8)$$

where T is the thermodynamic temperature, θ the reference temperature of 298.15 K and R the gas constant (8.31451 J mol^{-1} K^{-1}). Effects of non-ideality are accounted for by using an extended Debye-Hückel equation [10]:

$$\ln \gamma_i = -Az_i^2 I^{1/2}/(1 + BI^{1/2}) + \lambda \sum m_j \quad (9)$$

Table 2

Enthalpies of reaction of penicillin G to the equilibrium mixture of it and phenylacetic acid and 6-aminopenicillanic acid as a function of temperature and pH

The reactions were carried out in 0.1 M phosphate buffer and the initial concentration of penicillin G was 10 ± 1 mM. The pooled standard deviation of the measurements (two or three replicates for each data point) is 0.38 kJ mol^{-1}.

Observation	T (K)	pH	ΔH (kJ mol^{-1})
1	298.15	6.00	19.73
2	298.15	6.54	22.95
3	298.15	7.00	23.80
4	304.40	6.00	19.63
5	304.40	6.54	22.91
6	304.40	7.00	23.44
7	304.40	7.44	23.26
8	310.15	6.00	19.60
9	310.15	6.54	22.61
10	310.15	7.00	23.48

where γ_i is the activity coefficient of the i-th species and z_i its charge, A a Debye-Hückel constant, I the ionic strength, B an 'ion-size' parameter, λ an average interaction parameter and Σm_j the sum of the molalities of the other species in the solution. Initial estimates of 1.6 mol kg^{-1} and 0.0 kg mol^{-1} were made for B and λ, respectively. Improved values of these quantities were later regressed from the data (see below).

The experimental data are summarized in tables 2 and 3. They include both equilibrium and calorimetric data which have been determined as a function of temperature, pH and ionic strength. As indicated above, the primary object of this investigation was the determination of the thermodynamic parameters (ΔG^0, ΔH^0, and ΔC_p^0) for the reference reaction (process A). The model presented above allows one to calculate the observed or measured quantities under a variety of experimental conditions which include variations in the afore-mentioned experimental conditions. Consequently, we have used the above model and computational procedures similar to those previously described [10] to regress the desired parameters. The regression analysis was performed so as

Table 3

Equilibrium constants ($K_{A'} = [\Sigma APA][\Sigma \phi A]/[\Sigma PG]$) for the conversion of penicillin G to phenylacetic acid and 6-aminopenicillanic acid

Starting concentrations of penicillin G (for forward reactions) and 6-aminopenicillanic acid and phenylacetic acid (for reverse reactions) were 10–16 mmol (kg solution)$^{-1}$. All reactions were carried out in phosphate buffer (0.1 mol l^{-1}), with the exception of observations 11, 12, 17 and 18. The pooled standard deviation of the measurements (three to five observations for each reported value) is 4.7% of the value of $K_{A'}$. A test of material balance was applied to each set of observations; it was found to hold within the imprecision of the measurements.

T (K)	Forward reaction			Reverse reaction		
	Observation	pH	$K_{A'}$ (mol kg^{-1})	Observation	pH	$K_{A'}$ (mol kg^{-1})
292.15	1	6.67	0.330	2 [a]	6.68	0.275
298.15	3	6.71	0.445	4	6.59	0.382
304.15	5	6.68	0.579	6	6.54	0.469
310.15	7	6.10	0.221	8	6.02	0.194
310.15	9	6.30	0.416	10	6.07	0.247
310.15	11 [b]	6.54	0.694	12 [b]	6.43	0.590
310.15	13	6.61	0.756	14	6.39	0.524
310.15	15	6.68	0.644	16	6.59	0.565
310.15	17 [c]	6.74	1.051	18 [c]	6.46	0.536
310.15	19	7.05	1.71	20	6.85	1.13
310.15	21	7.55	7.46	22	7.32	4.16
316.15	23	6.65	0.879	24	6.59	0.725
322.15	25	6.75	1.19	26	6.53	0.776

[a] This observation was given zero weight in the correlation.

[b] 0.1 M phosphate buffer + 0.2 M KCl. The ionic strength was approx. 0.40 mol kg^{-1}.

[c] Buffer concentration was 0.05 mol l^{-1}. The ionic strength was approx. 0.11 mol kg^{-1}.

to minimize the quantity Σd_i^2, where d_i is the weighted difference between a measured and calculated data point. The weighting was performed so as to give unit weights to all points with the exception of a single outlier in the equilibrium data set. For the equilibrium data the percentage difference between measured and calculated quantities was used in the regression calculations. The weighting was carried out this way, since the measured values of the equilibrium constant ($K_{A'}$) are highly dependent upon pH while the measured enthalpies ($\Delta H_{A'}$) are not.

The results of this regression analysis are: $K = (7.35 \pm 1.5) \times 10^{-8}$ mol kg^{-1}, $\Delta G^0 = 40.7 \pm 0.5$ kJ mol^{-1}, $\Delta H^0 = 29.7 \pm 0.6$ kJ mol^{-1} and $\Delta C_p^0 = -240 \pm 50$ J mol^{-1} K^{-1} at 298.15 K for the reference reaction (process A). These uncertainties correspond to approx. 95% confidence limits. The parameters in the activity coefficient expression were also regressed; the results obtained were $B = 1.4$ mol$^{-1/2}$ kg$^{1/2}$ and $\lambda = -0.05$ mol^{-1} kg. In performing these calculations we initially used a value of pK_{2APA} equal to 4.6 from the work of Berezin et al. [7]. We were unable to obtain a satisfactory representation of our experimental data using this value. We therefore made this parameter an additional object of the regression. When this was done, we obtained a value of 5.4 for pK_{2APA}. In the absence of any data for ΔH^0_{2APA}, we have estimated it by assuming that ΔS^0 for the ionization is equal to zero. $\Delta C^0_{p,2APA}$ was estimated at -200 J mol^{-1} K^{-1} based upon known values of this quantity for other acid ionizations [8]. We also attempted a measurement of the second ionization constant of 6-aminopenicillanic acid using acid-base titrations of it with a combination pH electrode as the end-point indicator. The inflection point was difficult to determine from the experimental results and based upon these rather unsatisfactory experiments we were able to conclude only that pK_{2APA} is in the range 4.2–7.2. An accurate electrochemical determination of this quantity would be extremely useful. However, in its absence we have relied on a value of pK_{2APA} equal to 5.4 in the treatment of the experimental data.

We also regressed a value of ΔH^0_A using only the equilibrium data with a fixed value of -240 J mol^{-1} K^{-1} for $\Delta C^0_{p,A}$. The result was $\Delta H^0_A = 30.9 \pm 6.0$ kJ mol^{-1} at 298.15 K in good agreement with the result of 29.7 ± 0.5 kJ mol^{-1} obtained from the calorimetric data.

We now turn to a consideration of the precision and accuracy of the measurements on this system. Residual plots for both the enthalpy and equilibrium data are shown in figs. 2 and 3, respectively. The standard deviation of the fit to the enthalpy data is 0.40 kJ mol^{-1} which is close to the pooled standard deviation of the measurements themselves (0.38 kJ mol^{-1}). The standard deviation of the fit to the equilibrium data is 17%. This is significantly larger than the pooled standard deviation of the measurements of $K_{A'}$ (4.7%). However, K_A, is highly dependent on pH. Specifically, if the values of the pH in the data in table 3 are perturbed by ± 0.05, the calculated values of $K_{A'}$ are changed by ± 11%. Since the pH measurements have an uncertainty associated with them of approx. ± 0.05, the standard deviation of the fit to the equilibrium data can be accounted for by the random errors associated with the measurement of $K_{A'}$ and pH.

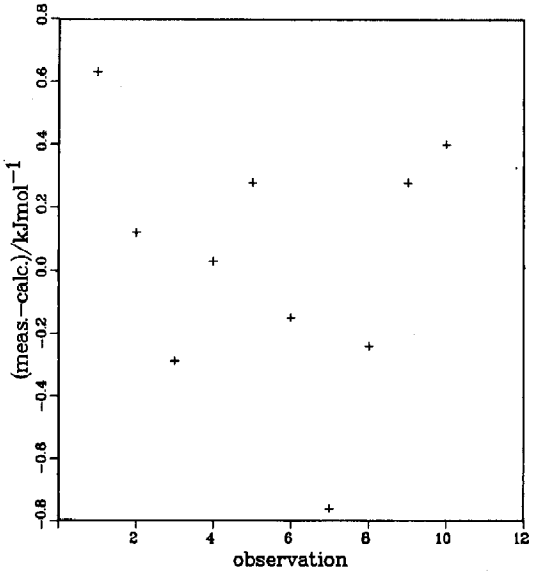


Fig. 2. Deviation plot for the calorimetric measurements. The differences (kJ mol^{-1}) between the measured and calculated enthalpies of reaction are given as a function of the observation number (see table 2).

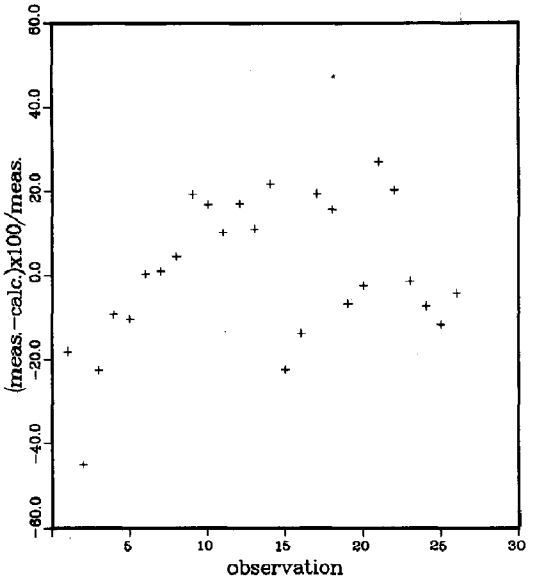


Fig. 3. Deviation plot for the equilibrium measurements. The percentage differences between the measured and calculated values of the equilibrium constant $K_{A'}$ are given as a function of the observation number (see table 3).

We have also examined the deviations for trends in pH, temperature, and ionic strength and find that there are no significant trends in the deviations that are attributable to these parameters. Also, examination of the deviations of the results obtained from both the forward and reverse reactions shows no significant effect due to this experimental factor. These items plus the fact that the van't Hoff enthalpy is in agreement with the calorimetric enthalpy strengthens our overall confidence in the accuracy of the measurements and of the model which has been used in the correlation.

The only other data in the literature with which our results can be compard are those of Svedas et al. [3]. They report three equilibrium data points (pH 5–6, $T = 298.15$ K, and $I \approx 0.1$ mol kg^{-1}; imprecision is 20–50%). We have compared their results with the values calculated from the model presented herein and find their results to be lower by a factor of 5–10. If pK_{2APA} is set equal to 4.6 in the model, the discrepancy is reduced to being a factor of 2–4. However, the overall discrepancy cannot be explained by any other such adjustments. Svedas et al. [3] used different methods of analysis for each of the reactants and products while we used a single direct method for all three substances with calibrations for each of the response factors performed on a daily basis [11]. We believe that a possible explanation for the discrepancy is a systematic error in one or more of the analytical methods used by Svedas et al. [3].

The model of this system can be used to calculate values of $K_{A'}$ as a function of pH, temperature, and ionic strength. Of these three variables, the pH has the greatest effect on $K_{A'}$ (see fig. 4). Thus, the direction, or extent of the overall reaction, can be controlled by appropriate adjustment of the pH. For example, using the value of $K_{A'}$, obtained from fig. 4, one can calculate that 99.90% of penicillin G will be converted at a pH of 8.0 to the products 6-aminopenicillanic acid and phenylacetic acid at 310.15 K and an ionic strength of 0.1 mol kg^{-1}. Changes of ionic strength and tem-

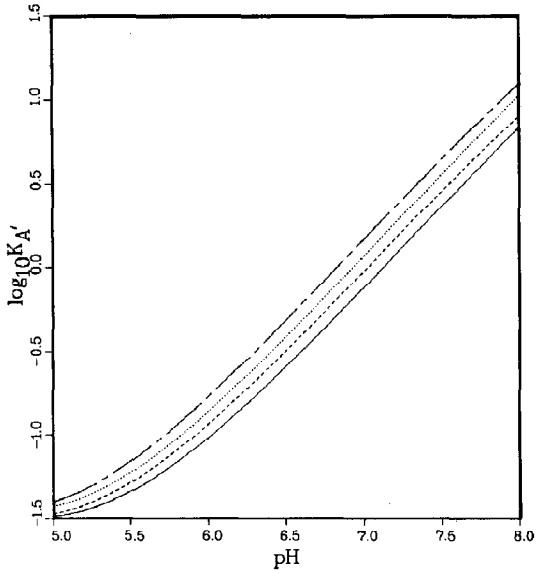


Fig. 4. Calculated values of the logarithm of the equilibrium constant $K_{A'}$ as a function of pH at different ionic strengths and temperatures: $T = 298.15$ K and $I = 0.0$ mol kg^{-1} (———); $T = 298.15$ K and $I = 0.1$ mol kg^{-1} (- - - - -); $T = 310.15$ K and $I = 0.0$ mol kg^{-1} (· · · · ·); and $T = 310.15$ K and $I = 0.1$ mol kg^{-1} (— - — - —). $K_{A'} = [\Sigma APA][\Sigma \phi A]/[\Sigma PG]$.

perature do not significantly affect the product yield. Thus, these environmental conditions can be adjusted to optimize the rate of reaction and stability of the enzyme used for the conversion.

Acknowledgments

We wish to thank Dr. Ewa Gajewski for her helpful discussions on this problem. The sample of soluble penicillin acylase was kindly provided by Boehringer Mannheim GmbH and Dr. Richard Virden of the University of Newcastle upon Tyne. The immobilized penicillin acylase and 6-aminopenicillanic acid were supplied to us by Dr. Dennis Clark of Beacham Laboratories. Mr. J.V. Advani of Ortho Pharmaceuticals Corp. advised us on the method of separation using HPLC.

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