CHEM 110L: EXP. 08

Introductory Biochemistry Laboratory

Determination of activation energy of amide bond hydrolysis

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The scientific goal of this experiment is to determine the activation enthalpy for the hydrolytic cleavage of the amide bond in 5-hydroxyisourate. The main educational goal of this experiment is to learn how to use UV-Vis spectrophotometers to measure reaction rates, and how to mathematically analyze reaction kinetics data.

1 | INTRODUCTION

The components of the full metabolic pathway that converts urate to allantoin in lower mammals have only recently been identified. [7] Although it was originally thought that urate oxidase was the sole enzymatic component of this pathway, recent studies have established that conversion of uric acid to allantoin occurs by sequential chemical modifications catalyzed by urate oxidase, 5-hydroxyisourate (HIU) hydrolase, and 2-oxo-4-hydroxy-4-carboxy-5-ureidoimidazoline (OHCU) decarboxylase.

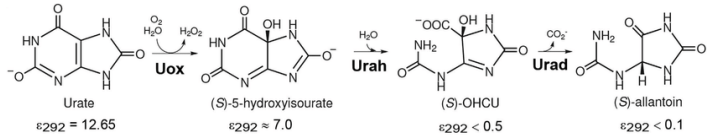


FIGURE 1 Scheme of the enzymatic conversion of urate into allantoin. [6]

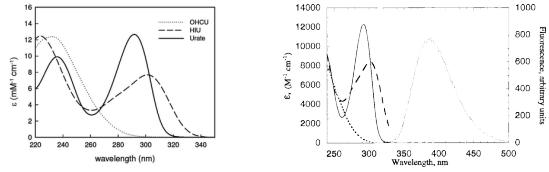


FIGURE 2 The experimentally determined UV absorbance spectrum of urate (thick solid line), the calculated spectra of 5-hydroxyisourate (dashed line), and decomposition products of 5-hydroxyisourate at short times (dotted line) at pH 7.85 and the experimentally determined fluorescence emission spectrum of 5-hydroxyisourate (thin solid line) at pH 7.5 with excitation at 306 nm. The calculated UV spectrum of 5-hydroxyisourate is corrected for contributions from decomposition products; the calculated UV spectrum of the decomposition products is dominated by absorbance due to 2-oxo-4-hydroxy-4-carbohydroxy-5-ureidoimidazoline. [5]

2 | MATERIALS AND METHODS

Uric acid was dissolved at a concentration of 100 μM in 100 μM potassium phosphate buffer, pH 7.0. The water bath was set to the assigned temperature (here, 308 K) and the cuvette was given time to equilibrate in the spectrometer. After blanking the spectrometer (Shimadzu UV-1900) with the buffer solution, potassium hexachloroiridate(IV) (K_2IrCl_6) was added directly to the cuvette at a 2:1 molar ratio to UA. Spectra were collected between 240 and 330 nm immediately after addition of K_2IrCl_6 and every subsequent minute for 40 minutes.

The resulting spectra were plotted in SpectraGryph (v2.1, [**?**]) and absorbance-time data at 304 nm underwent statistical analysis and non-linear fitting to rate-law models using Mathematica.

3 | RESULTS

The reaction scheme for the oxidation of UA by potassium hexachloroiridate(IV) is as follows:

$$\mathrm{UA} + \mathrm{H_2O} \underset{+2\,e^-,\,2H^+}{\overset{-2\,e^-,\,2H^+}{\rightleftharpoons}} \mathrm{HIU}$$

Recently, Kim et. al found the observed stoichometric ratio of UA and Ir_{OX} to be: $\frac{\Delta Ir_{OX}}{\Delta UA} \approx 2.8$. Thus, it is possible that the 2:1 ratio used in the experiment was not sufficient for UA to fully undergo oxidation. This would result in inflated absorbance values under 320 nm.

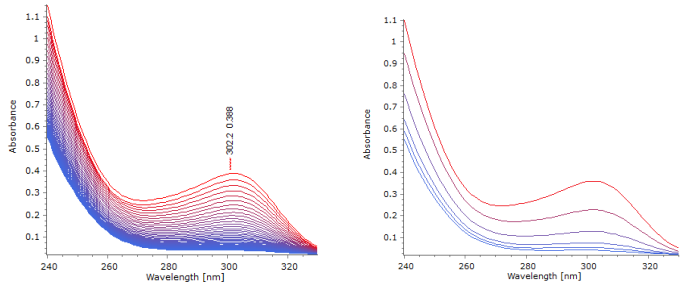


FIGURE 3 Spectra collected over a 2400s period (red → blue) after mixing 1:2 UA:oxidizing agent.

An initial estimate of the rate constant of HIU decomposition can be obtained by fitting absorbance data (between 304 and 330 nm) over time to a single-exponential model. The observed absorbance in this region of the spectrum will follow first-order kinetics due to the lack of contribution to absorbance by OHCU above 300 nm. However, OHCU does absorb below 300 nm, so data between 240 and 300 nm may be fitted to a model that accounts for its absorbance. Additionally, the instrument was blanked using only the potassium phosphate buffer, so the model must also account for any contributions to absorbance from the oxidizing agent. The fundamental equation in UV-Vis spectroscopy is the Beer-Lambert law:

$$A = \epsilon c L \tag{1}$$

where A denotes absorbance (dimensionless), ϵ is the molar absorption coefficient at specified wavelength (usually specified as a subscript; $\mathrm{mol^{-1}\ L\ cm^{-1}}$), c is the concentration of the absorbing species ($\mathrm{mol\ L^{-1}}$), and L is the cuvette length (cm). The absorbance of K_2IrCl_6 decreases linearly over time independent of its interactions with urate. [4] Absorbance-time data for K_2IrCl_6 collected by the instructor was fit to a zeroth-order rate law ($A = A_0 - \kappa\tau$), yielding:

$$A_{\mathrm{Ir_{OX}}} = 0.180959 - 2.89229 \times 10^{-6}\tau, \tag{2}$$

where τ is time in seconds (see Figure 4). Next, the Beer-Lambert law is applied again to the sequential process HIU → OHCU → allantoin, in a similar fashion to that done by Kahn et. al [5]:

$$A = \epsilon_{\mathrm{HIU}} C_{\mathrm{HIU}} + \epsilon_{\mathrm{OHCU}} C_{\mathrm{OHCU}} + \epsilon_{\mathrm{decomp}} C_{\mathrm{decomp}} + A_{\mathrm{Ir_{OX}}} \tag{3}$$

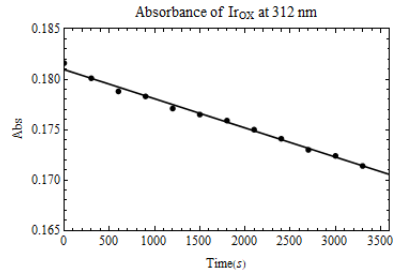


FIGURE 4 Linear (0^{th}-order) fit to experimental absorbance-time data (●) of 200 μM K_2IrCl_6 in 100 μM potassium phosphate buffer, pH≈7.1 (R^2 = 0.99999).

$$\frac{A}{[HIU]_0} = \alpha e^{-k_{HIU}\tau} + \beta e^{-k_{OHCU}\tau} + \gamma e^{-k_{allantoin}\tau} + A_{Ir_{OX}} \tag{4}$$

where:

$$\underbrace{\epsilon_{HIU} - \frac{\epsilon_{OHCU}\kappa_{HIU} - \epsilon_{allantoin}\kappa_{OHCU}}{\kappa_{HIU} - \kappa_{OHCU}}}_{\alpha} + \underbrace{\frac{\kappa_{HIU}}{\kappa_{HIU} - \kappa_{OHCU}}(\epsilon_{OHCU} - \epsilon_{allantoin})}_{\beta} + \underbrace{\epsilon_{allantoin}}_{\gamma} = \epsilon_{HIU}$$

Thus, by fitting the experimental absorbance-time data to Equation 4 using the experimentally-derived extinction coefficients for ϵ_{HIU}, ϵ_{OHCU}, and $\epsilon_{allantoin}$ [6], one may derive the rate constant κ_{HIU}. The analysis yielded the equation:

$$A^{Obs.} = \underbrace{1.44395e^{-0.002287\tau}}_{[HIU]} + \underbrace{0.843951e^{-0.00337095\tau}}_{[OHCU]} + \underbrace{0.1e^{-0.00171959\tau}}_{[allantoin]} + \underbrace{[0.180959 - 2.89229 \times 10^{-6}\tau]}_{[K_2IrCl_6]}$$

where τ is time in seconds. Therefore:

$$\kappa_{HIU} \rightarrow 2.287 \times 10^{-3}\ s^{-1} \quad \kappa_{OHCU} \rightarrow 3.37095 \times 10^{-3}\ s^{-1} \quad \kappa_{allantoin} \rightarrow 1.71959 \times 10^{-3}\ s^{-1}$$

From κ_{HIU}, we can solve for the first-order half-life of HIU decomposition at 308K:

$$t_{1/2,HIU} = \frac{ln(2)}{\kappa_{HIU}}$$
$$\rightarrow 303.082\ s\ (5.05136\ min)$$

This is in close agreement (<15% error) with the reference value of 4.6 minutes. As seen in Figure 5, the model of the reaction mixture agrees with the experimental data well and the rate law for HIU agrees with the reference rate law.

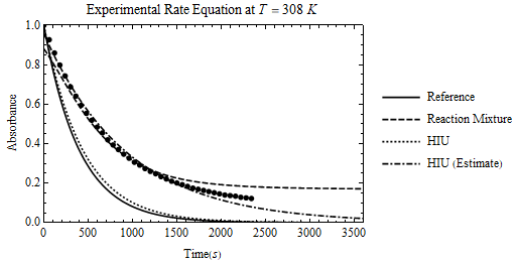


FIGURE 5 Fit to experimental absorbance-time reaction data (●) of 100 μM UA and 200 μM K_2IrCl_6 in 100 μM potassium phosphate buffer, pH≈7.0. Statistics of fit: R^2 = 0.991584; 0.997331 (Estimate).

4 | DISCUSSION

4.1 | Error Analysis

4.2 | Rationale for Choice of Oxidizing Agent

For this procedure, three oxidizing agents were provided: $KMnO_4$, K_2CrO_4, and K_2IrCl_6. We chose to use K_2IrCl_6 because:

- Groups in other sections had previously used K_2IrCl_6. Thus, K_2IrCl_6 was the best choice, as using another oxidant would potentially negate our ability to best compare between the results of different lab groups;
- In neutral conditions (the reaction conditions used here), potassium permanganate is a poor choice of oxidant, as it is only oxidized from $Mn^{[VII]}$ to $Mn^{[IV]}O_2$, which subsequently falls out of solution:

TABLE 1 Parameter confidence intervals of functions fitted to absorbance-time data via Mathematica.

	Estimate	Standard Error	t-Statistic	P-Value	Confidence Interval
κ_{HIU}	2.287×10^{-3}	9.89313×10^{-3}	0.23117	0.818456	1.77584×10^{-2}–2.23324×10^{-3}
κ_{OHCU}	3.37095×10^{-3}	1.58703×10^{-2}	0.212406	0.832956	2.87854×10^{-2}–2.23324×10^{-3}
$\kappa_{allantoin}$	1.71959×10^{-3}	1.77894×10^{-3}	0.0966637	0.923515	3.43251×10^{-2}–3.77643×10^{-2}
$\kappa_{HIU}^{Est.}$	1.06057×10^{-3}	1.94529×10^{-5}	54.5199	1.08093×10^{-37}	1.02119×10^{-3}–1.09995×10^{-3}

$$2\,H_2O + \underset{[VII]}{MnO_4^-} + 3\,e^- \longrightarrow \underset{[IV]}{MnO_2}\downarrow + 4\,OH^-$$

- Potassium chromate is a carcinogen that is quite corrosive and irritating to eyes and skin, and inhalation of even small amounts can be deadly. [1] In order to spare the students in our lab group from making a hasty hospital visit, we chose to use the relatively safe K_2IrCl_6.

4.3 | Determining the Activation Energy

As discussed in the experimental rationale, when the natural log of the rate constant (κ) is plotted versus the inverse of the temperature (T [K]), the slope is a straight line. The value of the slope (m) is equal to $-\frac{E_a}{R}$ where R is a constant equal to 8.314 $\frac{J}{mol \cdot K}$. The line of best fit to the reference dataset was found to be:

$$ln(\kappa) = 15.1 \pm 0.3 - 6496.1 \pm 88.7 \frac{1}{T},$$

making the $E_a \approx 54008 \pm 737$ $\frac{J}{mol}$ (or 54.0 ± 0.7 $\frac{kJ}{mol}$). The line of best fit to the experimental data was found to be:

$$ln(\kappa) = 13.1 \pm 7.4 - 5965.5 \pm 2193.5 \frac{1}{T}$$

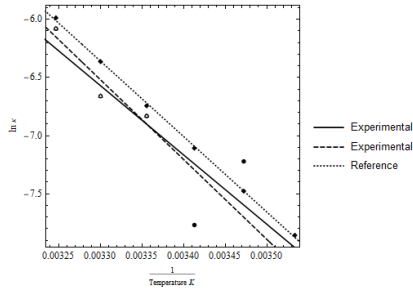
This makes the calculated $E_a \approx 49597.1 \pm 18236.8$ $\frac{J}{mol}$ (or 50 ± 18 $\frac{kJ}{mol}$). Additionally, if one chooses to ignore the odd results at 288 and 293K, fitting yields:

$$ln(\kappa) = 16.2 \pm 7.4 - 6870.4 \pm 2234.2 \frac{1}{T}$$

making the $E_a \approx 57120.2 \pm 18575.1$ $\frac{J}{mol}$ (or 57 ± 19 $\frac{kJ}{mol}$).

TABLE 2

T (K)	$\tau_{1/2}^{2014}$ (m)	κ^{2014} (s^{-1})	$\tau_{1/2}$ (m)	κ (s^{-1})	Group
283	29.8	3.88×10^{-4}	––	––	––
288	20.4	5.66×10^{-4}	15.8	7.29×10^{-4}	TR (1PM)
293	14.1	8.19×10^{-4}	27.3	4.23×10^{-4}	TR (6PM)
298	9.8	1.18×10^{-3}	10.7	1.11×10^{-3}	TR (1PM)
303	6.7	1.72×10^{-3}	9.02	1.28×10^{-3}	TR (1PM)
308	4.6	2.51×10^{-3}	5.05	2.29×10^{-3}	TR (6PM)
			8.46	1.37×10^{-3}	MW (6PM)



Calculated rate constants and half-life values for the first-order hydrolysis of HIU (A) by the instructor in 2014, and (B) by groups of students in 2019. The calculated activation energy of this reaction is derived from the plot ($\ln(\kappa)$ vs. $1/\tau$) on the right.

TABLE 3 Parameter confidence intervals calculated by Mathematica.

Dataset		Estimate	Standard Error	t-Statistic	P-Value	Confidence Interval
Reference	$ln(\kappa)_0$	15.0797	0.300649	50.1572	9.45515×10^{-7}	(14.245, 15.9144)
	$\frac{E_a}{R}$	-6496.07	88.7305	-73.2113	2.08593×10^{-7}	(-6742.42, -6249.71)
Experiment	$ln(\kappa)_0$	13.1177	7.367	1.7806	0.173014	(-10.3274, 36.5627)
	$\frac{E_a}{R}$	-5965.49	2193.51	-2.71961	0.0725732	(-12946.2, 1015.23)
Experiment*	$ln(\kappa)_0$	16.1547	7.3755	2.19032	0.272659	(-77.5599, 109.869)
	$\frac{E_a}{R}$	-6870.37	2234.17	-3.07513	0.200155	(-35258.2, 21517.4)

5 | CONCLUSION

Here, the activation energy for the hydrolytic cleavage of the amide bond in 5-hydroxyisourate, the first intermediate in the degradation pathway of uric acid, was determined to be 57 $\frac{kJ}{mol}$, a value that is in agreement with the previously reported value of 54 $\frac{kJ}{mol}$. Although our results indicate that K_2IrCl_6 was able to successfully oxidize UA to HIU at a 2:1 molar ratio, recent findings indicate that in order to fully oxidize UA, it would be necessary to increase this ratio to one approaching 3:1.

Additionally, the kinetics of decomposition of 5-hydroxyisourate may be studied further by monitoring the reaction by other techniques. For example, HIU is optically active, so circular dichroism spectra may be collected in a similar fashion to the procedure shown here. By observing signals at 312 nm (where only HIU has an appreciable ellipticity) and 257 nm (where only OHCU has an appreciable ellipticity), formation and decay of HIU or OHCU can be selectively monitored. [6]

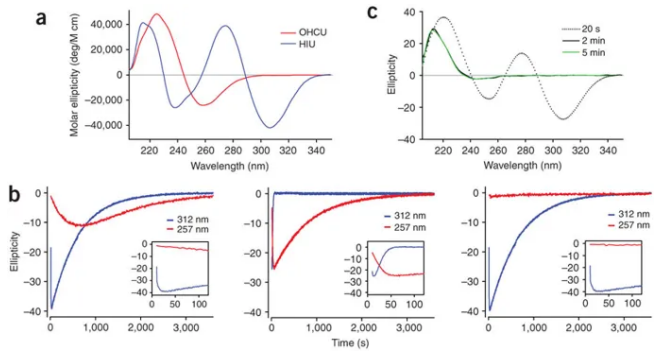


FIGURE 6 (a) Approximate CD spectra of HIU and OHCU in 0.1 M potassium phosphate, pH 7.6. (b) Time courses monitored by CD measurements at 312 nm (blue) and 257 nm (red) of urate degradation with urate oxidase (UO; left), UO+MuraH (middle), UO+MuraD (right); insets show the first 120 s of each time course. (c) Time-resolved CD spectra of the enzymatic product of urate degradation with UO+MuraH+MuraD. [6]

Appendix I: Rationale

TABLE 4 Materials available.

Name	Formula	M_w (g/mol)
Common laboratory chemicals[1]	N/A	N/A
Ascorbic acid		176.12
Uric acid		168.11
Potassium permanganate	$KMnO_4$	158.03
Potassium dichromate	K_2CrO_4	194.19
Potassium hexachloroiridate^{IV}	$[K_2IrCl_6]^{IV}$	483.13
Shimadzu UV-1900	N/A	N/A
Shimadzu UV-2500	N/A	N/A

[1]Except 5-isouric acid or 5-hydroxyisorate, which are unstable.

Preparation of 5-hydroxyisourate in buffered (pH $\approx$ 7) aqueous solution

5-hydroxyisorate (HIU) is relatively unstable in aqueous solutions and must therefore be generated *in situ* prior to kinetic analysis. Addition of urate oxidase (or a suitable oxidizing agent) to a solution of uric acid (UA) in a suitable buffer will result in the conversion of UA $\rightarrow$ HIU in under a minute.

1. Prepare 1 mL of KH_2PO_4 and K_2HPO_4 solutions by dissolving 0.136g of KH_2PO_4 and 0.174g of K_2HPO_4 in 1 mL dH_2O.
2. To make 10 mL of 0.1 M potassium phosphate buffer, combine 866 μL of 1.0 M K_2HPO_4 solution and 134 μL of 1.0 M KH_2PO_4 solution, then fill to 10 mL with dH_2O.

| Kinetic measurement with the UV-Vis Spectrophotometer

Conversion of HIU $\rightarrow$ OHCU can be measured by monitoring the decrease in absorbance at 293 nm from approximately 7.0 mM^{-1} cm^{-1} to 0.5 mM^{-1} cm^{-1}. Additionally, the production and hydrolysis of HIU may be measured over time at 312 nm (where only HIU has an appreciable signal) and 257 nm (where only OHCU has an appreciable signal). [3] [6]

Thus, I propose to measure the decomposition of HIU for about 20 minutes with the Shimadzu UV-1900, which can record absorbance values every 0.1s for a single wavelength, and every 8.5s for two wavelengths, as opposed to 1.5s and 12.5s for the Shimadzu UV-2600. It is customary to monitor first-order reactions for at least three half-lives. The rate of non-enzymatic hydrolysis of HIU was previously reported to be 2.7×10^{-3} by French and Ealick [2] and 2.1×10^{-3} by Kahn and Tipton.[5] From this, we can derive an estimate for the half-life; $t_{1/2}$ = 330.07s. Thus, our measurement should last around 990.21s (or 16.5m), and we should expect to see full decomposition of HIU around 660.14s (or 11m). We can set the number of repetitions to 17 and the repetition time interval to 60s.

For best results, the scan seed should be as fast as possible, and the spectral window should be no wider than necessary for analysis. Thus, we should set the spectral window to 240–330 nm, where HIU has λ_{max} at 302 nm and is the only absorbing species between 304 and 330 nm. HIU has an emission maximum located at 380–400 nm.

Calculation of the activation energy

In order to calculate the activation energy (E_a) of this reaction, we need an equation relating the rate constant k to the temperature. The Arrhenius Equation is as follows:

$$k = Ae^{-E_a/RT} \tag{5}$$

where A is the absorbance, k is the rate constant, and R is the gas constant (8.314 J/mol·K), and T is the temperature in degrees Kelvin. Taking the natural log of both sides yields:

$$ln(k) = -E_a[\frac{1}{R \cdot T}] + ln(A) \tag{6}$$

From this, we can easily find E_a by graphing ln(k) against 1/T. This means that we will need to repeat the experiment detailed above at multiple temperatures.

Appendix II. Additional Figures

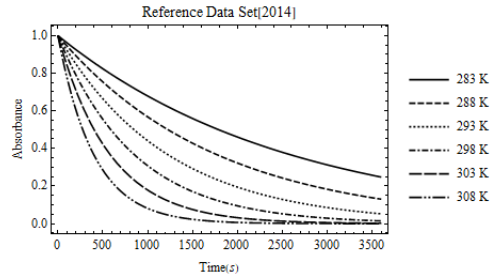


FIGURE 7 Reference data set collected by the instructor in 2014.

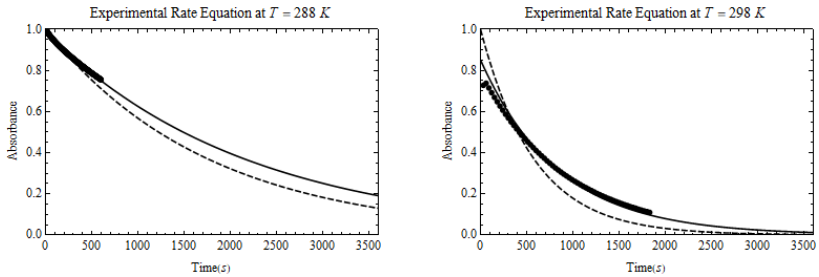


FIGURE 8 Absorbance-time data collected by other lab groups posted in the course website. Plots generated via Mathematica.

References

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[2] J.B. Frencha and S.E. Ealick, *Structural and kinetic insights into the mechanism of 5-hydroxyisourate hydrolase from Klebsiella pneumoniae*, Acta Crystallogr D Biol Crystallogr **67** (2011), 671–677.

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[7] I. Ramazzina, C. Folli, A. Secchi, R. Berni, and R. Percudani, *Completing the uric acid degradation pathway through phylogenetic comparison of whole genomes*, Nature Chemical Biol. **2** (2006), 144–148.