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Kinetics of Alcohol Dehydrogenase-Catalyzed Oxidation of Ethanol Followed by Visible Spectroscopy W

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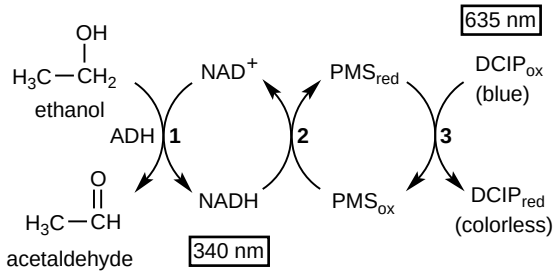
Alcohol dehydrogenase (ADH) has been extensively studied and reviewed *(1)* and often used in teaching undergraduate laboratories *(2–8)*. It naturally piques students' interest because of its function: ADH removes alcohol from the body by reaction **1** in Scheme I. Experiments involving the study of ADH allow faculty not only to teach important concepts of chemistry and biochemistry, but also to address the complicated issues of alcohol consumption on campus. Unfortunately, many previously published biochemistry experiments are not designed for a freshman class because they involve expensive instrumentation necessary to follow the reaction in the UV region at 340 nm *(2, 4–8)*. These experiments also require pipettors for delivering exact volumes of reagents or involve mathematical treatment of data, which are appropriate for higher-level courses *(3, 9)*. We have developed a two-week biochemistry experiment for the second semester of a general chemistry course that avoids these drawbacks and is suitable for an introductory biochemistry laboratory course. The biochemical emphasis of the laboratory is especially appropriate for the departments that choose to fulfill the ACS biochemistry requirement by integrating biochemical concepts in all chemistry courses instead of offering a separate biochemistry core course.

Experimental Overview

In this experiment, the effect of substrate concentration on the rate of enzymatic reaction was investigated and typical Michaelis–Menten kinetics were observed during the first week. The first-order reaction at relatively low concentrations of ethanol and the pseudo zero-order reaction at high concentrations of ethanol were emphasized. One of the solutions was allowed to reoxidize in air and recover the color of indicator in absence of additional ethanol to demonstrate the fact that open living systems tend to reach equilibrium with their oxidizing surroundings in the absence of an influx of energy in form of reducing agents. These reactions were monitored by visible spectroscopy at 635 nm. The switch from UV to visible spectroscopy was achieved by coupling reaction **1** with reactions **2** and **3** as shown in Scheme I. The ADH-catalyzed reaction of ethanol and NAD^+ produces acetaldehyde and NADH. NADH reduces the oxidized form of phenazine methosulfate (PMS_{ox}) to the reduced form, PMS_{red}, and then the PMS_{red} reduces 2,6-dichlorophenolindophenol ($DCIP_{ox}$), which is blue, to $DCIP_{red}$, which is colorless *(3, 10–12)*.



Scheme I. ADH–PMS–DCIP coupled reactions with the resulting color change in the visible region.

The effects of temperature, pH, the specificity of the enzyme to ethanol by comparison with several other alcohols, and the enzyme's inhibition by heavy metals were explored during the second week of the experiment. An apparent activation energy was calculated from rates near room temperature, and a typical decrease of activity was observed at high and low pH, at higher temperatures, with inferior substrates, and in the presence of lead. A combination of these experiments can be used in such a way that the lab is significantly shorter. For example, the zero order of reaction can be established at several relatively high concentrations of ethanol and the substrate specificity can be checked by comparing the rates of reaction with ethanol, methanol, and ethylene glycol in a two-hour experiment.

Procedure

A typical reaction was run in 0.01 M Tris–HCl pH 9.0, 0.3 M ethanol, 27 μM PMS, 6.7 μM DCIP, 0.25 mM NAD^+, and 0.16 μg/mL (387 units/mg) ADH (added last). The reaction was followed at 635 nm for 3 minutes. The cost of the reagents was approximately $0.40 per student per year (purchased at Sigma and Aldrich, 2003). Procedures were developed to avoid specialized equipment. Solutions were delivered with 1-mL syringes rather than micropipettors, and samples were incubated in Styrofoam cups. Data were collected with a Vernier LabPro colorimeter and imported into MS Excel spreadsheets for calculations and the display of graphics. Data can also be collected using a stopwatch and a simple spectrophotometer, such as a Spectronic 20.

Hazards

Hazards of the lab were minimized by limited exposure of students to very low concentrations of reagents present in aqueous solutions. In pure form, the following chemicals are dangerous and appropriate measures should be taken when preparing the reagent stocks: alcohols used are extremely flammable, Tris base is an irritant, DCIP is an irritant, PMS is an irritant and mutagen, and lead nitrate is toxic. Detailed information is provided in the Supplemental Materials.W

Results

The data presented throughout this article were collected by students. Every point represents an average of five runs and the error bars show the standard deviation. Even though the students worked in groups of two, every student in the lab was asked to perform a control run without ADH and a standard run with ADH on both weeks (Figure 1). This gave instructors an opportunity to explain the importance of controls, define the variables of the reaction, and point out the necessity of getting consistent and reproducible data before proceeding with further experimentation, where we allowed the group's data to be pooled. The reaction rate was determined by the slope on the linear stretch of the curve (from 0.7 min to about 2 min) of the run (Figure 1). The slope before 0.7 minutes resulted because reactions **2** and **3** were following ethanol oxidation, and it took time for them to "catch up" with reaction **1**. The lower slope after 2 minutes was due to depletion of the oxidized form of DCIP.

The effects of ethanol concentration on the reaction rate, defined as d*A*/d*t*, the change in absorption with time, are shown in Figure 2A. Students observed that the rate increases linearly with increasing ethanol concentration and determined that the order of reaction is 1 at ethanol concentrations less than 0.08 M. It was also noted that the rate did not change at ethanol concentrations greater than 0.2 M, confirming that the reaction is pseudo zero order in that region. This part of the experiment was designed so students could explore reaction kinetics. Special attention was paid to the zero order of reaction, as the first and second order of reactions were explored in two other experiments in our laboratories. The introduction of Michaelis–Menten kinetics was limited to the explanation of why the enzyme gets saturated at high concentration of ethanol by providing the students with an analogy of a bricklayer not being able to lay more bricks than physically possible even though a lot of bricks might be available. However, the work can be expanded to mathematical treatment of data and display of a Lineweaver–Burk plot (Figure 2B). Enzyme inhibition can be studied for advanced students or classes *(3)*.

Consideration of Figure 3 led to the thermodynamic interpretation of living systems. In this run, ethanol at a concentration of 3.33 mM reduced DCIP resulting in a colorless solution in about 20 minutes. DCIP was slowly reoxidized by oxygen present in the air and became blue again. We suggested to students that ethanol in this case was like food for any living system. It provided reducing power for a system to be removed from the equilibrium, which then existed in a steady state, since there was energy coming into the system. If energy (reducing substance in this case) is not provided to a living system, it dies, and returns to equilibrium with its

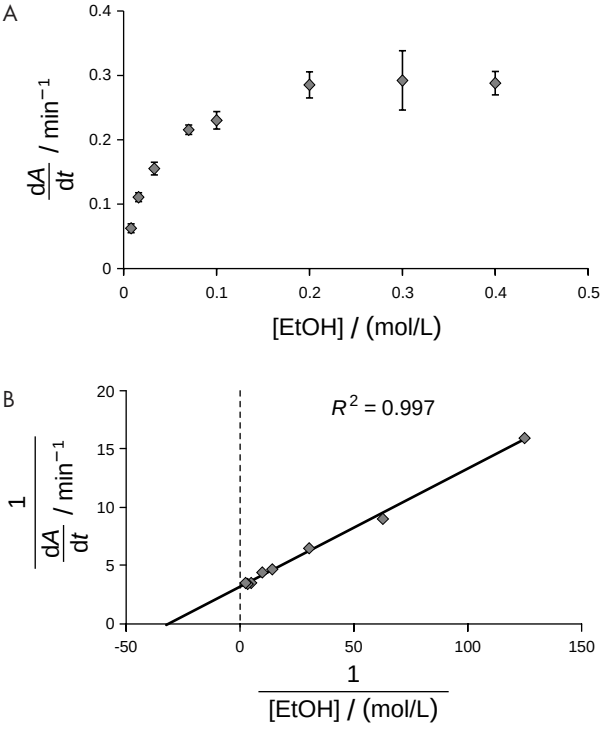


Figure 2. Effect of ethanol concentration on reaction rate.

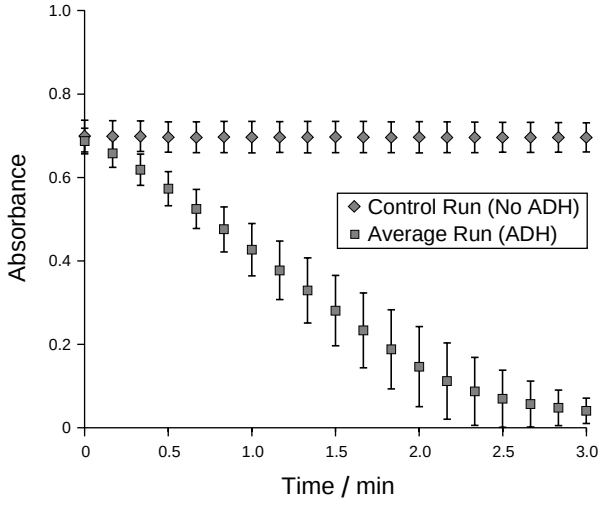


Figure 1. Control and regular run. Error bars show the standard deviation.

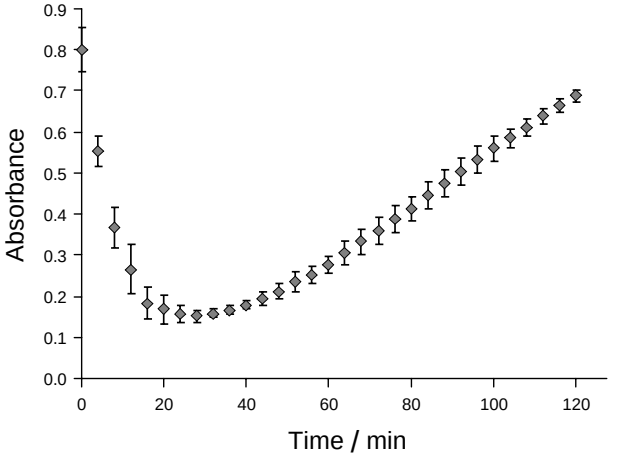


Figure 3. Recovery run: $DCIP_{red}$ slowly reoxidized by oxygen in the air.

oxidizing surroundings. That was exactly what students observed in Figure 3: when ethanol was exhausted, the color of the solution slowly turned back to blue, and the system returned back to equilibrium with the oxidizing environment.

An analogy of a key and a lock or a hand and a glove can be used to explain substrate specificity of this enzymatic reaction. It was observed that methanol, 1-propanol, 1-butanol, and ethylene glycol were all inferior substrates compared to ethanol (Figure 4). Instructors can use this opportunity to explain the toxicity of methanol and ethylene glycol and the treatment of methanol poisoning by ethanol in humans. The result of ADH poisoning by lead nitrate is also shown in Figure 4. Students were informed about zinc in ADH's active site and its replacement by Pb as illustration of one of many mechanisms of lead toxicity and the effects of leaded gasoline on the environment.

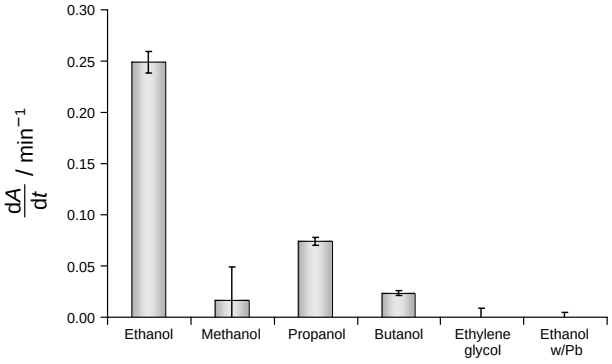


Figure 4. Reaction rates of different substrates with ADH.

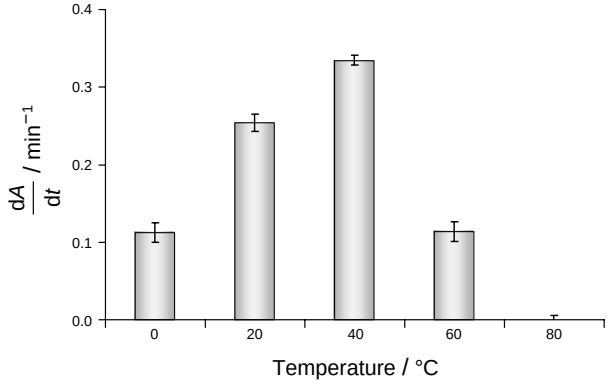


Figure 5. Reaction rates at varying temperatures.

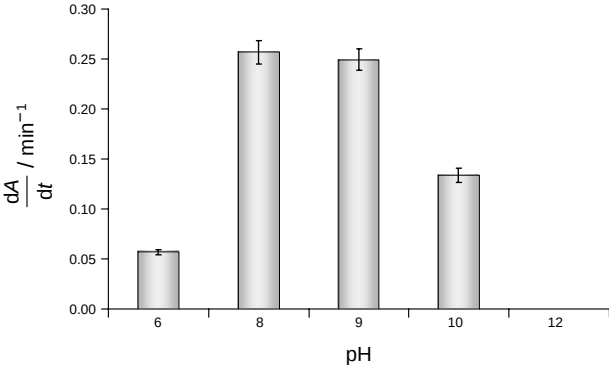


Figure 6. Reaction rates at varying pH.

The effects of temperature were explored and are displayed in Figure 5. Students should take into consideration that most of the reactions are accelerated by increased temperature and that the structure of the enzyme is affected by temperature as well. Thus, the effects on the rate of reaction are a composite of these two factors. The Arrhenius equation was used to calculate an apparent activation energy of 42 kJ/mol using rates measured at 0 °C and 20 °C. Students noted that high temperatures were effective in denaturing the enzyme.

Students observed a typical bell-like activity curve for ADH at different pH in Figure 6. This afforded instructors an opportunity to remind students about the effects of pH on reactions where H^+ is involved, talk about reasons for why a protein's tertiary structure is often extremely sensitive to changes in temperature, pH, and other environmental conditions and mention thermophiles and their adaptive proteins.

To evaluate our students' attitude towards this new two-week laboratory experiment, an anonymous voluntary survey was performed two weeks after the lab was over. About 47% of the class responded to the several statements, as fully disclosed in the survey document of Supplemental Materials.[W] An average student agreed that the material covered in the ADH kinetics laboratory was relevant, the laboratory materials were well written, and assignments to students were reasonable. The class recommended continuing ADH lab as part of the general chemistry course in the future.

[W]Supplemental Materials

Student handouts, note for the instructor, reagent preparation directions, detailed safety information, MS Excel templates for data collection and calculations, survey materials, Vernier LoggerPro files, WebCT student prelaboratory questions in both native and MSWord format, and answers to prelaboratory and postlaboratory questions are available in this issue of *JCE Online*.

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