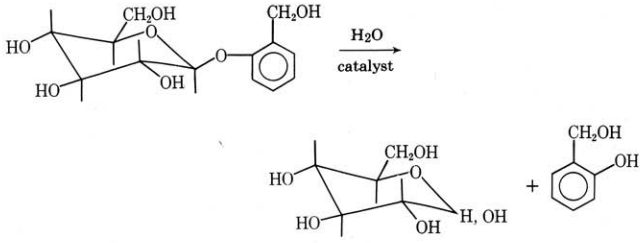
Activation Energies for an Enzyme-Catalyzed and Acid-Catalyzed Hydrolysis

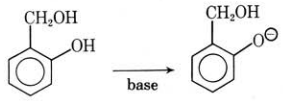
An Introductory Interdisciplinary Experiment for Chemists and Biochemists

K. R. Adams and M. B. Meyers
University of Ulster, Shore Road, County Antrim, Northern Ireland BT37 0QB

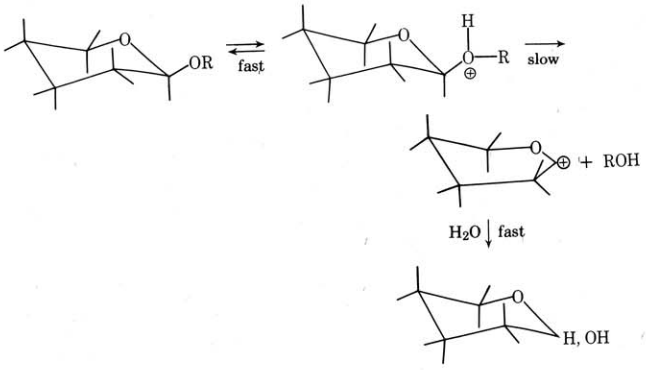
The astonishing catalytic power of enzymes is one of the earliest topics introduced to students in a first-year biochemistry course. Most commonly the catalysis is interpreted in terms of the transition state theory (*1–3*). A simple energy profile graph is often used to compare the course of an enzyme-catalyzed and a non-catalyzed reaction, and the rates of the two reactions are discussed in terms of their activation energies. We wished to reinforce the concept of activation energies in the laboratory course by allowing the student to determine and compare the Arrhenius activation energies, E_a, for the hydrolysis of salicin, a reaction that is subject to catalysis both by acid and by the enzyme emulsin (β-D-glucoside glucohydrolase EC 3.2.1.21).



The progress of the reaction is monitored spectrophotometrically. Aliquots of the reaction mixture are removed and quenched with excess alkali, which has the dual function of stopping the reaction and converting the salicyl alcohol to its anion which has a λ_{max} at 290 nm.

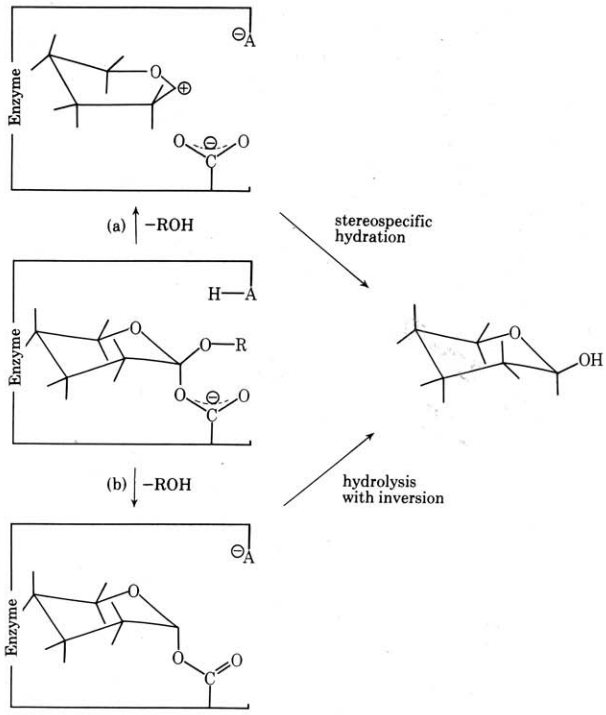


The acid-catalyzed reaction has been well studied (*4*) and proceeds by the A1 (unimolecular) mechanism. Initial protonation at the glycosidic oxygen is followed by the rate-determining cleavage of the O-glycosyl bond giving a cyclic carbonium ion that presumably exists in the half chair conformation:



A two-step mechanism has been proposed for the enzyme-mediated hydrolysis (*5*). The carboxylate group of an aspartic acid residue at the active site of the enzyme has been shown to be involved. The observed retention of the β-configuration arises from one of two pathways:

(1) the stereospecific addition of water to the intermediate carbonium ion which is stabilized by the carboxylate anion, or
(2) a double displacement involving initial formation of an ester and its subsequent hydrolysis with inversion:



In the procedure described below for the enzyme-catalyzed reaction, the salicin is greatly in excess of the catalyst, which is effectively saturated with substrate. Under these conditions virtually all of the enzyme is present as enzyme substrate complex and the rate is independent of the salicin concentration and therefore follows zero-order kinetics. In the acid-catalyzed reaction on the other hand, the concentration of the catalyst is much greater than that of salicin. The rate depends on the unimolecular decomposition of salicin protonated at its glycosidic oxygen, giving rise to first-order kinetics (*4*).

A suitable enzyme preparation can be obtained from almonds by a simple extraction procedure (*6*) that can be accomplished in less than 1½ hr. Alternatively a commercial sample of the enzyme may be used. The experiment is equally suited to students of biochemistry, organic, and physical chemistry. Sophisticated equipment is not required, and a single beam spectrophotometer is adequate for the assay.

Experimental

The whole experiment takes about 4 hr, but this time can be halved by having students do either the acid- or the enzyme-catalyzed reaction and exchange their data. The following quantities are sufficient for a group of 12 students. Emulsin type III (5 units per mg) was obtained from the Sigma Chemical Company.

Enzyme-Catalyzed Reaction

Solutions of emulsin (0.025 g) in 70 ml of 0.1 *M* phosphate buffer, pH 6, and of salicin (0.67 g) in 100 ml of the same buffer are equally divided into reagent bottles and placed in 30°C and 40°C water baths and allowed to equilibrate at the start of the class. Each student prepares a set of 14 numbered test tubes containing 10 ml of 2 *M* NaOH. The enzyme reaction is started by mixing 7.5 ml of the salicin and 2.5 ml of the emulsin solutions in a boiling tube at one of the two temperatures and the time is noted. A 0.3-ml sample is removed immediately and dispensed into the first test tube. Thereafter, 0.3-ml aliquots are removed at intervals of 3 min until a total of seven test tubes has been used. The entire procedure is repeated at 40°C using the remaining seven test tubes. The optical density at 290 nm of all the tubes is read against a blank of 7.5 ml of sodium hydroxide and 2.5 ml of distilled water. The zero-order rate constants for each temperature are obtained by plotting the optical density against time (see Fig. 1). The slope of the straight line obtained during the initial stages is the zero-order rate constant.

Acid-Catalyzed Reaction

A solution of salicin (0.67 g) in 100 ml of solution containing 80% water and 20% 1-propanol is divided between two water baths at 65°C and 75°C and allowed to equilibrate as before. A set of 15 numbered test tubes containing sodium hydroxide is prepared as described above. The reaction is started by mixing 7.5 ml of the salicin solution and 2.5 ml of concentrated hydrochloric acid in a lightly covered boiling tube in the bath at 65°C. Aliquots of the reaction mixture are removed and quenched as before except that 5-min intervals are necessary due to the slower rate of reaction. The whole procedure is repeated at 75°C using the next 7 test tubes, withdrawing samples at 3-min intervals. Finally, the reaction flask is placed in a boiling water bath for 15 min to obtain a wholly hydrolyzed sample. 0.3 ml of this solution is removed and placed in the last test tube. The first-order rate constants are obtained by subtracting all the optical density readings at each temperature from that of the fully reacted sample and plotting the logarithm of the resulting values against time (see Fig. 2). With careful planning the sampling can be speeded up by performing reactions at two temperatures simultaneously and interspersing sample withdrawals.

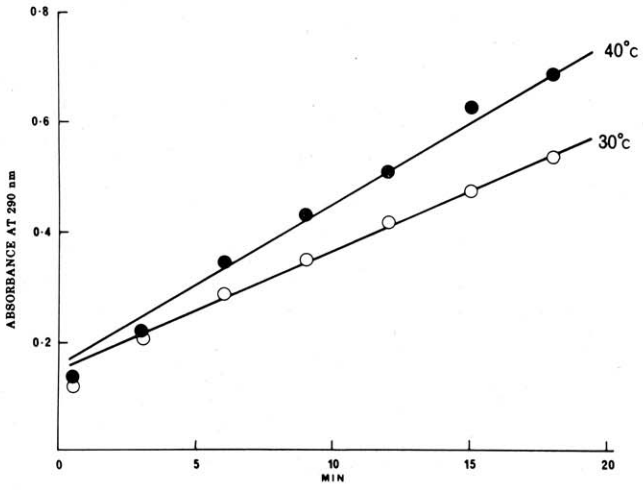


Figure 1. Zero-order plot for the enzyme-catalyzed reaction at 30°C and 40°C.

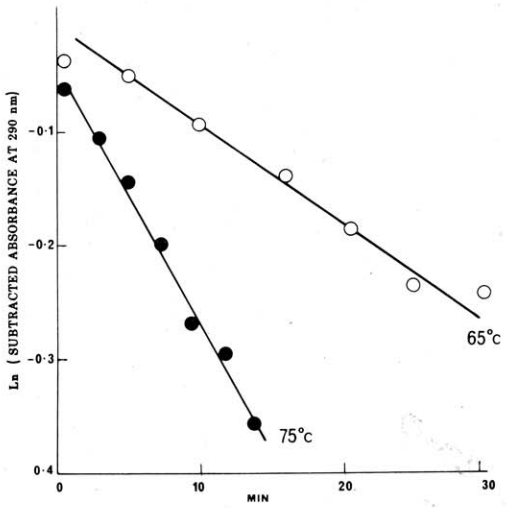


Figure 2. First-order plot of the acid-catalyzed reaction at 65°C and 75°C.

Results and Discussion

The energy of activation E_a for the enzyme and acid-catalyzed hydrolyses can be readily calculated from the following form of the Arrhenius equation:

$$E_a = \frac{RT_2T_1 \ln k_2/k_1}{T_2 - T_1}$$

where k_2 and k_1 are the rate constants at the upper and lower temperatures, T_2 and T_1, respectively. Values obtained for E_a were around 25 kcal/mole for the acid-catalyzed hydrolysis and 6 kcal/mole for the enzyme-catalyzed reaction. The latter value is typical of activation energies reported for diffusion-controlled reactions in water (*7*), thus the efficiency of emulsin as a catalyst is probably underestimated when present at the concentration used in the procedure described above. However, the experimentally determined values clearly demonstrate the remarkable effect of the enzyme on the activation energy. The values of activation energies obtained in this experiment are in broad agreement with early published data for these reactions (*8*) and are consistent with data for the acid hydrolysis of other glucopyranosides (*4*) and with the activation energies for other enzyme-catalyzed reactions (*8–12*). Students have consistently obtained good results. Accuracy in maintaining the temperature of the water baths and in the removal of samples was important for consistent results. We would recommend the use of an automatic pipet for this purpose. Thorough mixing of the quenched samples was also essential. The chore and inaccuracy of plotting kinetic data can be removed by the application of a least squares linear regression analysis computer program.

It is important to emphasize that the Arrhenius activation energy (E_a) takes no account of entropy effects. A useful extension of this experiment would be the calculation of the entropy ($\Delta S^\ddagger$) and other activation parameters from the rate constants using the expression (*13*)

$$k = \frac{e\mathbf{k}T}{h} \cdot e^{\Delta S^\ddagger/R} \cdot e^{-E_a/RT}$$

where **k** and h are, respectively, Boltzmann's and Planck's constants. Using the standard relationship

$$\Delta G^\ddagger = \Delta H^\ddagger - T\Delta S^\ddagger$$

and

$$E_a = \Delta H^\ddagger + RT$$

the free energy ($\Delta G^\ddagger$) and enthalpy ($\Delta H^\ddagger$) of activation can be readily obtained. In most cases the values of $\Delta H^\ddagger$ and $\Delta S^\ddagger$ are such that $\Delta G^\ddagger$ does not vary greatly for widely different enzymic reactions. Thus, if $\Delta H^\ddagger$ is large for a particular enzyme reaction the value of $\Delta S^\ddagger$ will tend to be large and positive whereas if $\Delta H^\ddagger$ is small then $\Delta S^\ddagger$ will be negative. Unfortunately, it is often not made clear in textbooks which ac-

tivation energy, $\Delta G^{\ddagger}$, $\Delta H^{\ddagger}$, or E_a, is referred to in graphs depicting energy profiles of enzyme-catalyzed reactions.

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