THE ACTIVATION ENERGY OF UREA HYDROLYSIS CATALYZED BY SOY BEAN UREASE

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The rate of most chemical reactions increases exponentially with temperature in accordance with the Arrhenius equation. The older literature on enzyme-catalyzed reactions (for references cf. Haldane (1930), Tauber (1937)) indicates that these do not conform, since the activation energy decreases with rise in temperature instead of being invariant. More recent studies do not confirm this general statement, however, but indicate that the reaction velocity of certain enzyme systems increases with temperature in conformity with the Arrhenius equation up to the inactivation temperature of the enzyme (Bodansky, 1939; Craig, 1936; Crozier, 1924; Gould and Sizer, 1938; Hadidian and Hoagland, 1939; Sizer, 1937, 1938, 1939). Bodansky (1937) has emphasized the fact that in much of the earlier work little attention was paid to pH control, and velocity constants were often improperly computed.

A comparison of the activation energies of catalytic systems in which the enzyme has been obtained from different species can yield valuable information concerning the biochemical relationships of enzymes. Sizer (1937) reported an activation energy of 13,000 calories per gm. mole for sucrose hydrolysis by malt invertase as compared with 11,000 calories for yeast invertase (1938). Bodansky (1939), on the other hand, obtained a value of 9940 calories for the hydrolysis of sodium β-glycerophosphate by either human or cat bone phosphatase. Activation energies of either 8700 or 11,700 calories were reported (Sizer, 1939) for urea hydrol-

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ysis by crude or crystalline jack bean urease. With this figure may be compared the calculation of von Euler (1920) of 20,800 calories, later corrected to 12,000 (1922), from the data of Van Slyke and Cullen (1914) on soy bean urease. The latter figure was checked by Sizer (1939) who obtained a value of 11,700 calories.

In this study the hydrolysis of urea catalyzed by soy bean urease has been investigated as a function of temperature in order to compare the activation energy with those of the jack bean urease system. If jack and soy bean ureases are comparable, it might be possible to obtain activation energies of both 8700 and 11,700 calories under the proper conditions for soy as well as jack bean urease.

EXPERIMENTAL

The urease was purified from either Arlco or Cellu soy bean flour. Enzyme solutions were prepared according to the method of Van Slyke and Cullen (1914) by suspending the flour in water and separating the urease solution from the insoluble material by filtration or centrifugation. In addition to this aqueous solution of urease a large number of more highly purified preparations were made. These were prepared by successive precipitations of the urease by various concentrations of acetone, alcohol, and ammonium sulfate. After each precipitation the enzyme was dissolved in water. In some cases the final urease solution was dialyzed free of non-colloidal material. Besides the studies which were made on the urease purified to various degrees from two different soy bean flours, tests were also performed in which unmanipulated and unmodified urease as present in the bean (yellow variety) was used. The temperature kinetics of unextracted urease in the soy bean were compared with jack bean urease in a similar state.

A stock solution was prepared which contained 3 per cent urea, 5.4 per cent Na₂HPO₄, and 4.25 per cent KH₂PO₄. The phosphate buffered the digest to pH 7.0 (optimum pH for urease activity) and kept the alkalinity produced by the liberated NH₃ from increasing by more than 0.1 pH unit while the reaction was being

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studied. To 2 ml. of urea-phosphate solution were added 1 ml. of enzyme solution and 1 ml. of stabilizer which was usually an oxidizing or a reducing solution. The stabilizer was added in view of the fact that the urease molecule is unstable and can be readily activated or inactivated by the addition of reducing or oxidizing agents (Hellerman, 1937). The activation energy of the jack bean urease-urea system is 11,700 calories when oxidizing agents are present, but is 8700 calories in the presence of neutral or reducing agents (Sizer, 1939).

During the course of urea hydrolysis the liberated NH₃ dissolves in the solution, while the CO₂ is evolved and can be measured manometrically (Van Slyke, 1927; Krebs and Henseleit, 1932). Hydrolysis follows the same apparent course whether studied by measurement of NH₃ production colorimetrically after nesslerization or CO₂ evolution manometrically with the Barcroft differential manometer (Sizer, 1939).

The 4 ml. of reaction mixture were placed in one cup of the manometer and 4 ml. of water in the control cup. From 2 to 3 minutes adaptation to the temperature of the water bath were allowed before the stop-cocks were closed. From ten to fifteen manometer readings were taken at each temperature during the time required for the evolution of 100 to 500 c.mm. of gas. The temperature of the water bath was controlled to ±0.05°. A pressure change of 1 mm. on the manometer was found to correspond to a change in volume of 2.6 c.mm. Since the number of molecules in a unit volume of gas at a given pressure varies with the temperature, it was necessary to convert the gas volumes to the standard temperature of 0° to make them comparable.

Results

In Fig. 1 is plotted CO₂ evolution as a function of time for a solution containing 2 ml. of urea-phosphate, 1 ml. of water, and 1 ml. of urease (saturated (NH₄)₂SO₄ precipitate of a 30 per cent aqueous extract of Cellu soy bean flour). From the figure it is apparent that the reaction follows a linear course during the first phase of the hydrolysis at all temperatures between 0.2-50°. Rates of hydrolysis were calculated from the slopes of the straight lines drawn through the plotted points and expressed as ml. of CO₂ evolved per minute. This proved to be an accurate method
of determining rate, since errors of separate readings are largely eliminated.

While the data of Fig. 1 are typical, there were occasional enzyme preparations which were so unstable that the plotted points fell off from a straight line after only 0.2 to 0.3 ml. of CO₂ had been evolved. In a few experiments a brief lag occurred at the start of the reaction. In all cases, however, it was possible to calculate rate of hydrolysis from the linear portion of the curve.

**FIG. 1.** Hydrolysis (as measured by ml. of CO₂ evolved) of 1.5 per cent urea (in phosphate buffer, pH 7.0) by soy bean urease is plotted as a function of elapsed time in minutes for several different temperatures. The reaction follows a linear course at all temperatures.

In Fig. 2 log rate of hydrolysis is plotted against the reciprocal of the absolute temperature. Since the plotted points are best fitted by a straight line, it is clear that the data are in accord with the Arrhenius equation

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\mu = \frac{2.303R \log (k_2/k_1)}{1/T_1 - 1/T_2}
$$

where \( \mu \) is the energy of activation in calories per gm. mole, \( R \) the gas constant, and \( k_1 \) and \( k_2 \) the rates at the respective absolute
Fig. 2. Log rate of urea hydrolysis by soy bean urease plotted against the reciprocal of absolute temperature. The distribution of the curves along the ordinate is arbitrary. Data of Van Slyke and Cullen on hydrolysis as measured by NH₃ formation, Curve 1; ○, dilute urease solution used; □, concentrated urease solution used. Hydrolysis as measured by CO₂ evolution, Curves 2 to 7. For details of each urease preparation see the text. In each experiment the digest contained 2 ml. of urea-phosphate, 1 ml. of urease, and 1 ml. of one of the following: ○, H₂O; ×, 0.2 M K₃Fe(CN)₆; ⊙, 0.2 M K₄Fe(CN)₆; □, 0.14 M sulfite; ●, 0.2 M Na₂S₂O₅; △, 0.2 M KCN.
temperatures $T_1$ and $T_2$. The distribution of the curves along the ordinate is purely arbitrary. The slope of Curves 1, 2, and 3 corresponds to an activation energy of 11,700 calories. In Curve 1 are presented the data of Van Slyke and Cullen (1914) who followed hydrolysis by distilling off and titrating the liberated NH$_3$. The velocity constants of the data for concentrated urease have been adjusted so that the 20$^\circ$ point coincides with that for the dilute urease. The 60$^\circ$ point is doubtless low because of temperature inactivation of the enzyme which occurs between 40–60$^\circ$, depending upon a variety of factors. In Curve 2 are presented the data of Fig. 1, as well as those procured when 1 ml. of 0.2 M K$_3$Fe(CN)$_6$ or 1 ml. of 0.2 M K$_4$Fe(CN)$_6$ was used as stabilizer instead of 1 ml. of H$_2$O. For Curve 3 the Arlco soy bean urease was purified from the aqueous extract by successive precipitation with saturated (NH$_4$)$_2$SO$_4$, 50 per cent alcohol, and 50 per cent acetone. Water or a 0.14 M mixture of Na$_2$SO$_3$ and NaIIISO$_3$ (pH 7.0) was used as stabilizer.

Strikingly different results are presented in Curves 4 and 5 of Fig. 2 where the data fit straight lines with a corresponding activation energy of 8700 calories. For the data of Curve 4 the urease was a 50 per cent acetone precipitate of a 25 per cent aqueous suspension of Arlco flour; 0.2 M Na$_2$S$_2$O$_3$ was the stabilizer. For Curve 5 the urease was purified by successive precipitation of a 10 per cent acetone extract of Arlco flour by 0.5 saturation with (NH$_4$)$_2$SO$_4$, 50 per cent acetone, 0.5 saturated (NH$_4$)$_2$SO$_4$, and 40 per cent alcohol. The stabilizers were H$_2$O, 0.2 M K$_3$Fe(CN)$_6$, and 0.2 M KCN.

Two straight lines intersecting at a critical temperature best fit the plotted points of Curves 6 and 7 of Fig. 2. The corresponding activation energies are 11,700 calories below and 8700 above the critical temperature. For Curve 6 the urease was the same as for Curve 3, but the stabilizers were 0.2 M K$_3$Fe(CN)$_6$ and 0.2 M K$_4$Fe(CN)$_6$. For Curve 7 the urease was a 0.5 saturated (NH$_4$)$_2$SO$_4$ precipitate of a 30 per cent suspension of Cellu flour. The stabilizers were 0.2 M K$_3$Fe(CN)$_6$ and 0.2 M KCN.

In a small number of experiments an activation energy of 7000 calories was obtained over the whole temperature range. It is not known whether this represents a new value for the familiar urease-urea system, or whether this figure is indicative of a differ-
ent urease enzyme which is sometimes present in soy beans. With regard to this it is interesting to note that the activation energy of 7000 calories was never obtained with Cellu soy meal, and never with Arlco soy bean urease after it had been highly purified.

From Fig. 2 as well as from 67 additional temperature studies not shown in the figure, it is apparent that the activation energies for the soy bean urease-urea system are 11,700 or 8700 calories over the whole temperature range, or 11,700 below and 8700 above a critical temperature. It appears that the particular value obtained depends upon the following factors: (1) The source of the soy bean flour. One encounters $\mu = 11,700$ calories more frequently with Cellu than with Arlco flour. (2) The method and degree of purification of the urease. Curves 3 and 5 (where water was the stabilizer) are characterized by respective $\mu$ values of 11,700 and 8700 calories; yet the only difference in the two series of experiments was the manner in which the urease was purified. (3) The stabilizer added to the reaction mixture. Data for curves 3 and 6 were procured simultaneously, the only difference being the stabilizer employed; yet the temperature effects are very different in the two cases.

Since either of two activation energies can be obtained for the soy bean urease-urea system, it is of interest to learn which of these two values characterizes the urease as it naturally occurs in the bean, where there is no possibility of the urease becoming modified by milling and extraction procedures. For this purpose the protective seed coat was removed from a single bean, the two halves (cotyledons) separated and soaked overnight in either 0.4 m K$_3$Fe(CN)$_6$, or 25 per cent acetone, or water saturated with toluene. These inhibitors suppressed respiration so that volume changes in the flask were due only to CO$_2$ liberated from urea. Each bean was placed in a separate Barcroft flask to which were added 2 ml. of urea-phosphate and 2 ml. of the respective inhibitor. After each temperature run the digest was discarded and the bean rinsed three times with water before being used again.

The CO$_2$ evolution was a linear function of time, just as was the case for hydrolysis by the various urease solutions prepared from the flour. In an Arrhenius plot of the data (Fig. 3) the points fall along a straight line which has a slope corresponding to $\mu = 11,700$
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calories, when water saturated with toluene is the stabilizer (Curve 1), and to \( \mu = 8700 \) calories when 12.5 per cent acetone or 0.2 M \( \text{K}_2\text{Fe(CN)}_6 \) is present (Curve 2). In Curve 3 are presented the data for the same experiment with the same inhibitors, but with one-fourth jack bean (one-half cotyledon) substituted for one soy bean. Scrutiny of the figure indicates that the data conform to the Arrhenius equation where \( \mu = 8700 \) calories.

**DISCUSSION**

Duplicate experiments in some cases were performed at all six temperatures. With different stabilizers two, three, or four separate studies were made on a single enzyme preparation at each

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**Fig. 3.** Log rate of CO\(_2\) evolution from urea catalyzed by urease not extracted from the bean. The Barcroft flasks contained 2 ml. of urea-phosphate plus 2 ml. of one of the following: O, water saturated with toluene; X, 25 per cent acetone; \( \Delta \), 0.4 M \( \text{K}_2\text{Fe(CN)}_6 \). Curves 1 and 2, one soy bean added to digest; Curve 3, one-fourth jack bean added to digest.
temperature. In duplicate experiments the manometer readings did not differ from the average by more than 5 per cent. The straight lines drawn through the plotted points were fitted by “eye,” a method which checks that of the “least squares” within about 2 per cent (Hoagland, 1936). The activation energies are accurate to about ±200 calories.

It appears significant that the activation mechanism for the urease system from two different genera of legumes, *Canavalia ensiformis* and *Glycine hispida*, is essentially identical for the two enzymes. In the case of crystalline jack bean urease an activation energy of 8700 calories was associated with neutral or reducing agents and \( \mu = 11,700 \) calories with oxidizing agents present in the digest. No such correlation between activation energy and oxidation-reduction potential is apparent for crude soy bean urease, however, in which other factors complicate the situation. For both enzymes a shift in activation energy could be elicited by modifying the composition of the digest. Under certain conditions with both enzymes two activation energies characterize the system with \( \mu = 11,700 \) calories below and \( \mu = 8700 \) calories above the critical transition temperature. Although enzymes from different species may not be immunologically identical (Northrop, 1939), they are very similar chemically and physically and may have the same mechanism of activation as is indicated by the urease and phosphatase systems.

A study of the activation energy of an enzyme from several unrelated species must be made to determine whether or not the identity of activation energy for a single enzyme from different species is a general phenomenon. For some enzyme systems it seems likely that the activation energy is independent of the species, since for a number of physiological processes the \( \mu \) values are the same for many different organisms and probably can be referred to the underlying pacemaker enzyme-catalyzed reactions which determine physiological rates (Gould and Sizer, 1938; Hadidian and Hoagland, 1939).

**SUMMARY**

The kinetics of urea hydrolysis have been studied with urease partially purified from two different samples of soy bean flour. The course of the reaction was followed by measuring \( \text{CO}_2 \) evolu-
tion with the Barcroft manometer. At all temperatures CO₂ liberation is a linear function of elapsed time.

Over the temperature range from 0.2–50° the data are in accord with the Arrhenius equation in which the energy of activation is either 8700 or 11,700 calories per gm. mole, depending on the composition of the digest. With certain enzyme preparations the activation energy was 11,700 calories below and 8700 calories above a critical temperature. The identity of activation energies for the soy bean and jack bean urease-urea system has been pointed out.

When a temperature study was made of hydrolysis by unextracted urease still present in the soy or jack bean, the activation energies were identical with those for the extracted and purified enzymes.

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