TEMPERATURE ACTIVATION OF THE UREASE-UREA SYSTEM USING CRUDE AND CRYSTALLINE UREASE*

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(Accepted for publication, April 5, 1939)

Many studies of the kinetics of enzyme action as a function of temperature have been made. The older work (for reference cf. Haldane, 1930; Tauber, 1937) indicates that while the velocity of an enzyme-catalyzed reaction increases with rise in temperature, up to the inactivation temperature of the enzyme, this change in rate is not an exponential function of the absolute temperature. It has been pointed out by Bodansky (1937), however, that in many cases the velocity constants for enzyme reactions have been improperly computed. Certain recent work, on the other hand, (Crozier, 1924; Craig, 1936; Sizer, 1937, 1938 a, b; Gould and Sizer, 1938) indicates that for the fat oxidase from Lupinus albus, for yeast invertase either partially purified or present in the living cells, and for the anaerobic dehydrogenase system of E. coli, the reaction velocity increases exponentially with temperature according to the Arrhenius equation

\[ K = e^{-\mu/RT} \]

where \( K \) is the rate of the reaction, \( z \) is a constant, \( e \) is 2.718, \( R \) is the gas constant, \( T \) is the absolute temperature, and \( \mu \) represents the energy of activation of the reaction in calories per gram molecule. The \( \mu \) value is constant over a wide range of temperature until an inactivation temperature is reached; it is characteristic of the specific enzyme used, but is independent of the substrate employed and is not influenced by the conditions under which the reaction is carried out (Sizer, 1937, 1938 a), and is the same whether the enzyme is active

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inside the cell or extracted from the cell (Sizer, 1938 b). A particular critical increment might not characterize a single enzyme under all environmental conditions, because the conditions for activation of participating groups of the enzyme may be altered in such ways that one or another of several characteristic values could be secured.

No studies of the temperature activation of enzyme systems as a function of the purity of the enzyme have been made. If it were demonstrated that the temperature characteristic of an enzyme system varied with the purity of the enzyme, it would no longer be possible to say that a single μ value characterizes a given enzyme system. In this investigation a study has been made of the relationship between enzyme purity and the temperature characteristic of the urease-urea system in the presence of a variety of salts, especially those active in oxidation-reduction phenomena. The following urease preparations were used: an aqueous extract of jack bean meal, commercial urease (Arlco), and crystalline urease prepared according to the methods of Sumner (1926; Sumner and Hand, 1928).

**EXPERIMENTAL**

**Enzyme Preparations**

(a) *Jack Bean Meal Urease.*—30 gm. Arlco jack bean meal were suspended in 0.068 M sulfite (0.6363 per cent Na₂SO₄ and 0.1416 per cent NaHSO₃) and filtered. The filtrate was diluted with 0.068 M sulfite until a solution with the proper activity was obtained.

(b) *Commercial Urease (Arlco).*—A stable glycerol suspension was prepared according to the method of Koch (1937). Other preparations of Arlco urease were suspensions (either after filtering or without filtering) in water or sulfite (0.068 M was always used). As will appear later, it made considerable difference in the case of the sulfite solution whether the dry urease powder was added to a sulfite solution directly, or to water to which sulfite was added subsequently.

(c) *Purified Commercial Urease.*—Arlco urease was purified according to the methods of Sumner for preparing crystalline urease, and was reprecipitated once, but the product did not appear crystalline.

(d) *Crystalline Urease.*—Sumner's method for preparing crystalline urease from jack bean meal was followed closely. The meal was suspended in 32 per cent acetone and filtered in the refrigerator. The crystals which separated overnight in the filtrate were centrifuged free of the fluid, washed with 32 per cent acetone, and centrifuged again. The urease was recrystallized by dissolving the crystals in a small volume of water; the solution was centrifuged free of insoluble material, and made up to 32 per cent with acetone. Finally, a few
drops of phosphate buffer, pH 6.0, were added slowly. The crystals which formed were separated by centrifugation. Five separate batches of crystalline urease were prepared. In preparing the last two, 300 gm. of meal were used instead of the customary 100 gm. In the case of preparations 3 and 4, urease fractions which had not been recrystallized were used in addition to the fraction which was once recrystallized. Preparation 5 was not recrystallized.

The activities of the crystalline urease preparations were not as high as those recorded by Sumner (100,000 to 130,000 units) ranging from about 1,000 to 55,000 units per gram. The lower activities may be attributed to the fact that high urease activities are recorded only when extremely concentrated urease solutions are used, but in these experiments only dilute urease solutions were suitable for use. It is also very likely that slight traces of metal impurities may have caused a slight inactivation or denaturation of the urease.

Estimation of Urease Activity

A solution was prepared which contained 3 per cent urea, 5.4 per cent Na₃HPO₄, and 4.25 per cent KH₂PO₄ and preserved with a few drops of toluene. 5 ml. urea-phosphate were added to 5 ml. urease solution after both solutions were adapted to the temperature of the water bath. At successive intervals 1 ml. samples were removed from the digest and added to 1 ml. normal HCl. The solution was diluted to about 75 ml. to which were added 10 ml. Nessler's solution, and it was finally diluted to 100 ml. The solution was then compared in a colorimeter with a standard {\(\text{NH}_4\text{SO}_4\)} solution treated in a similar manner containing 0.4 mg. nitrogen (only 0.2 mg. when the urease was very inactive) and also containing urea and phosphate so that the colors to be matched would be similar. In order to compensate for the fact that the weight of digest in the pipette varies slightly with the temperature of the digest, the standard was also adjusted to the temperature of the water bath. Thus the weight of digest and of standard were comparable at all temperatures. The temperature of the water bath was controlled to \(\pm 0.05^\circ C\).

The solution was buffered with phosphate to pH 7.0 with the result that the alkalinity did not increase by more than 0.1 pH unit during the reaction. The hydrolysis was usually terminated after 0.5 mg. ammonia nitrogen had been liberated. At each temperature the ammonia nitrogen was measured at seven different time intervals which elapsed before the 0.5 mg. ammonia nitrogen had been liberated.

Since both crude and crystalline urease decompose rather rapidly in water, a stabilizer was usually added. For this purpose a glycerol extract (cf. Koch, 1937) was used for crude urease, and a mixture of Na₂SO₃ and NaHSO₃ for crystalline urease (Sumner and Dounce, 1937). When it was discovered that the stabilizer might play an active rôlé in determining the effects of temperature upon the reaction, a number of other compounds were substituted; among these were KCN, \(\text{Na}_2\text{S}_2\text{O}_3\), CaCl₂, cystine, cysteine-HCl, \(\text{Na}_2\text{SO}_4\), \(\text{K}_2\text{Fe(CN)}_6\), \(\text{K}_4\text{Fe(CN)}_6\), \(\text{H}_2\text{O}_2\), FeCl₃, I₂, sodium iodoacetate, Cu₂O, and quinone.
RESULTS

The kinetics of urea hydrolysis catalyzed by urease have been analyzed by several workers (Van Slyke and Cullen, 1914; Lövgren, 1921), but there is no general agreement concerning the course followed by the reaction. Many believe that it is unimolecular for at least a part of the time. In this study determinations were made during only the first part of hydrolysis, since in most enzyme reactions this is the most significant part (Haldane, 1930; Nelson, 1933). For all the enzyme preparations both crude and crystalline, in the presence of a variety of salts, and at all temperatures, the rate of liberation of ammonia nitrogen did not vary with time (Fig. 1). This in general held true until 0.6–1.0 mg. nitrogen had been liberated. The rate of ammonia liberation remains constant for a greater or lesser proportion of the hydrolysis, depending upon the particular preparation used and upon the temperature; the constant rate is often of greater duration at higher temperatures. After the linear portion of the reaction the hydrolysis usually becomes retarded, although with one urease preparation an acceleration occurred.

Scrutiny of Fig. 1 indicates that there is no characteristic difference in the kinetics of urea deamination catalyzed by crude and by crystalline urease. All the experimental data were plotted in the manner illustrated and rate of hydrolysis was calculated from the slope of the straight line drawn through the plotted points. This proved a simple and accurate method of determining milligrams of ammonia nitrogen liberated per minute, and duplicate runs usually checked within 5 per cent. While the data might have been analyzed equally as well according to the unimolecular equation, the method used was much simpler.

Temperature Activation of Crude Urease.—The data obtained at different temperatures on urea hydrolysis using a jack bean meal filtrate suspended in sulfite were plotted and rate of reaction expressed as milligrams NH₃ nitrogen liberated per minute. When log rate is plotted against 1/T (Fig. 2, curve 1) the points are best fitted by a straight line over the temperature range of 0 to 40°C. Above the latter the points fall off from the curve indicating temperature inactivation of the urease. The slope of the line corresponds to an energy of activation of 11,700 calories for the urease-urea system. A
solution of Arlco urease was prepared by dissolving 0.67 gm. in 100 ml. sulfite and filtering free from insoluble material. An analysis of

![Graph](https://via.placeholder.com/150)

Fig. 1. Hydrolysis (as measured by mg. NH₃ N liberated) of 1.5 per cent urea in phosphate buffer, pH 7.0, catalyzed by urease dissolved in a sulfite solution, is plotted as a function of elapsed time in minutes for several different temperatures.

Upper Curves.—Obtained using crystalline urease 2.

Lower Curves.—Obtained using the filtrate from a suspension of jack bean meal.

the data on urea hydrolysis by this preparation yielded essentially similar results (Fig. 2, curve 2). Strikingly different results were obtained, however, if the Arlco urease was dissolved in a glycerine
FIG. 2. Log rate of NH₃ liberation from urea catalyzed by crude urease plotted against 1/T.
1. Jack bean meal filtrate in sulfite solution, \( \mu = 11,700 \).
2. Arlco urease in sulfite solution, \( \mu = 11,700 \).
3. Arlco urease in glycerine solution, \( \mu = 8,700 \).
4. Arlco urease in water solution, \( \mu = 8,700 \).
4a. Arlco urease in water + sulfite, \( \mu = 11,700; 8,700 \).
5. Purified Arlco urease in sulfite solution, \( \mu = 11,700; 8,700 \).
solution (cf. Koch, 1937) (Fig. 2, curve 3) or if 0.1 per cent Arlco urease was dissolved in water (Fig. 2, curve 4). The slopes of the plotted curves are much less and both correspond to $\mu = 8,700$ cal. When sulfite was added to the aqueous solution (Fig. 2, curve $4$) the results were distinctly different from those obtained when Arlco urease was added directly to a sulfite solution (Fig. 2, curve 2), or merely to an aqueous medium (Fig. 2, curve 4). A break appears in the curve at 25°C.; over the temperature range of 0–25°C. the slope of the straight line which fits the plotted points corresponds to $\mu = 11,700$, above 25°C. $\mu = 8,700$.

An attempt was made to prepare crystalline urease from 5 gm. of Arlco urease by Sumner's method. The purified product consisted largely of denatured protein, had little activity, and showed no evidence of a crystalline nature when examined microscopically. The purified urease was dissolved in sulfite and its kinetics studied as a function of temperature (Fig. 2, curve 5). An analysis of the data yielded results identical with those obtained with Arlco urease dissolved in water to which sulfite was added subsequently, namely a $\mu = 11,700$ below 26°C. and 8,700 above this temperature. Inactivation did not occur until 65°C. was reached.

It is clear that with crude urease preparations an activation energy of 8,700 calories is obtained when the enzyme is dissolved in water or a glycerine solution. When dissolved in sulfite solution, however, the situation is more complex; under certain conditions a $\mu$ value of 11,700 is obtained over the whole temperature range, while under other conditions the 11,700 value is obtained only below 25–26°C., while above this critical temperature $\mu = 8,700$. It is surprising to observe that the purified urease was inactivated above 65°C. while the crude jack bean meal urease lost its activity above 40°C.

**Temperature Activation of Crystalline Urease Dissolved in Sulfite Solution**

Crystalline urease preparations 1 and 2 were dissolved in sulfite solution and their kinetics studied as a function of temperature (Fig. 3, curves 1 and 2). The results obtained by making an Arrhenius plot of the data on rate of hydrolysis of urea as a function of temperature were identical with those procured with highly purified Arlco
Fig. 3. Log rate of NH₃ formation catalyzed by crystalline urease dissolved in sulfite solution plotted against 1/T.

1. Crystalline urease 1, \( \mu = 11,700; 8,700 \).
2. " " 2, \( \mu = 11,700; 8,700 \).
3. " " 3a, \( \mu = 11,700; 8,700 \).
4. " " 3, \( \mu = 11,700 \).
5. " " 3 (partially inactivated), \( \mu = 8,700 \).
6. " " 4a (dissolved first in water then SO₃), \( \mu = 11,700 \).
7. " " 4a in sulfite + 0.034 M CaCl₂, \( \mu = 11,700; 8,700 \).
8, 9. " " 4a in sulfite + excess CaCl₂, \( \mu = 11,700 \).
urease or Arlco urease dissolved in water to which sulfite was subse-
sequently added. Straight lines intersecting at 21° best fit the plotted
points; below this critical temperature \( \mu = 11,700 \), above it is 8,700.
Anomalous results were obtained with the third preparation of crys-
talline urease dissolved in sulfite solution. \( \mu = 11,700 \) was obtained
over the whole temperature range (Fig. 3, curve 4). 5 days later
when the preparation was used once more the urease activity was only
\( \frac{1}{2} \) of the former value. While the data obtained on this partially
inactivated enzyme were unreliable, there is little doubt that the \( \mu 
\) value has now completely changed to 8,700 (Fig. 3, curve 5). The
fraction of this same crystalline urease 3 which had not been re-
crystallized (called No. 3a) behaved exactly like No. 1 and No. 2
with the usual two \( \mu \) values of 11,700 and 8,700 below and above 22°C.
(Fig. 3, curve 3).

The studies on crystalline urease 4 in sulfite were made on that
fraction which redissolved in a small volume of water but did not re-
crystallize from solution on adding acetone and phosphate buffer
(this fraction is referred to as No. 4a). A small sample of No. 4a
stock solution was diluted with sulfite and a \( \mu = 11,700 \) was obtained
over the whole temperature range (Fig. 3, curve 6). Another sample
in sulfite was made up to 0.034 M with CaCl₂. A precipitate of CaSO₄
formed. The solution now appeared to exhibit the double \( \mu \) situation,
although the data are not very extensive (Fig. 3, curve 7). The next
day a \( \mu = 11,700 \) prevailed over the whole temperature range (Fig. 3,
curve 8). An addition of more than enough CaCl₂ to precipitate all
the sulfite caused no further change in the solution (except that the
activity fell somewhat), although it had stood for 2 days before being
tested again (Fig. 3, curve 9).

It appears from Fig. 3 (curve 6) that if 0.068 M sulfite solution is
added to an aqueous solution of crystalline urease the preparation is
characterized by a \( \mu = 11,700 \). A study was made to determine the
effects of changing the sulfite concentration upon the temperature
characteristic. The data obtained using crystalline urease 5 indicated
that over the sulfite concentration range from 0.0068 to 0.0306 M
\( \mu = 8,700 \), in the range from 0.034 to 0.272 M \( \mu = 11,700 \). A transition
from one value to the other occurs at a sulfite concentration be-
tween 0.0306 and 0.034 M.
In general, it may be stated that when crystalline urease is dissolved in a sulfite solution, \( \mu \) values of either 11,700 or 8,700 prevail over the whole temperature range, or 11,700 below and 8,700 above the critical temperature of 21–23°C. Modifications in the urease solution may occur which effect a change from one activation energy to the other.

**Temperature Activation of Crystalline Urease Dissolved in Sulfite Solutions**

The preceding experiments on crude and crystalline urease suggest that the activation energy of the urease-urea system is determined by the configuration of the active groups in the enzyme molecule. It is well known that urease contains labile sulfur (Sumner and Poland, 1933) and that urease is readily inactivated (presumably by the oxidation of the SH to S-S) by oxidizing agents (Hellerman, Perkins, and Clark, 1933). As in the case of other enzymes of this type (cf. Hellerman, 1937) if oxidation has not proceeded too far, the urease may be reactivated by the addition of reducing agents. Since the activity of crystalline urease is normally unaffected or only mildly increased by the addition of reducing agents, it appears that normally the urease is already in the reduced form. One interpretation of these experiments on the temperature activation of crude and crystalline urease is that when crude urease is dissolved in water or a glycerine solution, the urease active grouping is in a reduced state for which an activation energy of 8,700 is obtained, while when either crude or crystalline urease is dissolved in a sulfite solution, the enzyme may be in either a reduced form \( (\mu = 8,700) \) or a partially oxidized form \( (\mu = 11,700) \) depending on the temperature and other conditions. The change from one to the other is reversible, but never does urease behave kinetically as though both reduced and partially oxidized forms were active simultaneously at a single temperature and under the same conditions. This is evidenced by the fact that there is under no circumstance a blending of the two \( \mu \) values, the temperature characteristic is always either 8,700 or 11,700, never intermediate between the two.

In order to test the hypothesis that the activation energy is related to the configuration of certain active groups in the urease molecule, the urease medium was altered in a variety of ways calculated to stabilize urease in either its reduced \( (\mu = 8,700) \) or partially oxidized form \( (\mu = 11,700) \). In all these experiments crystalline urease 4
was used (fraction 4 a is the fraction which redissolved in a small portion of water, No. 4 b the fraction which did not redissolve in a little water, No. 4 c the fraction which was recrystallized once). The urease preparations were dissolved in a small volume of water. Samples of these stock suspensions were diluted in salt solutions so that the final solution was 0.068 M in salt. The following solutions completely inactivated the urease and their effects upon urease kinetics could not be measured: FeCl₃, cysteine-HCl, CuO (saturated solution), quinone (saturated solution), I₂ (saturated solution), sodium iodoacetate, and H₂O₂. The inactivating action of the cysteine-HCl may have been due to the unneutralized acid, of iodoacetate due to a trace of iodine present. A 0.0137 M H₂O₂ solution only partially inactivated the urease and so this preparation was used in a kinetic study.

Crystalline urease 4 a dissolved in a KCN solution yielded a $\mu = 8,700$ over the whole temperature range (Fig. 4, curve 1). This same value was unchanged after an interval of several days. Similar results were obtained with recrystallized urease 4 c (Fig. 4, curve 2). In this case, however, the temperature characteristic was not stable and changed overnight to 11,700 (Fig. 4, curve 3). A $\mu$ value of 8,700 was also obtained over the whole temperature range when crystalline urease 4 b was dissolved in solutions of the actively reducing agents, Na₂S₂O₄ (Fig. 4, curve 4), and K₄Fe(CN)₆ (Fig. 4, curve 8), the indifferent salt Na₂SO₄ (Fig. 4, curve 6), and the very weakly oxidizing reagent, cystine (Fig. 4, curve 5). An attempt was also made to study the activity as a function of temperature when crystalline urease 4 a was dissolved in water. This was very difficult since crystalline urease is extremely unstable in water (Sumner et al., 1938). By using three separate preparations, however, and studying hydrolysis at only two temperatures with each, it was possible to obtain data which clearly indicated a $\mu = 8,700$ for urease dissolved in water (Fig. 4, curve 7).

It appears that when dissolved in indifferent, actively reducing, or very mildly oxidizing solutions, crystalline urease is in a reduced form with an activation energy of 8,700 calories per gram mol of urease-urea complex.

Oxidizing agents were very difficult to use since most of them completely inactivated the crystalline urease. Reliable data were ob-
Fig. 4. Log rate of \( \text{NH}_3 \) formation catalyzed by crystalline urease dissolved in a variety of solutions, plotted against \( 1/T \). When the enzyme is dissolved in water or solutions of KCN, \( \text{Na}_2\text{S}_2\text{O}_3 \), cystine, \( \text{Na}_2\text{SO}_4 \), or \( \text{K}_4\text{Fe(CN)}_6 \), \( \mu = 8,700 \). In one instance the enzyme dissolved in a KCN solution became slightly inactivated, and \( \mu \) changed from 8,700 to 11,700. When the enzyme was dissolved in \( \text{K}_3\text{Fe(CN)}_6 \) or \( \text{H}_2\text{O}_6 \), \( \mu = 11,700 \).
tained, however, on crystalline urease dissolved in the active oxidizing solution of $K_4Fe(CN)_6$ (Fig. 4, curve 9) and of $H_2O_2$ (Fig. 4, curve 10). The analysis of the data suggests that when the urease is partly inactivated by oxidizing agents, the configuration of the urease molecule has undergone a change so that now the activation energy of the urea-urease complex is 11,700 calories.

The activity of crystalline urease was slightly greater in a sulfite solution than in water. With most of the other reducing reagents the activity was about the same or slightly less than in water. With the oxidizing solutions the urease activity fell to $1/3 (K_4Fe(CN)_6)$ or $1/10 (H_2O_2)$ of the original value. These results are consistent with those obtained by others (Hellerman, 1937).

**Temperature Characteristics of Crystalline Urease As Related to the Redox Potential of the Solution**

From the previous sections it is evident that $\mu = 11,700$ is in general correlated with the presence of oxidizing agents in the urease solution, while $\mu = 8,700$ is associated with reducing or indifferent agents in the solution. If the temperature characteristic of urease is determined by the redox potential of the enzyme solution and not by the type of electrolyte present, it should be possible to alter the $\mu$ value by changing the oxidation-reduction potential. This was done by using crystalline urease dissolved in varying mixtures of $K_4Fe(CN)_6$ and $K_4Fe(CN)_6$. In all cases the total salt concentration was 0.068 M.

The results obtained are not as quantitative as could be desired because of the fact that urease is very unstable in $K_4Fe(CN)_6$ solutions. The rates of urea hydrolysis were calculated in the usual way and an Arrhenius plot made of the data (Fig. 5). The distribution of the curves along the ordinate is purely arbitrary. When the $K_4Fe(CN)_6$ is three or more times as concentrated as $K_4Fe(CN)_6$ a $\mu = 11,700$ is obtained. When the ratio of the two salts is the same or when one was only twice as concentrated as the other $\mu = 11,700$ below and 8,700 above the critical temperature. When $K_4Fe(CN)_6$ is three or more times as concentrated as $K_4Fe(CN)_6$ $\mu = 8,700$ over the whole temperature range.

The oxidation-reduction potentials of the urease-urea solutions were measured with a Beckman pH meter at 26°C. using a platinum elec-
Fig. 5. Log rate of NH₃ liberation from urea catalyzed by crystalline urease dissolved in 0.068 M K₄ or K₄Fe(CN)₆ plotted against 1/T.
1. K₄Fe(CN)₆, E₀ = +0.611 volt, μ = 11,700.
2. K₂/K₄ = 3/1, E₀ = +0.474 volt, μ = 11,700.
3. K₂/K₄ = 2/1, E₀ = +0.459 volt, μ = 11,700; 8,700.
4. K₂/K₄ = 1, E₀ = +0.443 volt, μ = 11,700; 8,700.
5. K₂/K₄ = 1/2, E₀ = +0.420 volt, μ = 11,700; 8,700.
6. K₂/K₄ = 1/3, E₀ = +0.416 volt, μ = 8,700.
7. K₄Fe(CN)₆, E₀ = +0.280 volt, μ = 8,700.
trode. A progressive increase in $E_h$ from $+0.280$ to $+0.611$ volt accompanies an increase from 0 to 100 per cent in $K_+\text{relative to } K_{2\text{Fe(CN)}_6}$. It thus appears that in $-\text{Fe(CN)}_6$ solutions below $E_h = +0.42$ volt a $\mu = 8,700$ is obtained, above $E_h = +0.46 \mu = 11,700$, and when $E_h = +0.42 - +0.46$ volt $\mu = 11,700$ below a critical temperature and 8,700 above that point.

The critical oxidation-reduction potential at which the shift in the activation energy of the urease-urea system occurs will depend somewhat upon the manner in which the enzyme solution is prepared and also upon the particular compound added to stabilize the potential at a given value. Thus under the conditions of the experiment the sulfite solution was neither strongly reducing nor oxidizing; consequently the temperature characteristic was 11,700 or 8,700, or 11,700 and 8,700 depending upon the enzyme preparation used, the manner in which the solution was prepared, the age of the solution, etc. For the urea phosphate solution $E_h = +0.357$ volt; this value was not altered significantly by adding an equal volume of urease in such solutions as $\text{Na}_2\text{SO}_4$, $\text{KCN}$, cystine, glycerol, sulfite, and water, but was markedly lowered to $+0.28$ volt by $\text{Na}_2\text{S}_2\text{O}_3$ and $\text{K}_2\text{Fe(CN)}_6$. It was appreciably elevated to $+0.460 - +0.611$ volt by adding urease dissolved in 0.017 $\text{M H}_2\text{O}_2$ or 0.068 $\text{M K}_2\text{Fe(CN)}_6$. In general it appears that when the redox potential of the urease-urea phosphate system is $+0.36$ volt or lower the temperature characteristic is 8,700, but when the potential is greater than this $\mu$ is 11,700. At critical potentials (as in sulfite solutions) $\mu$ may be either 8,700 or 11,700, or 11,700 below and 8,700 above a critical temperature.

If urease exists in two forms at a given redox potential, then an Arrhenius plot of the data should give a curve that is concave upward. Since this is not the case, urease cannot exist in these two forms simultaneously. At a fixed but critical potential it is assumed that urease is in an unstable condition and can be easily shifted from one form to the other by slight alterations in the thermodynamic equilibria of the system produced by temperature changes or other factors.

**Urease Activity Measured by CO$_2$ Evolution As a Function of Temperature**

In the hydrolysis of urea both ammonia and carbon dioxide are liberated. The ammonia is very soluble and is not evolved until the
solution has become very alkaline. The carbon dioxide, some of which is used to neutralize the ammonia, is fairly soluble in phosphate buffer at pH 7. The solution soon becomes saturated, however, and CO₂ is evolved. This may be measured manometrically, and has been done in carbonate buffer at pH 5.0, by Krebs and Henseleit (1932) who used a Warburg manometer.

![Graph](attachment:image.png)

**Fig. 6.** CO₂ gas (measured in centimeters on the Barcroft differential manometer) liberated from urea catalyzed by 0.1 per cent Arlco urease in an aerated 0.068 M sulfate solution, plotted as a function of elapsed time in minutes. The pressures have not been corrected for temperature differences. 1 cm. = 26 c.mm. gas.

As was the case in the NH₃ determination an equal volume of urease solution was added to the urea-phosphate at pH 7.0. 2 ml. of each were placed in the flask of the Barcroft differential manometer, and adapted to the temperature of the water bath. Manometer readings were taken at successive intervals after closing the stopcocks. The control flask contained 4 ml. distilled water. In all experiments the enzyme solution was 0.1 per cent Arlco urease. A pressure change of
1 mm. on the manometer corresponds to a change in volume of 2.6 c.mm. Since all the determinations in a given series of temperatures were made within a period of 3–4 hours, no correction was made for the slight variations in atmospheric pressure which may have occurred during that time.

In Fig. 6 are plotted the data on CO₂ evolution as a function of time, in minutes. The enzyme solution was urease dissolved in 0.068 M sulfite aerated for 2 days before using. At all temperatures the

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**Fig. 7.** Log rate of CO₂ evolution from urea catalyzed by 0.1 per cent Arlo urease plotted against 1/T. X-duplicate experiment carried out in another manometer.

1. Urease dissolved in 0.068 M sulfite. \( \mu = 11,700 \).
2. Same solution after aeration for 2 days. \( \mu = 11,700 \) below and 8,700 above 22°C.
3. Urease dissolved in water. \( \mu = 8,700 \).
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CO₂ liberated is a linear function of time, as was the case for NH₃ production. Rate of evolution was calculated from the slopes of the lines and was multiplied by a factor which corrected for the fact that the gas evolved was measured at different temperatures. When log corrected rate is plotted against 1/T the points fall along two straight lines intersecting at 22°C. (Fig. 7, curve 2). The slope of the lower line corresponds to a μ = 11,700, for the upper μ = 8,700. For this same enzyme solution before aeration, the μ value is 11,700 over the whole temperature range (Fig. 7, curve 1). With 0.1 per cent Arco urease dissolved in water the data correspond to a μ = 8,700 over the whole temperature range (Fig. 7, curve 3). Similar results were obtained when other substances were added to the enzyme solution; the temperature characteristic was either 8,700 or 11,700, or 11,700 below and 8,700 above a critical temperature. It is clear that the analysis of the data as a function of temperature is the same whether urea hydrolysis is followed colorimetrically measuring NH₃ production, or gasometrically measuring CO₂ evolution.

DISCUSSION

The classical work on urease activity as a function of temperature was performed by Van Slyke and Cullen (1914) using soy bean meal; they followed the hydrolysis by distilling off the ammonia and titrating with acid. From their data Euler (1920) calculated a μ = 20,800 for urease from a Q₁₀ = 3 in the temperature range from 30-40°C. This is the value often quoted in text-books. Calculations made from the original data, however, indicate a constant μ = 11,700 between 10 and 50°C. In a later book Euler (1922) recalculated the same data and obtained a Q₁₀ of 1.91 and a μ of roughly 12,000. Observations by Euler and Brandting (1919) on the activity of urease in the temperature range from 30-40°C. yield a Q₁₀ of 1.92 corresponding to the same μ value. It seems very significant that the μ value for soy bean urease is identical with one of the two values associated with jack bean urease.

The phenomenon of a sharp break with one temperature characteristic above and another below a critical temperature when log rate is plotted against 1/T is common when physiological phenomena are involved (cf. Hoagland, 1936; Sizer, 1936). This sudden change in
temperature characteristic is usually explained by assuming that a change has occurred in the pace-maker for the catenary reactions controlling rate (Crozier, 1924). Thus, in a study of sucrose inversion by bakers' yeast, when a break was obtained with two different $\mu$ values above and below a critical temperature, it was assumed that there exist in yeast two entirely separate enzymes capable of hydrolyzing sucrose, one active below and the other above the critical temperature (Sizer, 1938 b). Such breaks have not been encountered here-tofore in simple enzyme systems, but have always been associated with vital phenomena. The data on urease cannot be explained by the suggestion that two separate ureases with different $\mu$ values are present, since crystalline urease has been used which has been recently shown to consist chiefly of a single homogeneous protein with a molecular weight of 483,000 (Sumner et al., 1938). The only explanation of the results which seems likely is that the configuration of the active groups in the urease molecule determines the activation energy. Labile sulfhydryl groups are probably involved; it is suggested that when they are reduced the activation energy is 8,700 calories, but when oxidized (partially or perhaps completely) it is 11,700 calories. This oxidation or reduction accompanied by a change in $\mu$ is reversible. A similar explanation might apply equally well to a change in temperature characteristic for certain physiological systems. A shift in $\mu$ value could be accounted for equally well by a shift in pace-maker, or a change in the activation energy of a single pace-maker caused by an alteration in the configuration of the catalyst for that step in the reaction.

The actual mechanism by which the activation of the urease molecule is modified by the redox potential is not clear. The effective peripheral groups responsible for the urease activity are probably involved in this activation. While the redox potential may determine the configuration of these groups it must not be thought that at a given potential there is an equilibrium set up between those radicals which are in a reduced and those in an oxidized form. Under such conditions with two different forms of urease active independently, an upward concavity should appear in the Arrhenius plot. The degree of curvature would depend upon the redox potential and a break at a critical temperature could not occur. The results could be explained,
however, on the assumption that under a given environmental condition urease exists exclusively in one form or the other. At critical redox potentials the urease may be very labile, and slight changes

TABLE I

The temperature characteristics ($\mu$ in the Arrhenius equation) are tabulated for urea hydrolysis (as measured by NH$_3$ production) catalyzed by a variety of crude and crystalline enzyme preparations dissolved in a number of different solutions.

<table>
<thead>
<tr>
<th>Urease preparation</th>
<th>Enzyme medium</th>
<th>$\mu$ value</th>
<th>Critical temperature</th>
<th>Inactivation temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>Jack bean meal</td>
<td>Na$_2$SO$_4$ + NaHSO$_3$</td>
<td>11,700</td>
<td>--</td>
<td>40°C</td>
</tr>
<tr>
<td>Arlco</td>
<td>Na$_2$SO$_4$ + NaHSO$_3$</td>
<td>11,700</td>
<td>--</td>
<td>45°C</td>
</tr>
<tr>
<td></td>
<td>Glycerine</td>
<td>8,700</td>
<td>--</td>
<td>50°C</td>
</tr>
<tr>
<td></td>
<td>H$_2$O</td>
<td>--</td>
<td>&gt;40°C</td>
<td></td>
</tr>
<tr>
<td></td>
<td>H$_2$O, then SO$_3$</td>
<td>11,700</td>
<td>25°C</td>
<td>&gt;40°C</td>
</tr>
<tr>
<td>Purified</td>
<td>Na$_2$SO$_4$ + NaHSO$_3$</td>
<td>&quot;</td>
<td>26°C</td>
<td>65°C</td>
</tr>
<tr>
<td>Crystalline 1 &quot; 2</td>
<td>Na$_2$SO$_4$ + NaHSO$_3$</td>
<td>11,700</td>
<td>21°C</td>
<td>45°C</td>
</tr>
<tr>
<td>&quot; 3 a</td>
<td>&quot;</td>
<td>&quot;</td>
<td>&quot;</td>
<td>&quot;</td>
</tr>
<tr>
<td>&quot; 3</td>
<td>&quot;</td>
<td>11,700</td>
<td>22°C</td>
<td>&quot;</td>
</tr>
<tr>
<td>&quot; 3 (partly inactive)</td>
<td>&quot;</td>
<td>8,700</td>
<td>40°C</td>
<td>&quot;</td>
</tr>
<tr>
<td>&quot; 4 a</td>
<td>H$_2$O, then SO$_3$</td>
<td>11,700</td>
<td>--</td>
<td>&gt;40°C</td>
</tr>
<tr>
<td>&quot; 4 a (partly inactive)</td>
<td>SO$_3$ + 0.034 M CaCl$_2$</td>
<td>11,700</td>
<td>23°C</td>
<td>&quot;</td>
</tr>
<tr>
<td>&quot; 4 a</td>
<td>KCN</td>
<td>8,700</td>
<td>--</td>
<td>&quot;</td>
</tr>
<tr>
<td>&quot; 4 c</td>
<td>&quot;</td>
<td>&quot;</td>
<td>&quot;</td>
<td>&quot;</td>
</tr>
<tr>
<td>&quot; 4 c (partly inactive)</td>
<td>&quot;</td>
<td>11,700</td>
<td>--</td>
<td>&quot;</td>
</tr>
<tr>
<td>&quot; 4 b</td>
<td>Na$_2$SO$_4$</td>
<td>8,700</td>
<td>--</td>
<td>&quot;</td>
</tr>
<tr>
<td>&quot; 4 c</td>
<td>Cystine</td>
<td>&quot;</td>
<td>&quot;</td>
<td>&quot;</td>
</tr>
<tr>
<td>&quot; 4 a</td>
<td>Na$_2$SO$_4$</td>
<td>&quot;</td>
<td>&quot;</td>
<td>&quot;</td>
</tr>
<tr>
<td>&quot; 4 a</td>
<td>H$_2$O</td>
<td>&quot;</td>
<td>&quot;</td>
<td>&quot;</td>
</tr>
<tr>
<td>&quot; 4 a</td>
<td>K$_2$Fe(CN)$_6$</td>
<td>&quot;</td>
<td>&quot;</td>
<td>&quot;</td>
</tr>
<tr>
<td>&quot; 4 a</td>
<td>H$_2$O</td>
<td>&quot;</td>
<td>&quot;</td>
<td>&quot;</td>
</tr>
</tbody>
</table>

in the system such as a shift in temperature might cause a conversion of urease from the reduced to the oxidized form or the reverse. With such a mechanism, a shift in the $\mu$ value might occur at a critical temperature. Dr. W. J. Crozier\textsuperscript{1} has suggested the possibility

\textsuperscript{1} Personal communication.
that in order to be activated the effective peripheral groups of urease must first be oxidized and then reduced, cyclically. With such a system oxidizing or reducing agents would modify one or the other phase of the cycle and in this manner fix the activation energy at 8,700 or 11,700 calories per gram mol.

There are no significant differences between the kinetics as a function of temperature for urease in widely different stages of purity, ranging from a suspension of jack bean meal to crystalline urease which has been once recrystallized (see Table I). In previous experiments on temperature activation of invertase it appears that the medium played no rôle (Sizer, 1937), but this is far from true for crystalline urease, where the addition of certain substances can cause a complete change in the activation energy. The oxidation-reduction potential of the medium may function in fixing the \( \mu \) value of an enzyme system by determining which of the active groups of the enzyme will determine the course of the reaction. Recent work on monomolecular films indicates that urease activity is associated with the hydrophobic surfaces of the urease molecule, the hydrophilic surfaces exerting no measurable activity (Langmuir and Schaefer, 1938). This is consistent with the general point of view that certain groupings in the enzyme molecule are responsible for the enzyme activity. From this present work on the temperature activation of urease it appears that by modifying such active groups not only the enzyme activity is altered but also the activation energy of the enzyme may be changed as well.

SUMMARY

1. The hydrolysis of urea catalyzed by jack bean meal has been followed by determining colorimetrically after Nesslerization the ammonia nitrogen, and volumetrically the carbon dioxide liberated at successive intervals during the reaction. During the early part of hydrolysis the rate of ammonia or carbon dioxide liberation is constant for all the urease solutions which were used.

2. When log rate of \( \text{NH}_3 \) or \( \text{CO}_2 \) formation was plotted against \( 1/T \), the points fell along a straight line, the slope of which corresponded to an activation energy of either 8,700 or 11,700 calories per gram mol. Frequently urease, when dissolved in sulfite solution, was
characterized by an activation energy of 11,700 below and 8,700 above the critical temperature of about 23°C. At high temperatures the plotted points fell off from the curve due to temperature inactivation.

3. Essentially the same results on temperature activation were obtained with crude jack bean meal, Arlco urease, crystalline urease not recrystallized, and crystalline urease once recrystallized. The temperature characteristic which was obtained depended in part upon the composition of the medium. When dissolved in water, or aqueous solutions of glycerine, KCN, Na₂S₂O₇, cystine, Na₂SO₄, and K₄Fe(CN)₆, the temperature characteristic or μ of urease is 8,700. On the other hand, when urease is dissolved in solutions of K₄Fe(CN)₆ or H₂O₂ the μ value is 11,700. When dissolved in a solution containing Na₂SO₄ and NaHSO₃ the μ value may be either 8,700 or 11,700 over the whole temperature range, or 11,700 below and 8,700 above 23°C.

4. When crystalline urease is dissolved in varying mixtures of K₄Fe(CN)₆ and K₃Fe(CN)₄, the temperature characteristic depends upon the oxidation-reduction potential of the digest. When Eₜ is greater than +0.46 volt μ = 11,700, when less than +0.42 volt μ = 8,700, when between +0.42 − +0.46 μ = 11,700 below and 8,700 above the critical temperature.

5. It is suggested that in reducing or in indifferent solutions the configuration of the urease molecule (as determined especially by SH groups present) is such that the activation energy is 8,700 calories. In oxidizing solutions the urease molecule has been so altered (perhaps by the oxidation of the SH groups) as to be partly inactivated and now has an activation energy of 11,700. Such changes in the urease molecule are reversible (unless oxidation has proceeded too far) and are accompanied by a corresponding change in the activation energy.

CITATIONS

Bodansky, O., J. Biol. Chem., 1937, 120, 555.