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A STUDY OF CHLORIDE ION REACTIVATION OF L-AMINO ACID OXIDASE

by

Edward Arthur Weiskopf

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Ames, Iowa

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INTRODUCTION

One of the main interests in the field of biochemistry is concerned with protein structure and its relation to enzyme activity. Among the various aspects of protein structure are composition, sequence of amino acids, stereochemical configuration, and size. Analytical methods are now available to determine total amino acid composition, and methods of sequential analysis have been developed in the organic chemical approach to protein structure. However, physical chemical methods must be relied on for the study of size, shape, and configuration of proteins.

It has been said that since denaturation is such a fundamental property of a large group of proteins, a theory of denaturation is essentially a general theory of the structure of native and denatured proteins (14). In fact, denaturation may be defined as a physical or intramolecular rearrangement rather than a chemical alteration of native protein structure and that it leads to a change in specific spatial configuration without hydrolysis of primary covalent bonds (14).

Many methods have been used to study protein denaturation and renaturation. Among the most important of these are: (1) decrease in solubility, (2) loss of biological activity, (3) increased reactivity of constituent groups, and (4) changes in molecular size and shape. Recently, measurements

of optical rotation and U.V. difference spectra have aided in the study of denaturation and renaturation.

Neurath (12) pointed out that "measurement of any single change is an insufficient means to characterize a denatured protein or for that matter, to estimate the extent to which a protein has become denatured." However, practical considerations often demand the selection of but one, especially in kinetic analysis. Any criterion may be singly sufficient to establish the fact of denaturation though inadequate to characterize the altered protein (14).

The kinetics of protein denaturation and biological inactivation show distinct similarities, and are regarded as involving the same type of process. It should be noted that in almost all cases the thermodynamic parameters such as $\Delta H^{\neq}$, the enthalpy of activation for the denaturation process, are of the same order of magnitude for protein denaturation and enzyme inactivation, indicating a close correlation between the two processes.

The problem of the reversibility of denaturation occupies a key position in consideration of protein denaturation and protein structure. The fact that some proteins are reversibly denatured indicates that the tertiary structure of the protein is disturbed in some manner upon denaturation and that upon renaturation the localized displaced groups are placed back in their original position.

Three extensively studied cases of reversible denaturation which support this are these of trypsin (24), soybean trypsin inhibitor (14) and chymotrypsinogen (14).

In 1951, Kearney and Singer (21) reported that L-amino acid oxidase from moccasin venom undergoes a spontaneous inactivation in water and dilute phosphate. This inactivation was reported to be entirely reversible, and the apparent equilibrium between active and inactive forms of the enzyme was found to be determined by pH. The inactivation was prevented by 10^{-2} M univalent anions and by low concentrations of riboflavin and those of its analogs which have a high affinity for the enzyme. Concentrations of those substances which prevented inactivation were also found to reactivate the enzyme. It should be noted that monovalent anions are not required for activity.

In their series of papers dealing with L-amino acid oxidase (17-23) Singer and Kearney dealt mainly with the inactivation process. The reactivation process was left in a more or less qualitative state. The enzyme used in their study was only partially purified. Subsequently, Wellner and Meister (25) prepared crystalline L-amino acid oxidase from the venom of the eastern diamondback rattlesnake (Crotalus adamanteus).

It is the purpose of this study to investigate the reactivation process using crystalline L-amino acid oxidase.

The study will deal with the effect of chloride ion concentration, pH, and temperature on the rate of reactivation.

L-amino acid oxidase catalyses the oxidation of certain amino acids to the corresponding imino acids which subsequently undergo spontaneous hydrolysis to the α-keto acids. The α-keto acids thus formed may then be decarboxylated by the peroxide formed during the reoxidation of the enzyme by oxygen or other suitable oxidant.

It has been shown that under standard condition, oxygen uptake is proportional to enzyme concentration (21). Therefore, oxygen uptake can be used to determine the concentration of active enzyme in any given solution. This, of course, assumes that the inactive form of the enzyme present does not affect the oxygen uptake.

EXPERIMENTAL

Materials

Enzyme

L-amino acid oxidase was purchased from Worthington Biochemical Corp. in the form of a lyophilized yellow solid. It was prepared according to the procedure of Wellner and Meister (25). The enzyme was kept in a freezer when not in use. Solutions for the pH dependent studies were made by dissolving about 10 mg. of the enzyme weighed to the nearest 0.2 mg. in 100 ml. of cold 2.5×10^{-3} M phosphate buffer of the desired pH. Enzyme solutions for the chloride dependence studies were made by dissolving about 10 mg. enzyme weighed to the nearest 0.2 mg. in 100 ml. redistilled water. The pH was adjusted to 7.0 with 10^{-2} M NH_4OH. The solutions were kept refrigerated when not in use and were used within a day or two after preparation.

Amino acid

0.800 g. L(-)Leucine, purchased from Pfanstiehl Laboratories, Inc., was dissolved in 100 ml. 0.575 M cacodylic acid buffer, pH 7.0. A drop of toluene was added as a bacteriocide, and this solution was also kept refrigerated when not in use.

Phosphate buffer

Potassium Phosphate Monobasic (Mallinckrodt analytical reagent) was dissolved in redistilled water to give a 0.1 M stock solution. 50 ml. of this stock solution was diluted to 100 ml. with more redistilled water and various amounts of 0.1 N NaOH to give secondary 0.05 M phosphate stock solutions of various pH's, ranging from 5.6 to 6.9. These solutions were then diluted to produce 2.5×10^{-3} M phosphate buffers in the pH range given above.

Potassium chloride

Mallinckrodt analytical reagent grade potassium chloride was dissolved in redistilled water to give a 0.10 M solution.

Standard buffer

A solution was prepared in redistilled water which was 0.01 M in KH_2PO_4 and 0.01 M in Na_2HPO_4. According to Bates (1) this solution has a pH of 6.96.

Apparatus

All pH measurements were made with a Beckman model GS extended scale pH meter. All inactivations and reactivations were carried out in a water bath in which the temperature was controlled to within T $\pm$ 0.07°C.

Experimental Procedure

Inactivation

From 20 to 25 ml. of the enzyme solution contained in a 25 ml. vol. flask was placed in the water bath at the temperature of which the reactivation was to be run. One drop of toluene was added as a bacteriocide. The length of time that the enzyme solutions were allowed to inactivate depended upon the temperature. Four hours inactivation at 38°C, 2 hours at 45°C and 1 1/2 hours at 50°C was sufficient to insure equilibrium between the active and inactive forms of the enzyme. The pH was measured at the end of inactivation. The inactivated solutions were kept refrigerated until reactivated usually less than 48 hours.

Reactivation

Reactivation at 38°C was carried out in a 25 ml. volumetric flask. Either 15 ml. enzyme and 2 ml. 0.1 M KCl or in some cases 20 ml. enzyme solution and 2.66 ml. KCl solution were mixed in a flask and placed in the water bath. One drop of toluene was added. Two ml. samples were withdrawn from the flask at suitable intervals over a period of about 7 hours. Samples were withdrawn with 2 ml. volumetric pipettes and were discharged immediately into Warburg flasks. The Warburg flasks were kept in an ice bath until there was sufficient time to assay the samples for activity.

Reactivations at the higher temperatures were carried out in 25 ml. conical flasks, stoppered with an aluminum foil covered rubber stopper. The amounts of enzyme and KCl were the same as the reactivations at 38°C. However, the procedure was slightly different.

About 10 ml. of the KCl solution contained in a test tube, was placed in the water bath. The enzyme solution in a conical flask was also placed in the water bath. The two solutions were allowed to come to equilibrium with the temperature of the bath. After about 15 minutes, the correct volume of 0.1 M KCl was injected into the enzyme solution by means of an automatic pipett. The glass portion of the pipett was scratched at the proper positions such that either 2.0 or 2.66 ml. of solution was delivered.

Upon the injection of the KCl solution, the timer was started, and the flask shaken momentarily to insure mixing of the two solutions. Samples were withdrawn from the flask by 2 ml. automatic pipettes; a different pipett was used for each sample. These were adjusted to draw slightly more than 2 ml. The samples were immediately introduced into small test tubes held in place in an ice bath by a wire screen. After all samples had been taken, 2 ml. volumetric pipettes were used to transfer a sample to the Warburg flask for analysis.

Activity assay

The oxidation of leucine was carried out in a Warburg

apparatus at 25°C. The activity was followed manometrically by the drop in pressure due to the uptake of oxygen at constant volume.

Two ml. of enzyme solution taken from the reaction flask was added to the main compartment of the Warburg flask; 0.5 ml. of 8 mg./ml. leucine solution was added to the side arm. In the center well was placed 0.2 ml. of 10% potassium hydroxide solution and a strip of filter paper 40 x 12 mm. to absorb any carbon dioxide given off by the decarboxylation of the alpha-keto acid.

The Warburg reaction vessel was then attached to an open end manometer, placed on the Warburg shakers and temperature equilibrated for 15 minutes. The leucine was then tipped into the main compartment at time zero. From 2 to 8 such reaction flasks were run simultaneously along with a blank which served as a thermo-barometer.

The reaction flasks were constantly shaken to facilitate gas exchange at the liquid vapor interface. The shakers were stopped every 3 minutes when readings were taken. Readings were taken over a period of from 18 to 24 minutes, and oxygen uptake was linear over that length of time.

RESULTS AND DISCUSSION

Effect of Chloride Ion Concentration

In an attempt to discover more about the mechanism of chloride ion reactivation of L-amino acid oxidase, the reactivation was followed using different concentrations of chloride ion.

One-hundred ml. of an enzyme solution containing 10 mg. enzyme at pH 7.0 was inactivated for 8 hours at 37.8°C. Two ml. of potassium chloride solution of various concentrations were added to 15 ml. aliquots of the inactivated enzyme solution, and the reactivations were followed at 37.8°C. The potassium chloride solutions used were adjusted to pH 7.0 with 10^{-2} M NH_4OH.

The activity of the solutions increased with time up to from 8 to 12 hours, then leveled off. In some cases a drop in activity was noted after 24 hours. This was almost certainly due to bacterial activity because when one drop of toluene was added no drop in activity was noted.

Initial slopes from activity versus time plots for various chloride ion concentrations are listed in Table 1. Also listed in Table 1 are the reciprocal values, $\frac{1}{v}$ and $\frac{1}{(KCl)}$. The results are also plotted in Figures 1 and 2.

It can be seen from the initial rate versus chloride concentration plot that at low chloride ion concentrations, doubling the chloride concentration, doubles the rate. This,

Table 1. Initial rates of reactivation and reciprocals at various chloride ion concentrations

$(Cl^-)x10^4M$	$\frac{1}{Cl^-}M^{-1}$	Initial rate $(\frac{\mu l O_2/30 \text{ min.}}{\text{hr.}})$	1/Initial rate $(\frac{\mu l O_2/30 \text{ min.}}{\text{hr.}})^{-1}$
56.0	179	9.6	0.104
28.0	358	9.0	0.111
14.0	715	6.6	0.152
7.00	1430	4.2	0.238
3.50	2860	2.1	0.476

then, indicates a first order relationship for chloride ion in the reactivation.

At higher chloride concentrations the initial rate of reactivation levels off to a maximum. This can be interpreted simply as a result of saturation of the binding site on the enzyme molecule by chloride. If the binding site is filled for all enzyme molecules, any additional chloride ions serve no useful purpose toward reactivation.

In order to interpret Figure 2 in a similar manner, some additional background may be useful. Starting from the general assumption that any number of chloride ions can combine with the enzyme molecule and that each particular species formed undergoes reactivation at its own rate, we have for the rate of reactivation

(1) Rate = k_1 (DCl) + k_2 (DCl_2) + ...

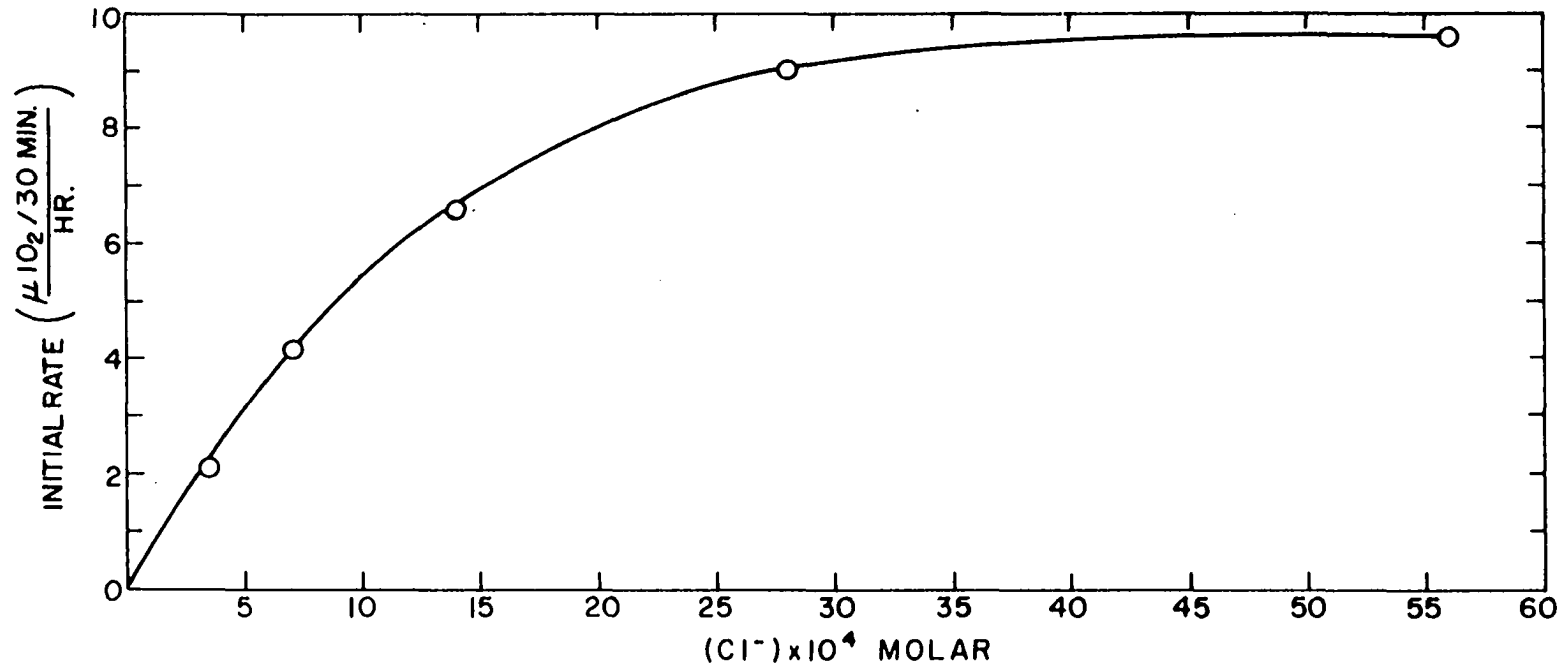

Figure 1. Plot of initial rate data vs. (Cl^-) at 37.8°C.

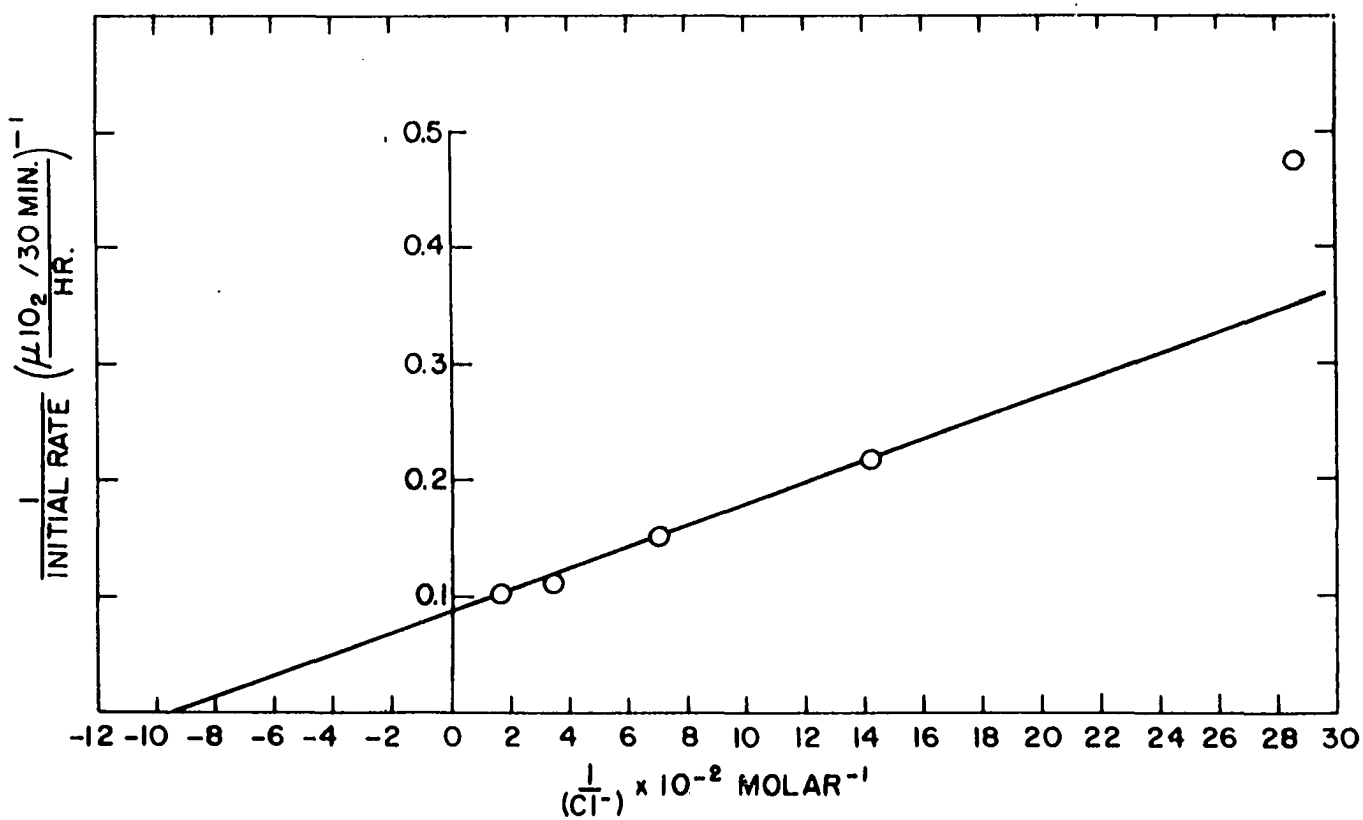

Figure 2. Plot of reciprocal rate data vs. $\frac{1}{(Cl^-)}$ at 37.8°C.

where DCl_n is the inactive (denatured) form of the enzyme combined with n chloride ions, and k_n is the reactivation rate constant for DCl_n. Associated with each species of the enzyme is an equilibrium constant.

(2) $K_1 = \frac{(DCl)}{(D)(Cl^-)}$

(3) $K_2 = \frac{DCl_2}{(DCl)(Cl^-)} = \frac{DCl_2}{K_1(D)(Cl^-)^2}$.

Rearranging the equilibrium expressions gives

(4) $DCl = K_1 (D)(Cl)$

(5) $DCl_2 = K_1 K_2 (D)(Cl)^2$.

Substituting expressions 4 and 5 into 1 yields

(6) $Rate = k_1 K_1 (D)(Cl) + k_2 K_1 K_2 D (Cl)^2 + \ldots$

(7) $Rate = D \left[k_1 K_1 (Cl) + k_2 K_1 K_2 (Cl)^2 + \ldots\right]$.

The total Inactive Enzyme present can be expressed as

(8) $D\ total = D + DCl + DCl_2 + \ldots$.

Substituting expressions 4 and 5 into 7 yields

(9) $D\ total = D + K_1 (D)(Cl) + K_1 K_2 (D)(Cl)^2 + \ldots$

(10) $D\ total = D \left[1 + K_1 (Cl) + K_1 K_2 (Cl)^2 + \ldots\right]$

Solving expression 10 for D and substituting into 7 yields

$$(11)\quad \frac{\text{Rate}}{\text{D total}} = \frac{k_1K_1(Cl) + k_2K_1K_2(Cl)^2 + \ldots}{1 + K_1(Cl) + K_1K_2(Cl)^2 + \ldots} \cdot$$

Inverting 11 and omitting D total as it remains the same in all reactivations yields

$$(12)\quad \frac{1}{\text{Rate}} = \frac{1 + K_1(Cl) + K_1K_2(Cl)^2 + \ldots}{k_1K_1(Cl) + k_2K_1K_2Cl^2 + \ldots} \cdot$$

By plotting $\frac{1}{\text{rate}}$ vs. $\frac{1}{Cl}$ at low chloride ion concentrations, and assuming that at these low chloride ion concentrations terms involving higher power chloride combinations are negligible, a straight line is obtained. The ordinate intercept of this plot is $\frac{1}{k_1}$, and abscissa intercept is $-K_1$.

If at higher chloride ion concentrations, higher power chloride combinations are important, the plot of $\frac{1}{\text{rate}}$ vs. $\frac{1}{Cl^-}$ will not be linear over the full range.

Figure 2, however, shows linearity over the full range of chloride ion concentration. It can be concluded, therefore, that a single chloride ion combines with the inactive enzyme. This combination in turn undergoes a change such that the enzyme is reactivated. Thus, for the reactivation process, can be written

$$(13)\quad D + Cl^- \overset{K_1}{\rightleftharpoons} DCl$$

$$(14)\quad DCl \overset{k_1}{\rightleftharpoons} ACl$$

$$(15)\quad ACl \overset{K}{\rightleftharpoons} A + Cl^-$$

where A is the active form of the enzyme.

The fact that only one ion is needed for reactivation should not be regarded as being unusual. Chervenka (2) showed that only one calcium ion was needed to stabilize chymotrypsinogen against urea denaturation. In several other cases, notably pancreatic ribonuclease, (11) prostatic phosphatuse, (9) and glutamic dehydrogenase, (5) good evidence for such a simple stabilization has been obtained. In their study of urea denaturation of pancreatic ribonuclease, Nelson and Hummel (11) attribute the stabilization effects of the various polyvalent anions to complex formation involving specific groups at the active center of the enzyme.

Although these references are mainly concerned with stabilization of enzymes against denaturation, the same general mechanism can be applied to chloride ion reactivation of L-amino acid oxidase, as is shown in Equations 13 to 15.

Inactivation Equilibrium

Singer and Kearney (21) determined the variation of extent of inactivation with pH for L-amino acid oxidase in moccasin venom. It seemed desirable to repeat the determination using the crystalline enzyme.

The activity of several freshly prepared enzyme solutions at various pH's was determined. The solutions were then inactivated, and the activity again measured.

It was noticed, however, that solutions at the lower pH

region when reactivated with chloride ion, showed a higher activity than the original solution when dilution effects were taken into account. This was taken to mean that the initial solution was slightly inactive. Wellner and Meister (25) pointed out that the crystalline enzyme looses activity even if kept at -20°C. At pH 5.70 reactivation showed the initial solutions to be from ten to eighteen per cent inactive. The initial activity of all solutions was then adjusted to coincide with the reactivated activity at low pH. These adjusted activities were used, then, to calculate per cent inactivation for the various pH values. Table 2 shows the values for per cent inactivation at various pH values and three temperatures. Figure 3 shows the same data.

Two qualities are observed in Figure 3. The first is the general S shape of the curves. The second is the appearance of temperature dependence on the extent of inactivation.

The S shape of the curves is reminiscent of a weak acid or base titration curve, indicating the involvement in the reversible inactivation of an amino acid residue with an ionization in the range indicated. The pH corresponding to 50% inactivation for 38°C. is 6.10. The same general S shaped curve was also found by Singer and Kearney (21). However, the pH corresponding to 50% inactivation at 38°C. is 6.55 in their study.

Table 2. Per cent inactivation at various pH values for 38.0, 44.8 and 50.0°C.

Temperature °C.	pH	Per cent inactivation
38.0	5.66	29.0
	5.70	35.5
	5.80	36.0
	5.85	37.0
	5.91	39.5
	6.01	45.5
	6.11	53.5
	6.36	65.5
	6.45	77.5
	6.49	79.0
	6.67	86.5
	6.72	89.0
	7.00	94.0
44.8	5.71	24.0
	6.02	34.5
	6.10	43.5
	6.32	58.5
	6.50	66.0
	6.70	79.6
50.0	5.60	18.3
	5.76	22.2
	6.03	35.6
	6.19	51.1
	6.30	57.5
	6.50	62.5
	6.69	77.0
	6.96	88.4

The temperature dependence of the extent of inactivation is of interest because it is seen that higher temperatures favor the active form of the enzyme. It appears, then, that this inactivation process is different from the irreversible

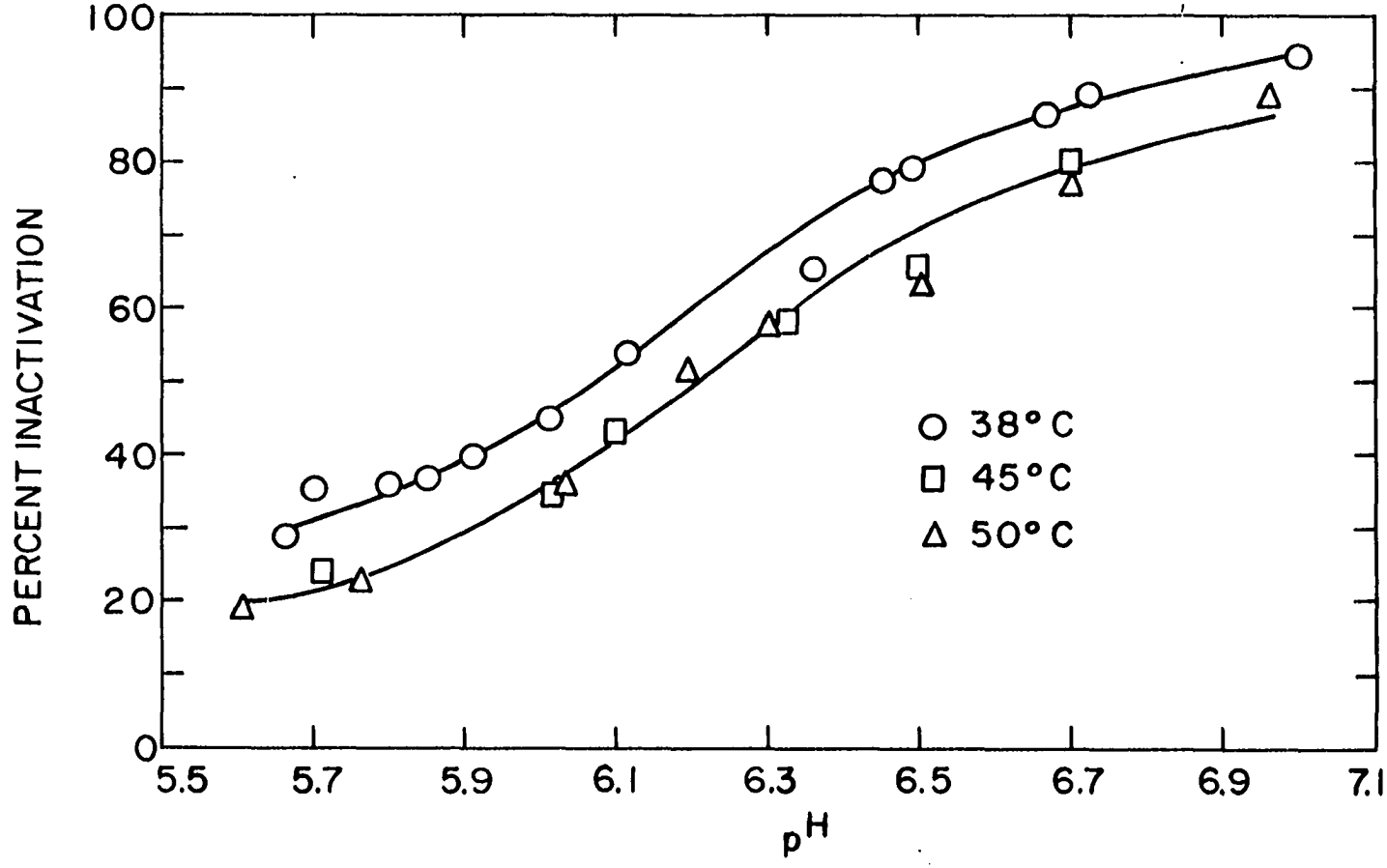

Figure 3. Plot of per cent inactivation vs. pH at 38.0, 44.8, and 50.0°C.

denaturation that occurs above 70°C.

Quantitative comparisons of equilibrium constants at the three temperatures, were not made due to some uncertainty in the data. This uncertainty was primarily due to two factors.

(1) The enzyme is partially inactive at the beginning of the runs, and estimation of initial activity after reactivation compounds the experimental errors involved in the activity analysis.

(2) There may be some partial irreversible denaturation occurring in some runs, especially at the higher limit of pH.

An attempt was made to circumvent the first difficulty. If the enzyme solutions could be activated to full activity by chloride ion, for example, and the chloride ion removed immediately before inactivation, no initial inactivity would exist.

Preliminary experiments with a Sephadex (G-25 Medium of Pharmacia Corp.) column showed that chloride ion could be separated from the L-amino acid oxidase activity in crude rattlesnake venom. Also, the total units of activity coming through the column equaled the number put into the column. With solutions of crystalline enzyme, the chloride ion could also be separated from the enzyme. However, there was a loss in total activity coming through the column. In fact, no activity came through unless a crude venom solution had first been sent through the column. The study was dropped at that

point.

Rate of Inactivation

It was seen previously that there is an equilibrium between active and inactive enzyme, and this equilibrium depends on pH. Therefore, the following expression can be written;

$$\text{Active enzyme} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \text{Inactive enzyme}$$

As was the case with the inactivation equilibrium studies, it was thought advisable to repeat part of Singer and Kearney's (21) rate of inactivation work using the crystalline enzyme. Therefore, the rate of inactivation was followed at several pH values at 38.0°C.

When the inactivation is followed, the activity drops with time to an equilibrium value and remains there. The reaction order can be demonstrated using the above equilibrium as shown in the following rate expression development (6)

Let:

k_1 = the first order rate constant for active $\longrightarrow$ inactive form.

k_2 = the first order rate constant for inactive $\longrightarrow$ active form.

a = concentration of active enzyme at time t (proportional to enzyme activity at time t).

a_0 = concentration of active enzyme at time t = 0.

A_0 = total amount of enzyme.

(A_0-a) = concentration of inactive enzyme

a_e = concentration of active enzyme at equilibrium.

K = equilibrium constant = $\frac{k_1}{k_2} = \frac{(A_0 - a_e)}{a_e}$.

(16) $-\frac{da}{adt} = k_1 \qquad \frac{da}{(A_0 - a)\,dt} = k_2$

The expression for the reversible inactivation is given by the sum of the two previous equations, i.e.,

(17) $\frac{-da}{dt} = (k_1 + k_2)\, a - k_2\, A_0$.

At equilibrium,

(18) $k_2\, A_0 = (k_1 + k_2)\, a_e$.

Substitution of 18 into 17 yields,

(19) $\frac{-da}{(a - a_e)\,dt} = k_1 + k_2$.

Integrating this yields

(20) $\frac{-d \ln (a - a_e)}{dt} = k_1 + k_2$.

If k_1 and k_2 are first order rate constants, and ln $(a - a_e)$ is plotted against time, a straight line of slope $-(k_1 + k_2)$ is obtained. From a knowledge of the equilibrium constant, $K = \frac{k_1}{k_2}$, the rate constants for the two processes can be separated.

When log $(A - A_e)$ is plotted against time, where A is

the enzyme activity at time t and A_e is the equilibrium activity, a straight line is obtained. Thus, both the inactivation and the reactivation are first order with respect to the starting form of the enzyme.

From the slopes of the curves of log $(A - A_e)$ versus time, $k_1 + k_2$ was obtained at several pH values. This quantity was reasonably independent of pH; the average value was 7.30×10^{-4} sec^{-1}.

Table 3 gives values of the equilibrium constants, K, and rate constants k_1 and k_2 at various pH values. The rate constants are also plotted in Figure 4.

Figure 4 shows that k_1 and k_2 both depend on pH but in

Table 3. Equilibrium and rate constants at various pH values from the rate of inactivation at 38°C.

pH	K	$k_1 \times 10^4$ sec^{-1}	$k_2 \times 10^4$ sec^{-1}
5.84	0.614	2.03	3.31
5.91	0.834	3.01	3.62
6.25	1.19	3.94	3.31
6.36	2.33	4.95	2.12
6.45	3.75	6.91	1.84
6.72	7.20	9.86	1.37
5.81	0.620	2.27	3.67
5.80	0.563	2.59	4.62
6.01	0.840	3.33	3.97
6.11	1.16	3.61	3.11
6.36	1.88	4.96	2.64
6.49	3.81	5.74	1.51
6.67	6.50	6.22	0.96

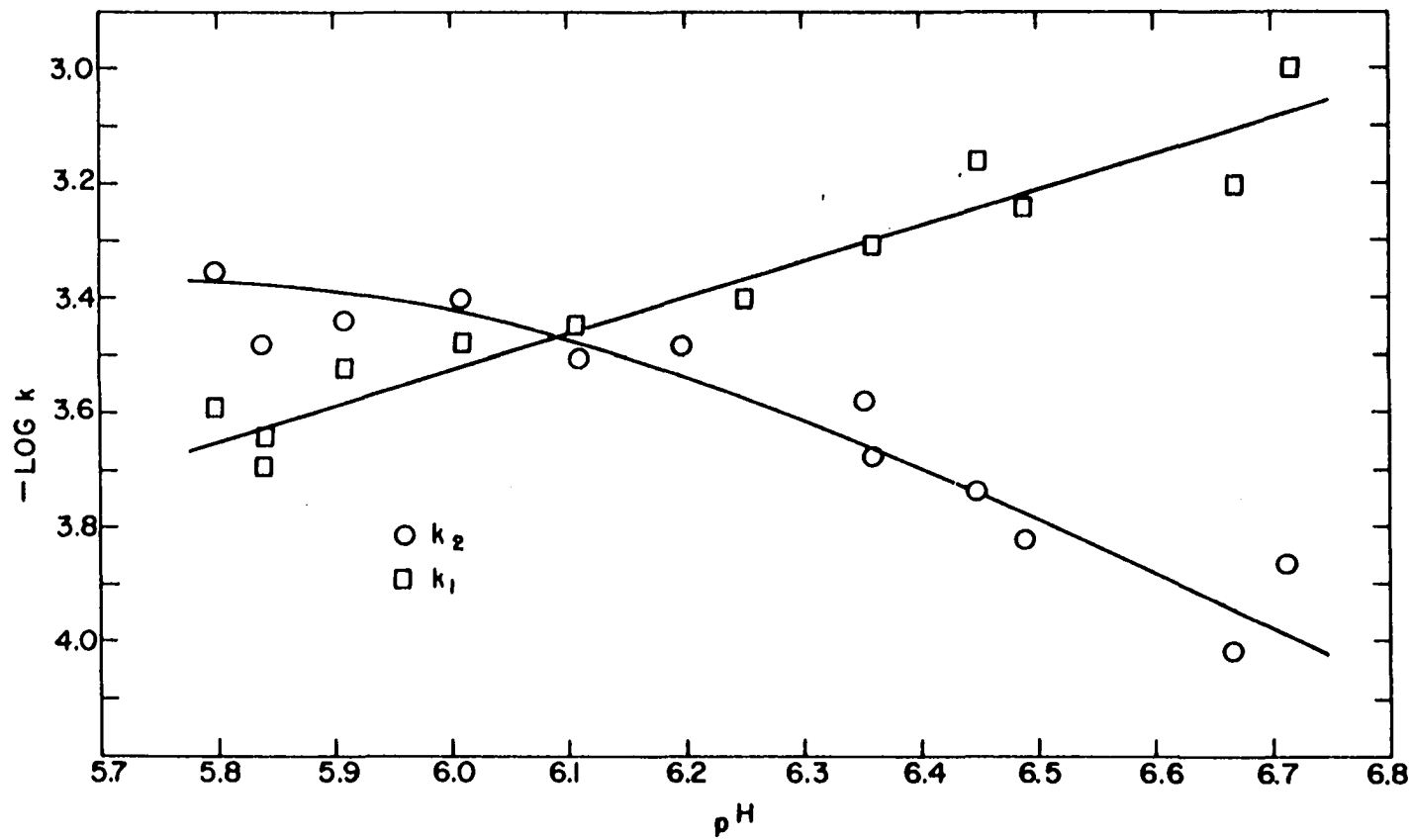

Figure 4. Plot of rate data from inactivation studies at 38.0°C. vs. pH

inverse ways. This suggests that the role of H^+ ion is not a simple catalytic one, i.e. H^+ ion must combine with protein in the process of reactivation, and must be dissociated from the protein in the process of inactivation.

Reactivation Equilibrium

Turning to the reactivation process, a pH dependence of both rate and extent of reactivation was found. As the reactivation process was followed, the enzyme activity increased with time until it reached a maximum value. A plot of log (Am - A) versus t, where Am is the maximum activity reached upon reactivation (assumed to be an equilibrium value), and A is the activity at time t, gave a slope of $k_1 + k_2$ as with inactivation. The individual rate constants were separated by substitution of the $k_1 + k_2$ value into $K = \frac{k_1}{k_2}$, where K is the calculated equilibrium constant.

The fact that an equilibrium was present upon reactivation was shown by changing the pH of an inactivated sample to a lower pH, more favorable to reactivation, using 2.5×10^{-3} M KH_2PO_4 and dilute HCl. When the original solution was reactivated along with the sample in which the pH had been changed, the solution at low pH showed the same final activity as the solutions before inactivation. However, the final activity of the sample at higher pH showed much less activity. In addition the pH of some samples which had been reactivated

at the higher pH values were adjusted to lower values and reactivated again. The activity rose substantially.

Data for the reactivation equilibria are listed in Table 4, and a graphical representation is shown in Figure 5.

The initial enzyme activities for all pH values were obtained in the same manner as in the inactivation equilibrium study. The reactivation activity maximum at around pH 5.60 was used in estimating the true initial activity.

As was the case with the per cent inactivation versus pH data, no good quantitative comparison of equilibrium constants at different temperatures could be made, due to the uncertainty in the data. The uncertainty was again due to the uncertainty in the initial activity and to the possibility of irreversible denaturation.

That irreversible denaturation was a factor, can be seen by the following facts. A sample (15 ml.) of a solution which had been inactivated for 90 minutes at 50°C. and pH 6.90 was adjusted to pH 5.65 with 2.5×10^{-3} KH_2PO_4 and 10^{-2} M HCl. This sample, and a portion of the inactivated solution at pH 6.90 were both reactivated using 10^{-2} M KCl for 20 minutes, enough time to completely reactivate the solutions. One drop of toluene had been added to each solution. After dilution effects were taken into consideration, the sample reactivated at pH 5.65 showed twice the activity of the same solution reactivated at pH 6.90, yet only about 60% of the activity

Table 4. Per cent reactivation at various pH values

Temperature °C.	pH	Per cent reactivation
38.0	5.72	100
	5.93	100
	6.02	94.7
	6.22	91.4
	6.34	82.4
	6.55	75.6
44.8	5.63	100
	5.81	96.6
	5.97	87.7
	6.20	85.7
	6.36	80.0
	6.61	66.6
50.0	5.55	100
	5.66	94.4
	5.90	92.6
	6.08	72.2
	6.24	68.2
	6.40	63.9
	6.59	46.5
	6.88	21.4

of a sample inactivated and reactivated at pH 5.65.

Thus certainly at 50°C., and possibly at other temperatures, some irreversible denaturation occurs at the higher limits of pH.

The possibility of this denaturation makes conjecture on the nature of Figure 5 highly questionable. What can be said is that a pH dependence of the extent of reactivation does exist, i.e., there is an equilibrium between inactive and

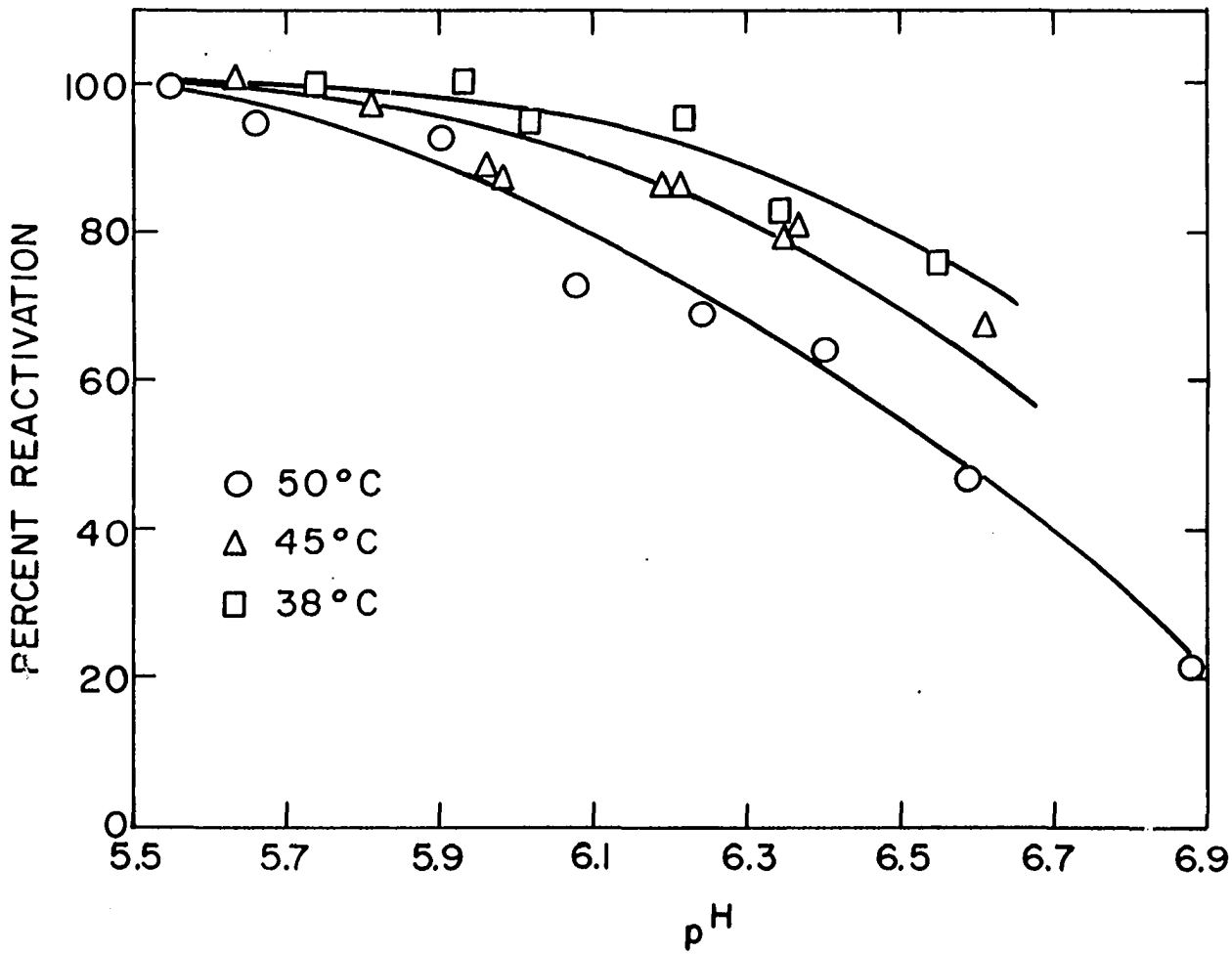

Figure 5. Plot of per cent reactivation vs. pH at 38.0, 44.8 and 50.0°C.

active forms of the enzyme, even in the presence of chloride ion. That chloride ion shifts the equilibrium from the inactive to the active form, can be seen by comparison of Figures 3 and 5. This equilibrium for the reactivation process (i.e., in the presence of chloride) cannot be obtained much above pH 7.0 due to irreversible denaturation.

Rate of Reactivation

It was noted in the previous section that an equilibrium existed between active and inactive forms of the enzyme in the presence of 1.18×10^{-2} M KCl. Using these equilibria and $k_1 + k_2$ calculated from the slope of log (Am - A) versus time plots, values of the first order reactivation rate constant were determined at 38.0, 44.8 and 50.0°C. at various pH values. The data obtained are listed in Tables 5, 6 and 7, along with log k. Figure 6 shows the relationships between log k, pH and temperature.

The data illustrated in Figure 6 shows that an increase in H^+ ion concentration brings about an increase in reactivation rate. The pH range in which the study was done was limited because at higher pH values irreversible denaturation would occur. Below pH 5.5, little or no inactivation would occur.

If straight lines were drawn through the points of Figure 6 at each temperature, they would have a slope of less than one, indicating one H^+ ion involved in a partial

Table 5. Rate constants and logarithms at various pH values at 38°C.

pH	k x 10^4 sec^{-1}	-log k
5.74	3.48	3.458
5.93	2.42	3.616
6.02	2.46	3.609
6.22	1.70	3.770
6.34	1.10	3.959
6.55	0.85	4.071
5.95	2.09	3.680
6.10	2.13	3.672
6.45	0.95	4.021
6.31	1.42	3.848
6.09	1.91	3.719
6.62	1.44	3.842
6.79	0.76	4.119
6.52	0.61	4.215
6.75	0.89	4.051
6.00	2.02	3.695
6.62	7.59	4.120

dissociation. (The least squares slopes of the lines were 0.63, 0.53, and 0.41 for 38.0, 44.8, and 50.0°C. respectively.)

The following expression can then be written for the rate of reactivation. (All charges are dropped.)

(21) $\text{rate} = k_1 (E) + k_2 (EH)$

EH represents the inactive enzyme molecule with one more proton than E. An equilibrium constant is associated with E and EH.

(22) $K = \frac{(EH)}{(E)(H)}$

Table 6. Rate constants and logarithms at various pH values at 44.8°C.

pH	k x 10^3 sec^{-1}	-log k
5.63	3.04	2.517
5.81	2.31	2.636
5.96	1.53	2.815
6.21	1.08	2.967
6.36	1.12	2.951
6.61	0.85	3.070
6.35	1.05	2.979
6.19	1.28	2.893
5.98	1.38	2.860
6.24	1.41	2.851
6.71	0.50	3.301
5.95	1.58	2.801
6.19	1.84	2.735
6.68	0.66	3.177
6.10	1.50	2.824
6.20	1.54	2.813
6.63	0.82	3.087
6.50	1.09	2.963
6.38	1.03	2.987
6.32	1.41	2.851
6.01	1.42	2.848
5.90	1.52	2.818
6.03	1.35	2.870
6.10	2.16	2.666
6.30	2.48	2.606

The total enzyme concentration can be expressed as

$$(23)\quad E_T = E + EH$$

Solving Equations 22 and 23 for E and EH in terms of E_T and substituting in Equation 21 gives

$$(24)\quad \text{rate} = k_1 \frac{E_T}{K(H) + 1} + k_2 \frac{E_T\, K(H)}{K(H) + 1}$$

Table 7. Rate constants and logarithms at various pH values at 50.0°C.

pH	k x 10^3 sec^{-1}	-log k
5.55	7.67	2.115
5.66	7.39	2.131
5.90	5.01	2.300
6.08	3.47	2.460
6.24	4.52	2.345
6.40	4.60	2.337
6.59	3.70	2.432
6.88	1.43	2.845
6.55	1.11	2.955
6.01	6.98	2.196
6.34	3.67	2.435
5.98	1.48	2.830
6.50	1.61	2.793
6.02	1.58	2.801
6.19	4.56	2.341
6.10	3.81	2.499
6.24	5.07	2.295
6.11	5.94	2.226
6.30	4.45	2.352

Rearranging 24 gives

$$(25)\quad \frac{\text{rate}}{E_T} = \frac{k_1 + k_2\,K(H)}{K(H) + 1}$$

Inverting 25 yields

$$(26)\quad \frac{E_T}{\text{rate}} = \frac{K(H) + 1}{k_1 + k_2\,K(H)}$$

If it is assumed that $k_1 = 0$, equation 26 can then be written,

$$(27)\quad \frac{E_T}{\text{rate}} = \frac{K(H) + 1}{k_2\,K(H)} = \frac{1}{k_2} + \frac{1}{k_2\,K(H)}$$

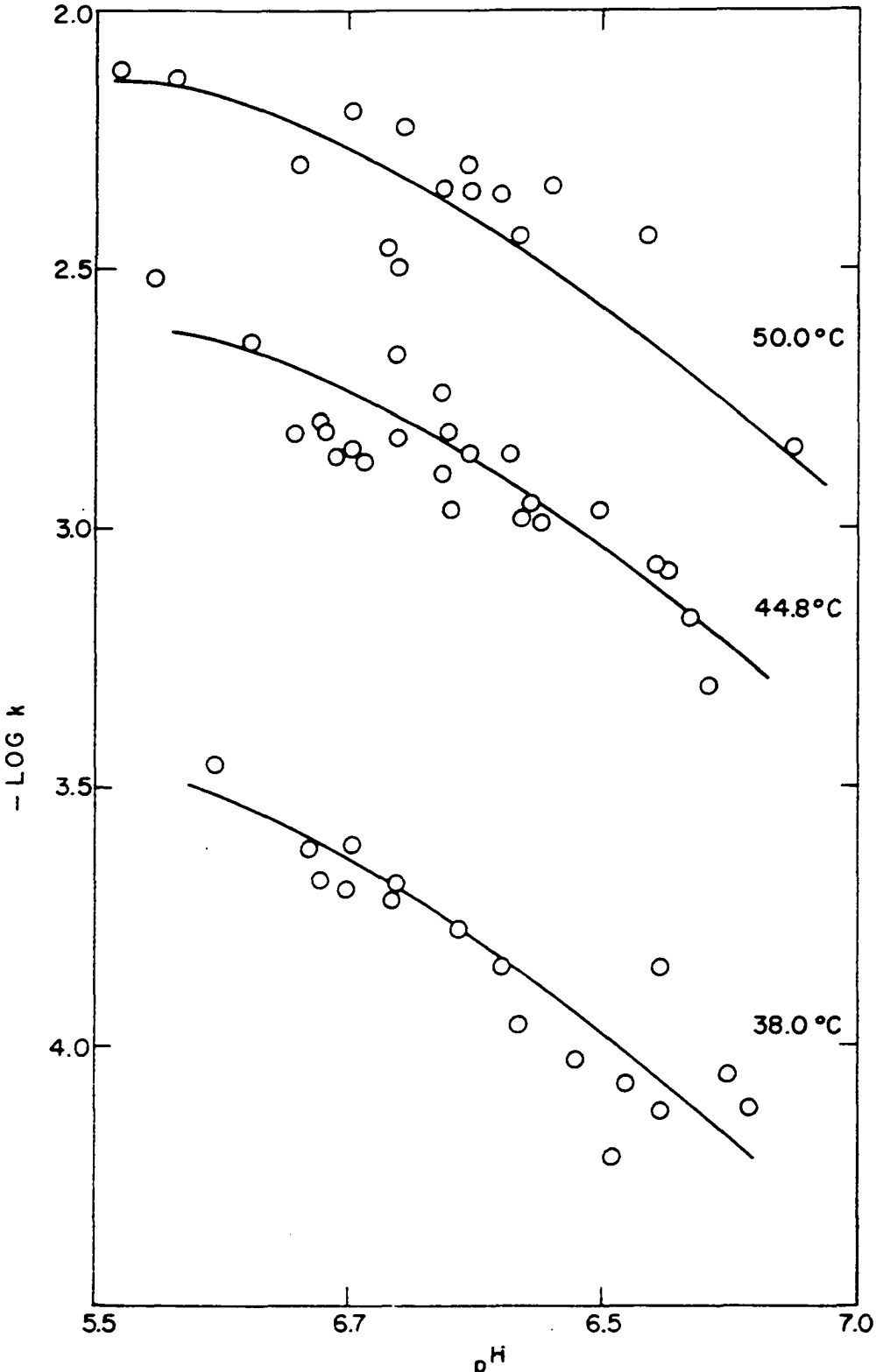

Figure 6. Plot of log k vs. pH at 38.0, 44.8 and 50.0°C.

From Equation 27 it can be seen that if $\frac{1}{\text{rate}}$ is plotted against $\frac{1}{(H^+)}$ a straight line is obtained. The ordinate intercept of the line is $\frac{1}{k_2}$; the abscissa intercept is -K.

More will be mentioned about the assumption that k_1 is zero in the conclusions.

The first order rate constants determined at 38.0, 44.8 and 50.0°C. are listed in Tables 8, 9 and 10 along with the reciprocal of k. The values of the reciprocal of k at each temperature were plotted against the reciprocal of hydrogen ion concentration. This procedure is illustrated in Figures 7, 8 and 9.

Table 8. Rate constants and reciprocals for various pH values at 38.0°C.

pH	$(H^+) \times 10^7$	$\frac{1}{H^+} \times 10^5$	$k \times 10^4$	$\frac{1}{k} \times 10^{-3}$
5.74	18.2	5.49	3.48	2.87
5.93	11.8	8.47	2.42	4.13
6.02	9.55	10.5	2.46	4.06
6.22	6.03	16.6	1.70	5.88
6.34	4.57	21.9	1.10	9.09
6.55	2.82	35.5	0.85	11.8
5.95	11.2	9.09	2.09	4.78
6.10	7.95	12.6	2.13	4.69
6.45	3.55	28.2	0.95	10.5
6.31	4.90	20.4	1.42	7.04
6.09	8.13	12.3	1.91	5.24
6.62	2.40	41.7	1.44	6.94
6.79	1.62	61.7	0.76	13.2
6.52	3.02	33.1	0.61	16.4
6.75	1.78	56.2	0.89	11.2
6.00	10.0	10	2.02	4.95
6.62	2.40	41.7	0.76	13.2

Table 9. Rate constants and reciprocals for various pH values at 44.8°C.

pH	$H^+ \times 10^7$	$\frac{1}{H} \times 10^5$	$k \times 10^3$	$\frac{1}{k} \times 10^{-2}$
5.63	23.5	4.26	3.04	3.29
5.81	15.5	6.45	2.31	4.33
5.96	11.0	9.09	1.53	6.54
6.21	6.17	16.2	1.08	9.26
6.36	4.37	22.9	1.12	8.93
6.61	2.46	40.6	0.85	11.8
6.35	4.47	22.4	1.05	9.52
6.19	6.46	15.5	1.28	7.81
5.98	10.5	9.52	1.38	7.25
6.24	5.76	17.4	1.41	7.09
6.71	1.95	51.3	0.50	20.0
5.95	11.2	8.93	1.58	6.33
6.19	6.46	15.5	1.84	5.43
6.68	2.09	47.8	0.665	15.0
6.10	7.95	12.6	1.50	6.67
6.20	6.31	15.8	1.54	6.49
6.63	2.35	42.6	0.819	12.2
6.50	3.16	31.6	1.09	9.17
6.38	4.17	24.0	1.03	9.71
6.32	4.79	20.9	1.41	7.09
6.01	9.77	10.2	1.42	7.04
5.90	12.6	7.94	1.52	6.58
6.03	9.33	10.7	1.35	7.41
6.10	7.95	12.6	2.16	4.63
6.30	5.01	20.0	2.48	4.03

The values of k_2 and K obtained at these temperatures are listed in Table 11.

From the temperature dependence of k_2, the energy of activation for the reactivation process can be calculated. From an Arrhenius plot of log k_2 versus $\frac{1}{T}$, Figure 10, a slope of $1.104 \times 10^4 \pm 0.065 \times 10^4$ was obtained. This corresponds to an energy of activation, Ea, of 50.5 ($\pm$ 3)

Table 10. Rate constants and reciprocals for various pH values at 50.0°C.

pH	$H^+ \times 10^7$	$\frac{1}{H} \times 10^5$	$k \times 10^3$	$\frac{1}{k} \times 10^{-2}$
5.55	28.2	3.55	7.67	1.30
5.66	21.9	4.57	7.39	1.35
5.90	12.6	7.94	5.01	2.00
6.08	8.32	12.0	3.47	2.88
6.24	5.76	17.4	4.52	2.21
6.40	3.98	25.1	4.60	2.17
6.59	2.57	38.9	3.70	2.70
6.88	1.32	75.7	1.431	6.99
6.55	2.82	35.5	1.11	9.01
6.01	9.77	10.2	6.98	1.43
6.34	4.57	21.9	3.67	2.72
6.50	3.16	31.6	1.61	6.21
6.19	6.46	15.5	4.56	2.19
6.10	7.94	12.6	3.81	2.62
6.24	5.76	17.4	5.07	1.97
6.11	7.76	12.9	5.94	1.68
6.30	5.01	20.0	4.45	2.25

k Cal.

The equilibrium constants given in Table 11 show no variation with temperature, within experimental error. It can then be concluded that the change of enthalpy in the reactivation is close to zero.

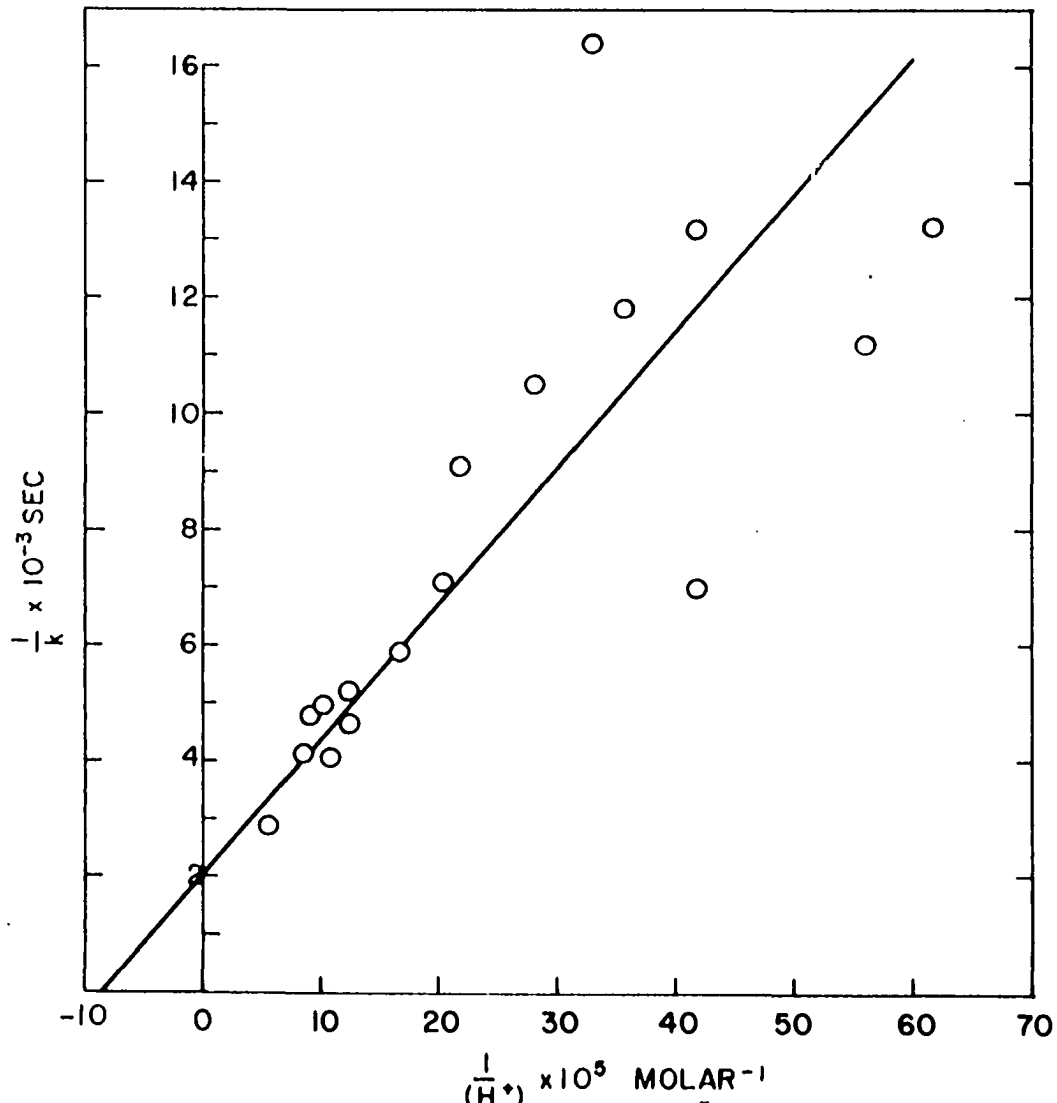

Figure 7. Plot of reciprocal rate data vs. $\frac{1}{(H^+)}$ at 38.0°C.

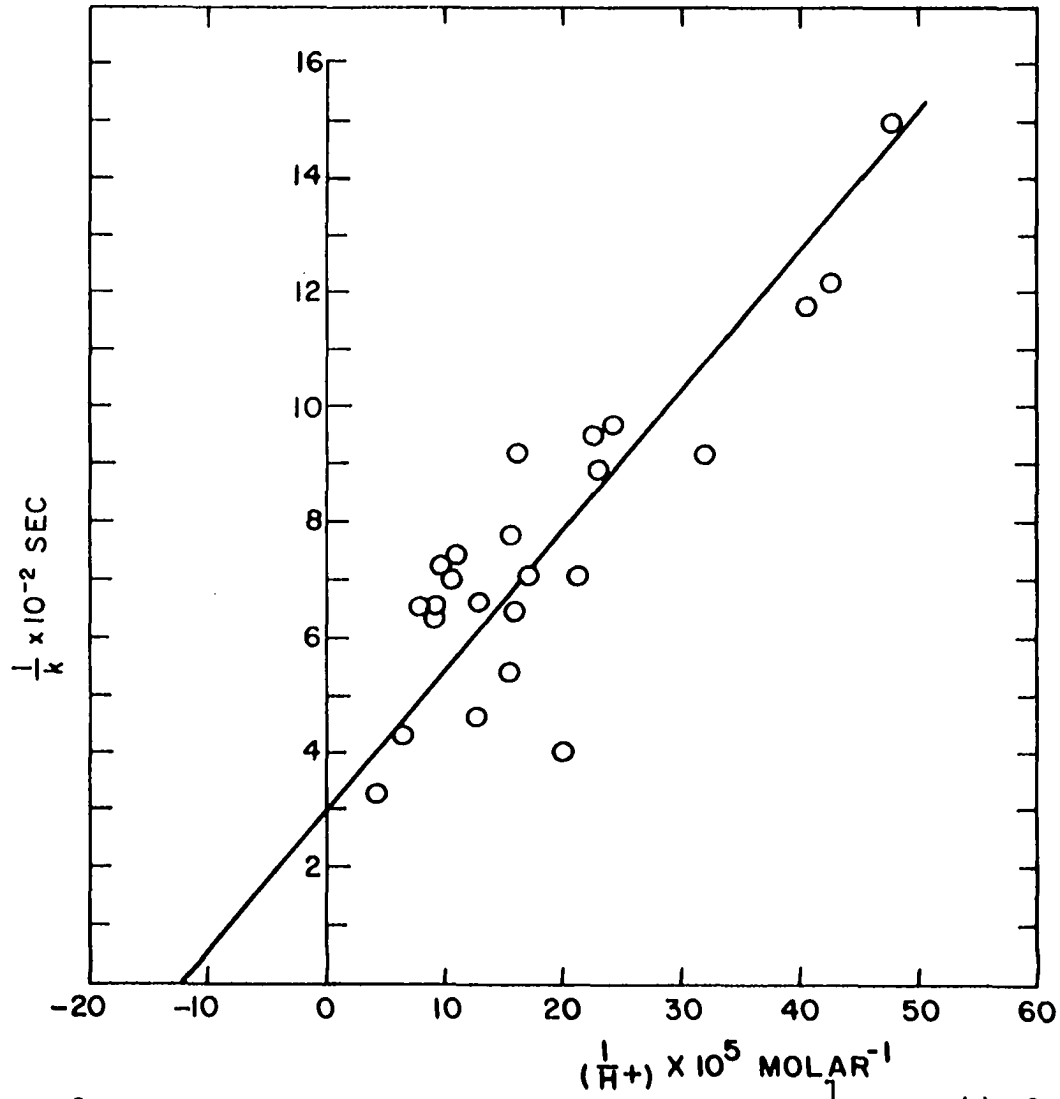

Figure 8. Plot of reciprocal rate data vs. $(\frac{1}{H^+})$ at 44.8°C.

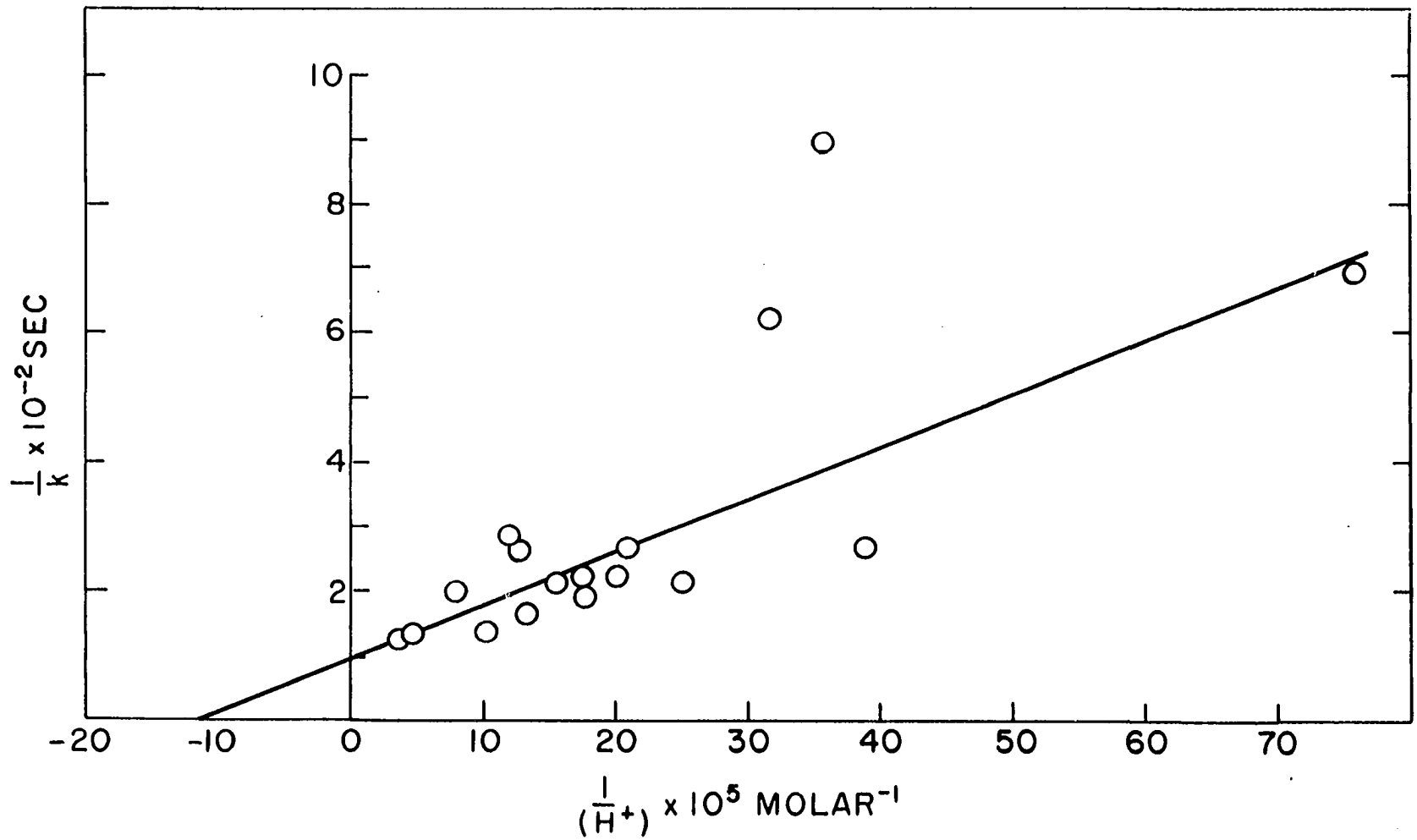

Figure 9. Plot of reciprocal rate data vs. $\frac{1}{(H^+)}$ at 50.0°C.

Table 11. Specific rate and equilibrium dissociation constants for chloride reactivation

Temperature °C.	$k_2 \times 10^4$	$K \times 10^7$
38.0	5.0 ± 1	11.8 ± 4
44.8	33 ± 9	8.3 ± 4
50.0	100 ± 30	8.7 ± 4

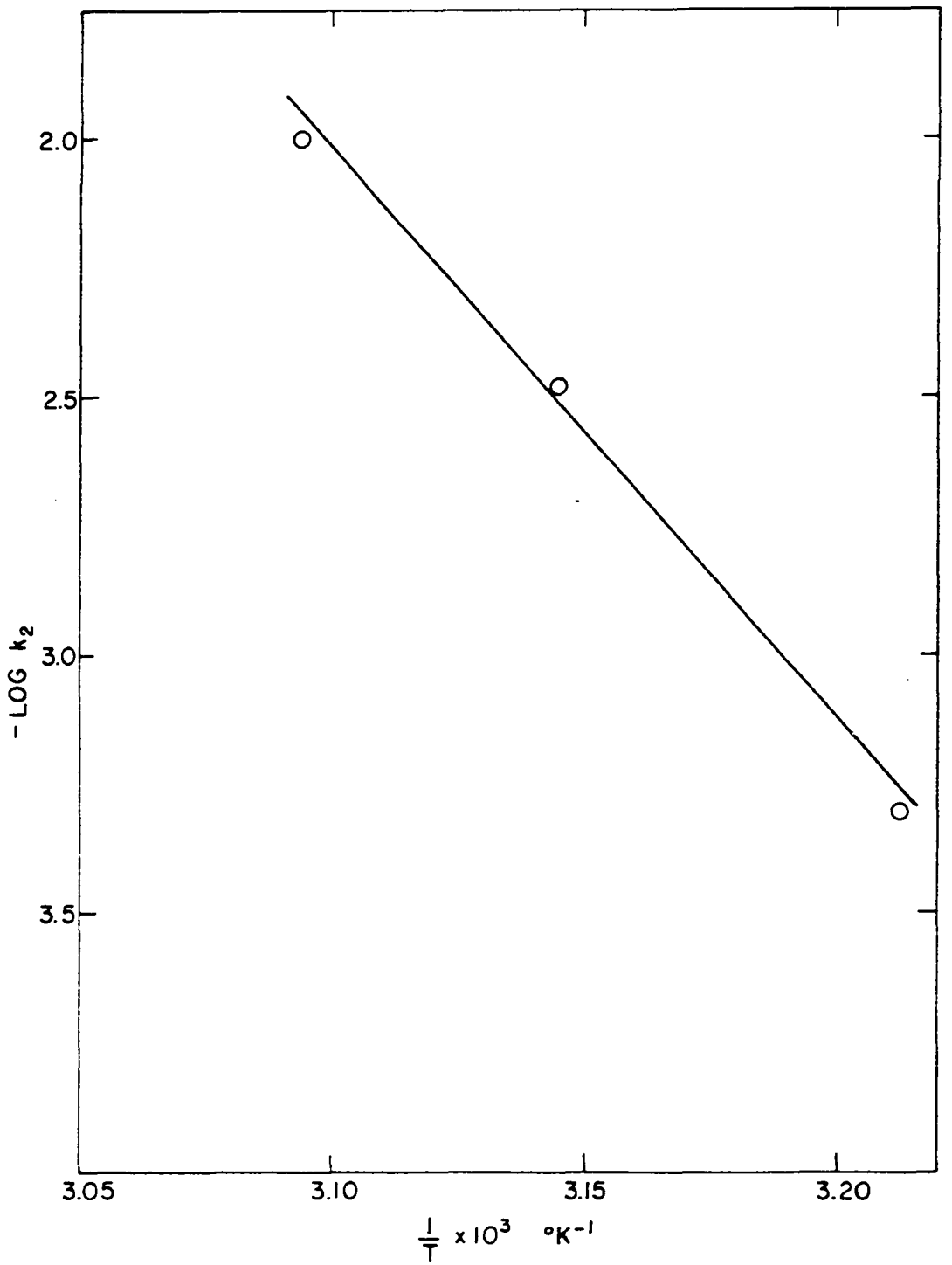

Figure 10. Plot of log k_2 vs. the reciprocal of the absolute temperature

CONCLUSION

In many studies of protein denaturation the postulated mechanism of denaturation is essentially the same. (For example, see papers or reviews by Neurath (12), Scheraga (15, 16), Pauling (13), Putnam (14), Stearn (24), Levy and Benaglia (8) and Gibbs (4).) The native protein (active enzyme) is considered to be an assembly of α-helices, cross linked by covalent bonds and side chain hydrogen bonds. The denatured protein consists of randomly coiled chains in which hydrogen bonds are ruptured but the covalent cross links still persist. The pH of maximum stability occurs in a region which depends on the pH's of the donor and acceptor groups involved in hydrogen bond cross linking and bears no relationship to the isoelectric point.

The activation process for denaturation envisaged by Scheraga and Laskowski (16) occurs in two steps. "(1) when the native protein is subjected to some stress such as changes in temperature, pressure, pH, urea concentration, etc., the native molecules instantaneously equilibrate with the new environment by ionization, rupture or formation of intramolecular hydrogen bonds, etc., (2) among the re-equilibrated native molecules there exists a small concentration of molecules in which all side-chain hydrogen bonds, responsible for stabilization of the native molecule, are broken. These molecules are considered to be in the activated state."

The pH dependence of protein denaturation is not due to an acid-base type catalytic effect, but results from a preliminary ionization process in the transition to the activated state. It is concluded that only a few ionization processes are required to prepare the activated complex, five for pepsin (7), six for ricin (8), two for hemoglobin (10), and only one for egg albumin (14).

It was pointed out previously that the pH dependence on the rates of inactivation and reactivation were approximately equal and opposite for L-amino acid oxidase. The denaturation (inactivation) process corresponding to the above arguments can then be written

(a) $EH \rightleftharpoons E + H$

(b) $E \longrightarrow E^{\neq}$

(c) $E^{\neq} \longrightarrow E_D$

Where EH is the native enzyme, E is the ionized form of the enzyme, $E^{\neq}$ is the activated ionized complex, and E_D is the inactive form of the enzyme. The rate limiting step in expressions a, b, and c is the formation of $E^{\neq}$, and this step is pH dependent. Associated with E_D is another ionization

(d) $E_D + H \rightleftharpoons E_D H$

This is the same ionization as expression a. One of the

two forms, E_D or E_D H, or both undergoes reactivation. If E_D only undergoes reactivation, the pH dependence as measured would be opposite to what was found. If both E_D and E_D H undergo reactivation at about the same rate, no pH dependence of the rate of reactivation would be found at all. The fact that at higher H^+ ion concentration, the rate of reactivation is enhanced, leads to the conclusion that E_D H is the dominating form involved in the reactivation.

It was assumed in the derivation of the rate expression that E_D H is the only form of the enzyme involved in reactivation (i.e. $k_1 = 0$). This is equivalent to saying that the hydrogen ion that comes off before inactivation can occur, must be replaced before the reactivation process.

The dissociation constant calculated from Figures 7-9 agrees reasonably well with the pH indicated by 50% inactivation in Figure 3. The dissociation constant of the ionizing group in the enzyme molecule is about 10^{-6}.

The inactivation and reactivation processes may be thought of as occurring in the following manner.

In any given enzyme solution in the pH range where reversible inactivation takes place, there is a certain fraction of what has been called E and EH, determined by the equilibrium 22. There are certainly other ionization processes taking place in the enzyme molecule, but as these do not affect the inactivation process, they are not included

in the discussion. As the temperature is raised above 10°C. some of the dissociated molecules (E) have enough energy such that some localized bonds holding the molecule in its specific configuration are broken, leading to the inactive form of the enzyme.

In the inactive form of the enzyme there is also an ionization, with the same pK, and assumed to be concerned with the same group in the enzyme as the inactivation. There is then, a certain amount of inactive enzyme in the associated form EH. This form of the inactive enzyme can overcome an energy barrier and return to the active form of the enzyme in a manner similar to the inactivation process.

The energy of activation for the reactivation process (50.5 k cal./mole) is consistent with other processes of this nature. Typical values range from 40,000 to 100,000 cal./mole for protein denaturation (14). Such reactions should not have measurable velocities at 50-100°C. according to simple collision theory of molecular reactions. Yet protein denaturation is generally too rapid for kinetic study at temperatures above 65°.

This kinetic paradox can be explained when the entropy of activation is taken into account. In general, values for $\Delta S^{\neq}$ for simple molecules are only about $\pm$ 5 entropy units. However, for complex molecules, considerable entropy changes are possible. With protein denaturation there is a large

entropy increase during the activation process, commonly running from 50 to as high as 500 units. Thus, in denaturation, the large increase in entropy is sufficient to make the reaction go despite the high energy of activation.

In the case of L-amino acid oxidase, the overall free energy change in going from inactive to active form is close to zero. Also, taking Singer and Kearney's value for $\Delta H^{\neq}$ for the inactivation process of 42.5 k cal., the overall enthalpy change is about 8 k cal. Therefore the overall entropy change is also slight. However, a large entropy change in the activation process can be envisaged.

Localized portions of the active enzyme can "open up" slightly in the activation process and "close up" again in a different position, forming the inactive state. The reactivation process would be similar, and upon the "closing up" would be in the original active state.

SUMMARY

The reversible inactivation and reactivation of L-amino acid oxidase by chloride ion was studied in an attempt to determine more about the nature of this reaction.

Results obtained by studying the effect of chloride ion concentration on the rate of reactivation indicated that one chloride ion is bound to the enzyme in the reactivation process. The exact nature of the chloride binding is still uncertain.

From equilibrium data of the inactivation process, it was concluded that a group in the enzyme, probably near the active site, with a dissociation constant of about 10^{-6}, dissociates as a first step in the inactivation process.

In the light of the pH dependence of both the rate of inactivation and reactivation, a mechanism of these two processes was proposed whereby the preliminary ionization of a particular group in the enzyme was important for inactivation, and the preliminary recombination of the H^+ ion with this same (or possibly very similar) group, was an important step in the reactivation process.

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