The Role of Zinc in Alcohol Dehydrogenase

IV. THE KINETICS OF THE INSTANTANEOUS INHIBITION OF HORSE LIVER ALCOHOL DEHYDROGENASE BY 1,10-PHENANTHROLINE*

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The alcohol dehydrogenase crystallized from horse liver contains 2 atoms of zinc per molecule and the activity of this enzyme is inhibited by metal-binding agents (1, 2). The inhibition of liver alcohol dehydrogenase by one of these agents, 1,10-phenanthroline, differs from that which is observed when the alcohol dehydrogenase isolated from yeast is exposed to 1,10-phenanthroline (3). The enzyme is inhibited by 1,10-phenanthroline only instantaneously and reversibly (2); under identical experimental conditions, the yeast enzyme, although showing a similar instantaneous type of inhibition, in addition exhibits a slow, irreversible inhibition (4). Direct experimental evidence (5) indicates that 1,10-phenanthroline acts at a zinc atom of these enzymes. These differences in the 1,10-phenanthroline inhibitions may reflect differences in the mechanism of reaction, in the structure of the active loci of these enzymes, or in both.

The present communication examines the kinetics of the inhibition of liver alcohol dehydrogenase by 1,10-phenanthroline, and the effects of coenzyme and substrates thereon. Mechanisms of action of this enzyme have been postulated based on the experimental findings.

EXPERIMENTAL

Alcohol dehydrogenase of horse liver, purified and twice crystallized (6), was obtained commercially (Worthington Biochemical Corporation), and was monodisperse upon ultracentrifugation in 0.06 M phosphate buffer, pH 7.15, at 7°. The zinc content was 1660 µg of Zn per g of protein, corresponding to 2.11 gram atoms of zinc per mole of enzyme, based on a molecular weight of 83,000 (7). The protein concentration was determined spectrophotometrically at 280 mp, with an absorption coefficient of 2.2 mg of protein per ml per cm² (6).

Reagents were used as previously described (1). 1,10-Phenanthroline hydrochloride (G. Frederick Smith Company) was dissolved in 0.06 M phosphate buffer, pH 7.15, and adjusted to a final pH of 7.15 with NaOH.

Enzymatic activity was determined spectrophotometrically by measurement at 340 mp of the change in the DPNH concentration. The optical density was recorded continuously with a Cary recording spectrophotometer within 8 seconds after the reaction was started and for the next 30 seconds. Initial rate of change were measured, and a steady state was assumed since the reaction was linear over this period. Activities, v, are expressed as moles of DPNH produced or oxidized per second per mole active site of enzyme, assuming 2 active sites per molecule. The use of a 5-cm path length absorption cell, with a volume of 15 ml, permitted the measurement of activities of the order of 0.1 sec⁻¹.

For the OP inhibition studies all measurements were made in 0.1 ionic strength phosphate buffer, pH 7.15, at 23°; this buffer was chosen to allow comparison of results with those obtained elsewhere (8). The reaction mixture contained varying concentrations of coenzyme or of substrate, of OP (including zero), and liver alcohol dehydrogenase. When the coenzyme concentration was varied, the substrate concentration was held constant at a nonrate-limiting value, determined experimentally. When the substrate concentration was varied, the coenzyme concentration was held constant at a high and almost rate-limiting value, since activity continues to increase slightly with increases in the highest coenzyme concentrations that were feasible. In measuring the rate of DPN → DPNH, hereafter described as the "forward" reaction, the enzyme concentration was 1.82 x 10⁻⁵ M; in measuring the rate of DPNH → DPN (the "backward" reaction), the enzyme concentration was 3.65 x 10⁻⁵ M; 0.5 ml of the enzyme solution was added to the reaction mixture last to start the reaction. Enzyme and OP were in contact for approximately 8 seconds before activity was measured.

The activity data were plotted as reciprocal activities versus reciprocal substrate or coenzyme concentrations, according to Lineweaver and Burk (9). Lines were fitted to the experimental points by the method of least mean squares and the degree of linearity was expressed as a correlation coefficient r, as previously described (10).

RESULTS

Instantaneous Reversible Inhibition of Liver Alcohol Dehydrogenase Activity—The enzyme is inhibited by OP, as reported previously (2). The partial activity, v/v₀, is plotted versus the logarithm of the OP concentration in the reaction mixture (Fig. 1) to demonstrate the differences in the magnitude of the inhibition when the forward and back reaction are measured. For 1 The abbreviations used are: OP, 1,10-phenanthroline; Tris, tris(hydroxymethyl)aminomethane; LADII and YADII, liver and yeast alcohol dehydrogenase, respectively (used in reactions only).
FIG. 1. The instantaneous, reversible inhibition of liver alcohol dehydrogenase activity by OP; partial activity of crystalline liver alcohol dehydrogenase versus the negative logarithm of the concentration of OP. The inhibition is in the reaction mixture at pH 7.15, in 0.1 ionic strength phosphate buffer; 23°. For the reaction $DPN \rightarrow DPNH$ ($\bullet \bullet \bullet$), $(DPN) = 3.3 \times 10^{-4}$ M, $(CH_3OH) = 1.67 \times 10^{-2}$ M, liver alcohol dehydrogenase = 200 $\mu$g. The points (X) represent partial activity when a reaction mixture containing $2 \times 10^{-3}$ M OP is diluted after activity is measured ($v_0/v_c = 0.19$) to produce a final OP concentration of $4 \times 10^{-4}$ M; only enzyme and OP are diluted. For the reaction $DPNH \rightarrow DPN$ ($\circ \circ \circ$), $(DPNH) = 5 \times 10^{-3}$ M, liver alcohol dehydrogenase = 4 $\mu$g. Activity is measured upon the addition of liver alcohol dehydrogenase; uninhibited activity is $v_0$, inhibited activity is $v_i$.

FIG. 2. Kinetics of the inhibition of liver alcohol dehydrogenase activity by OP, when DPN concentration is varied in the reaction mixture. All the lines are calculated by the method of least mean squares, and $r$ = the correlation coefficient. Activity measurements: $(CH_3OH) = 2 \times 10^{-2}$ M; the initial rate of $DPN \rightarrow DPNH$ is measured in 0.1 ionic strength phosphate buffer, pH 7.15; 23°.

the forward reaction, the concentration of DPN is $3.33 \times 10^{-4}$ M, and the concentration of ethanol, $1.67 \times 10^{-2}$ M. Inhibition of 50% occurs at $4.5 \times 10^{-4}$ M OP, to be compared with a value of $4.8 \times 10^{-4}$ M OP, obtained previously in 0.1 M pyrophosphate buffer, pH 8.8, with similar concentrations of reactants (2). For the back reaction, the DPNH concentration is $1 \times 10^{-4}$ M and the acetaldehyde concentration, $5.0 \times 10^{-3}$ M; 50% inhibition is observed in the presence of $2.2 \times 10^{-5}$ M OP.

The OP inhibition under these conditions is completely reversible by dilution, as shown by the following experiment. The forward activity was measured in the presence of $2 \times 10^{-5}$ M OP; after 30 seconds an aliquot of 3 ml was removed and added to 12 ml of solution containing sufficient DPN and ethanol to result in final concentrations of $(DPN) = 3.33 \times 10^{-3}$ M, ethanol = $1.67 \times 10^{-2}$ M. This accomplished a 5-fold dilution of enzyme and inhibitor without change of the substrate or coenzyme concentrations. Assuming complete reversibility, the predicted fractional activity after dilution would be 0.56; 0.57 is observed experimentally. Similar results have been obtained in 0.1 M pyrophosphate buffer, pH 8.8 (2).

Effect of Varying DPN Concentrations—Reciprocal enzymatic activities, $v^{-1}$, are plotted versus reciprocal DPN concentrations at four OP concentrations, including zero (Fig. 2). The ethanol concentration is constant at $2 \times 10^{-2}$ M. The calculated lines converge at a common intercept, where $v^{-1}$ is 0.37 second; this corresponds to a maximum activity of 2.7 sec$^{-1}$. The slopes of the $v^{-1}$ versus $(DPN)^{-1}$ lines increase with increasing OP concentration.

Effect of Varying Ethanol Concentration—Fig. 3 shows reciprocal activity plotted versus reciprocal ethanol concentration for four OP concentrations, including zero. The DPN concentration is $4 \times 10^{-3}$ M. The calculated lines intersect at $(CH_3OH) = 3.8 \times 10^{-4}$ M and $v^{-1} = 0.21$ second. Both the slopes and the intercepts of the $v^{-1}$ versus $(CH_3OH)^{-1}$ lines increase with increasing OP concentration.

Effect of Varying DPNH Concentration—The plot of reciprocal activity versus reciprocal DPNH concentration at two OP concentrations and at $(OP) = 0$ is shown in Fig. 4. The acetaldehyde concentration is $5 \times 10^{-3}$ M. The calculated lines meet at a common intercept, $v^{-1} = 0.55$ second, corresponding to a maximum activity of 1.81 sec$^{-1}$. The slopes of the $v^{-1}$ versus $(DPNH)^{-1}$ lines increase with increasing OP concentration.

Effect of Varying Acetaldehyde Concentration—In Fig. 5, reciprocal activity versus reciprocal acetaldehyde concentration is plotted, in the absence of OP and for three OP concentrations. The $DPNH$ concentration is $1 \times 10^{-4}$ M. The calculated lines are parallel, and the intercepts of the $v^{-1}$ versus $(CH_3CHO)^{-1}$ lines increase with increasing OP concentration.

FIG. 3. Kinetics of the inhibition of liver alcohol dehydrogenase activity by OP, when ethanol concentration is varied in the reaction mixture. The lines are calculated as in Fig. 2. Activity measurements: $(DPN) = 4 \times 10^{-3}$ M; the rate is measured as in Fig. 2.
DISCUSSION

The data indicate that both oxidized and reduced coenzyme compete with OP (Figs. 2 and 4) for an enzymatic site, which apparently involves zinc. Neither ethanol nor acetaldehyde competes with OP (Figs. 3 and 5). The combination of OP with the enzyme at a zinc atom is reversible (Fig. 1). Based on these data, and on previous explicit assumptions (3), the following mechanism of enzyme action and OP inhibition is proposed for examination:

\[
\text{LADH-Zn + DPNH} \xrightleftharpoons[k_7]{k_1} \text{LADH-Zn-DPNH} \quad (1)
\]

\[
\text{LADH-Zn-DPNH + CH}_3\text{CHO} + \text{H}^+ \xrightleftharpoons[k_8]{k_4} \text{LADH-Zn-DPN} + \text{C}_2\text{H}_5\text{OH} \quad (2)
\]

\[
\text{LADH-Zn-DPN} \xrightarrow{k_3} \text{LADH-Zn + DPN} \quad (3)
\]

\[
\text{LADH-Zn + OP} \xrightarrow[K_{op}]{k_5} \text{LADH-Zn-OP} \quad (4)
\]

In this formulation LADH-Zn represents each zinc atom of liver alcohol dehydrogenase. \(K_{op}\) is the dissociation constant of the dissociable LADH-Zn-OP complex. This reaction mechanism is based on and similar to that presented by Theorell and Chance for the liver enzyme (11) and with that postulated for the yeast enzyme (3).

The velocity constants for the control reactions (Figs. 1 to 3) may be calculated from the Lineweaver-Burk plots in which substrate or coenzyme concentrations are varied in the absence of OP. The calculations are similar to those of Theorell et al. (8) (Table I). The numerical constants derived do not differ very greatly from those obtained by these authors and recalculated in Table I, except for \(k_8\). Variations of the numerical values by a factor of 2 were observed in some duplicate experiments.

\(K_{op}\), the apparent dissociation constant of the enzyme-OP complex, may be determined from the velocities of the inhibited reaction, as previously described (3). In Fig. 4, reciprocal activity is plotted versus OP concentration at 4 DPNH concentrations (see Fig. 4). The lines intersect at \(-OP = 4 \times 10^{-6} \text{ M}\), giving the apparent \(K_{op}\) for the back (DPNH + DPN) reaction. In Fig. 5, reciprocal activity is plotted versus OP concentration at 4 DPNH concentrations (see Fig. 2). The lines intersect at \(-OP = 7 \times 10^{-6} \text{ M}\), giving the apparent \(K_{op}\) for the forward (DPN + DPNH) reaction.

**TABLE I**

<table>
<thead>
<tr>
<th>Constant</th>
<th>Units</th>
<th>From Figs. 2 to 3</th>
<th>From Theorell et al. (8)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(k_1)</td>
<td>(\text{M}^{-1} \times \text{sec}^{-1} \times 10^{-4})</td>
<td>6.8</td>
<td>7.4</td>
</tr>
<tr>
<td>(k_3)</td>
<td>(\text{sec}^{-1})</td>
<td>3.7</td>
<td>3.2</td>
</tr>
<tr>
<td>(k_4)</td>
<td>(179)</td>
<td>4.8</td>
<td>4.8</td>
</tr>
<tr>
<td>((\text{H}^+)_4k_4)</td>
<td>(\text{M}^{-1} \times \text{sec}^{-1} \times 10^{-2})</td>
<td>4.8</td>
<td>4.8</td>
</tr>
<tr>
<td>(k_5)</td>
<td>(\text{M}^{-1} \times \text{sec}^{-1} \times 10^{-4})</td>
<td>5.1</td>
<td>60</td>
</tr>
<tr>
<td>(k_6)</td>
<td>(1.8)</td>
<td>1.8</td>
<td>0.7</td>
</tr>
</tbody>
</table>

**Fig. 6.** Determination of \(K_{op}\) from the velocities of liver alcohol dehydrogenase activity inhibited by OP. (A) \(v^{-1}\) is plotted versus OP concentration at 4 DPNH concentrations (see Fig. 4). The lines intersect at \(-OP = 4 \times 10^{-6} \text{ M}\), giving the apparent \(K_{op}\) for the back (DPNH + DPN) reaction. (B) \(v^{-1}\) is plotted versus OP concentration at 4 DPNH concentrations (see Fig. 2). The lines intersect at \(-OP = 7 \times 10^{-6} \text{ M}\), giving the apparent \(K_{op}\) for the forward (DPN + DPNH) reaction.
intersect at (OP) = 4.0 \times 10^{-8} M, which corresponds to the apparent \( K_{OP} \) for the back (DPNH → DPN) reaction, independent of DPNII concentration. Similarly, with the use of the data of Fig. 2, reciprocal activity is plotted versus OP concentration at four DPN concentrations in Fig. 6B. The lines intersect at (OP) = 7.0 \times 10^{-8} M, corresponding to an apparent \( K_{OP} \) for the forward (DPN → DPNH) reaction independent of DPN concentration. The given definition for \( K_{OP} \) (Equation 4) sets its value as a function only of enzyme and OP concentrations, implying that \( K_{OP} \) is independent of substrate and coenzyme concentrations. This 17-fold difference in the apparent \( K_{OP} \) values is well outside the experimental error, and suggests that the postulated mechanism (Equations 1 to 4) is not a full representation of the inhibition of liver alcohol dehydrogenase by OP.

The value for \( K_{OP} \), determined spectrophotometrically (5) in the absence of substrate and coenzyme, is 3.3 \times 10^{-8} M, with a standard deviation of ±0.58 \times 10^{-8} M in 13 determinations, as measured in 0.1 M Tris buffer, pH 7.5, 23°C. It was found to be 3.1 \times 10^{-8} M ± 1.7 \times 10^{-8} M in 14 determinations in phosphate buffer pH 7.15, 23°C. Although the \( K_{OP} \) for the forward reaction, 7.0 \times 10^{-8} M, may not be significantly different from this value, the \( K_{OP} \) derived from the back reaction, 4.0 \times 10^{-8} M, is. It thus appears that both the coenzyme and the substrate may affect this inhibition, as expressed by the apparent \( K_{OP} \), by mechanisms different from those in Equations 1 to 4.

This discrepancy between the apparent values for \( K_{OP} \) in the presence of ethanol or acetaldehyde, and that between those and the \( K_{OP} \) determined spectrophotometrically, which has an intermediate value, indicates a possible effect of the substrates on \( K_{OP} \). Ethanol (Fig. 3) is not completely noncompetitive with OP, although both intercepts and slopes of the lines describing the inhibition do increase with OP concentration. Acetaldehyde (Fig. 5) is uncompetitive with OP. If a substrate has no effect on the inhibitory binding of OP to LADH-Zn, the kinetics of inhibition would be expected to be purely noncompetitive, as Equations 2 and 4 imply. It is possible to estimate the effects of the substrates on the inhibition, and empirically to obtain a value for \( K_{OP} \) which is common both for the forward and back reaction, by extrapolating the data in Figs. 3 and 5. For this purpose, a first approximation of \( K_{OP} \) as a function of substrate concentrations may be estimated.

The data in Figs. 6A and B indicate that one molecule of OP reacts with each zinc atom to form the enzyme-inhibitor complex. Direct spectrophotometric data confirm this (5). Thus, that concentration of OP which inhibits 50% is directly related to \( K_{OP} \). A plot of the logarithm of this OP concentration versus the logarithm of the (C\(_2\)H\(_5\)OH) and (CH\(_3\)CHO) concentrations, to maximize the fit of the data to linearity, is shown in Fig. 7. This plot does arrange the points, derived from the data in Figs. 3 and 5, linearly. Changing concentrations of ethanol or acetaldehyde have opposite effects on the concentration of OP required to inhibit activity 50%: decreasing (C\(_2\)H\(_5\)OH) decreases the (OP) required, while decreasing (CH\(_3\)CHO) increases it. The two lines showing this relationship intersect at (OP) = 7 \times 10^{-8} M, and approximately at 10^{-4} M substrate concentration. Thus, at this relatively low substrate concentration, an apparent \( K_{OP} \) value is obtained which is common both for the forward and back reactions, and is in agreement with that obtained from Figs. 3 and 6B. Thus there are three possible numerical values for \( K_{OP} \): 7 \times 10^{-8} M, 3.1 \times 10^{-8} M (5), and 4 \times 10^{-4} M (Figs. 5 and 6A).

A value for \( K_{OP} \) which adequately represents the experimental data on inhibitions should lead to the derivation of velocity constants which agree with those derived from the uninhibited control reactions in Table I. The velocity constants in the reaction scheme shown in Equations 1 and 3 were calculated from the inhibition kinetics by a previously described graphic method of solution (3). Fig. 8A shows the data on (C\(_2\)H\(_5\)OH) variations in which the values of the slopes of the lines in Fig. 3 are plotted as a function of (OP) concentration. Fig. 8B is a plot of the intercepts from the same data, versus (OP) concentration. The equations for solution for values of \( k_2, k_3, k_4 \), and \( k_5 \) are indicated.

With the use of these data, and each of the three possible values for \( K_{OP} \) discussed above, the results shown in Table II were calculated. \( K_{OP} = 7 \times 10^{-8} M \) is an unacceptable value, since no solution is then available for \( k_3 \) or \( k_6 \). When \( K_{OP} = 3.1 \times 10^{-8} M \), real values are obtained for all the velocity constants which are in good agreement with those shown in Table I, except for \( k_5 \) which is low. When \( K_{OP} = 4 \times 10^{-8} M \), both \( k_3 \) and \( k_4 \) are lower than the values in Table I, whereas \( k_5 \) is high.

**Fig. 7.** Empirical plot of the negative logarithm of the concentration of OP inhibiting 50% versus the negative logarithm of the substrate (ethanol or acetaldehyde) concentration. Data are derived from Figs. 3 and 5, respectively. For the details, see the text.

**Fig. 8.** Calculation of the velocity constants \( k_2, k_3, k_4 \), and \( k_5 \), from the data in Figs. 3 and 5, respectively. For the details, see the text.
Obviously, the significance of these deviations is questionable. It may be concluded, however, that the $K_{op}$ value of $3.1 \times 10^{-5}$ m gives consistent values for velocity constants, and being determined independently of activity measurements (5), is the most objective of the acceptable values for $K_{op}$.

The inhibitory kinetics when ethanol concentration is as high as $2 \times 10^{-3}$ m (Fig. 3) seem to be consistent with Equations 1 to 4, and this value for $K_{op}$. High acetaldehyde concentrations, on the other hand, are not susceptible to a similar kinetic analysis. The data in Fig. 5, where (CH$_3$CHO) was varied to as high as $5 \times 10^{-2}$ m, produce lines which are parallel; no similar solution for velocity constants is available, therefore. The apparent value of $K_{op}$, however, is affected by the (CH$_3$CHO) concentration (Fig. 7). Thus, while the forward reaction scheme and the chosen value for $K_{op}$ seem consistent with the postulated reaction scheme, the back reaction scheme does not. Further modifications thus may be examined.

The apparent effect of the substrates on $K_{op}$ may imply the formation of a ternary complex involving the coenzyme, the substrate, and zinc, since the inhibitor, OP, combines specifically with zinc under the conditions employed. Several observations, however, contradict this hypothesis. Consistent values for the velocity constants in Equations 1 to 3 can be calculated from noninhibited (Table I) or inhibited (Table II) reaction kinetics without including equations for the formation of such ternary complexes in the calculations. Further, if a substrate actually were bound to zinc atoms, it should compete with OP; neither substrate does. Alternatively, however, it might be postulated that a ternary complex forms which does not involve a zinc atom as a coordinating center, but with the substrates so bound to the coenzyme that the binding of the latter to the zinc is affected, and thereby the inhibition by OP. Since OP acts only at a zinc atom, the present data on inhibitions are not adequate to test this hypothesis directly. It may be concluded, however, that such a ternary complex does not form at the zinc atom.

Such modifications of the reaction scheme invoke actions of the substrates extrinsic to the enzyme molecule itself to explain disparities in the inhibitory effects of OP exerted at its zinc atoms. These are obviously of limited value in the absence of further experimental evidence as to their mode of interaction with this metallodehydrogenase. For the purpose of constructing a kinetic model, however, some indirect evidence exists which may with equal justification be used to support the hypothesis of a change in the enzyme molecule itself, to account for the observed differences.

Horse liver alcohol dehydrogenase may be postulated to exist in two forms with different degrees of association with the coenzyme, or with OP, or with both. These forms might be in an oxidation-reduction equilibrium; this would be consistent with the observation that this enzyme can reduce DPN in the absence of added ethanol (12). Alternatively, such forms might differ in structure; another zinc metallodehydrogenase, the glutamic dehydrogenase of beef liver (13, 14), when crystallized has a molecular weight of $10^6$ (15), but is cleaved to smaller molecules by DPNH or by OP (16). Recent findings (17-19) indicate that the yeast alcohol dehydrogenase (YADH) also may exist as several molecular moieties. A kinetic scheme incorporating such reactions has been constructed, and leads to a formulation which rationalizes the apparent disparities in the data on inhibition shown here, while including the competitive behavior of coenzyme and OP. Direct evaluation of structural changes in the liver enzyme are in progress to test such a model.

The differences in the interaction of the yeast and the liver enzymes with OP may reflect the mode of binding of the zinc to the apoenzyme, as well as the chemical and steric organization of the apoenzyme protein molecule proximate to the zinc atoms.

**Summary**

The kinetics of the instantaneous reversible inhibition of horse liver alcohol dehydrogenase by 1,10-phenanthroline have been studied. The data indicate that diphosphopyridine nucleotide (DPN) and its reduced form (DPNH) interact with the enzyme at or near a zinc atom, whereas ethanol and acetaldehyde do not. The inhibition is mediated through the formation of an enzyme-inhibitor complex in which 1 molecule of 1,10-phenanthroline is bound to each zinc atom. This is consistent with the results of studies using spectrophotometric rather than enzymatic material (5). The kinetically determined values of $K_{op}$, the dissociation constant of this complex, extrapolated to (DPN) = 0 or to (DPNH) = 0, are $7.0 \times 10^{-5}$ m and $4.0 \times 10^{-4}$ m, respectively.

This compares with a value of $3.1 \times 10^{-5}$ m obtained by spectrophotometric determinations of the dissociation constants (5). This discrepancy, together with certain anomalies in the inhibition kinetics when (C$_2$H$_5$OH) or (CH$_3$CHO) are varied, has been the basis for several model schemes of enzyme reaction, including the assumption that liver alcohol dehydrogenase may exist in oxidation-reduction forms capable of interconversion through the oxidation-reduction reaction between diphosphopyridine nucleotide and its reduced form. The inhibition kinetics imply that [(LADH)Zn] and [(YADH)Zn] may have different modes of action.

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| Table II

| Velocity constants for liver alcohol dehydrogenase calculated (see fig. 8) from inhibition data in fig. 8 and with different values for $K_{op}$ |
|-----------------|-----------------|-----------------|
| Constant       | Units           | 4.0 X 10^-6 m   | 3.1 X 10^-6 m   | 7.0 X 10^-6 m   |
| $k_1$          | sec^-1          | 4.1             | 4.4             | 5.0             |
| $k_2$          | sec^-1          | 27              | 61              | Negative        |
| $k_3$          | M^-1 X sec^-1 X 10^-4 | 9.7              | 1.3             | 0.56            |
| $k_4$          | M^-1 X sec^-1 X 10^-4 | 0.83            | 1.9             | Negative        |
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