A MECHANISM FOR THE INACTIVATION OF YEAST PYRUVATE KINASE FORMULATED FROM THEORETICAL ANALYSIS OF KINETIC DATA

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ABSTRACT

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by Norbert Anthony Feliss

This thesis evaluates the kinetic mechanism of the inactivation of yeast pyruvate kinase as a function of temperature and FDP concentration. The data analyzed were abstracted from the Ph.D. thesis of Ronald T. Kuczenski at Michigan State University, 1970. Differential rate equations have been formulated on the basis of the following model representing the mechanism of inactivation.

$$T \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} 2D^*$$

$$D^* \xrightarrow{k_2} D$$

$$D \xrightarrow{k_3} 2M$$

T = Tetramer
D = Dimer
M = Monomer

The solution of the rate equation yields an equation in which the decay in activity of tetramer and dimer is a function of the initial protein concentration, the rate constants k_1, k_{-1}, k_2, k_3, and a theoretical number N which represents the percentage of the activity that the dimer contributes to the overall activity.

$$T' = (1/\zeta)[\{(\sigma^\circ-1)\mathrm{EXP}(-k_1t/\sigma^\circ)+1\}^2-1] + N(k_2^2/4k_{-1})(\sigma^\circ-1)t\mathrm{EXP}(-k_3t)$$

$$(1/\zeta) = [16(k_1k_{-1}/k_2^2)]^{-1}$$

$$\sigma^\circ = (1+16k_1k_{-1}T^\circ/k_2^2)^{1/2}$$

The above equation gives a successful approximation to the experimental decay curves at temperatures of 0° and 23° and for FDP concentrations between 5.36 mM and 97.2 mM.

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By

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A THESIS

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To my parents, Jim and Evelyn Feliss

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INTRODUCTION

This thesis encompasses an extensive and explicit mathematical interpretation of the mechanism of the FDP-enhanced inactivation of yeast pyruvate kinase. From independent studies of Kuczenski and Suelter (1,2,3) it has been conclusively shown that yeast pyruvate kinase is susceptible to inactivation at low temperatures. Addition of fructose 1,6-diphosphate (FDP), an allosteric activator, markedly enhances the rate of loss of activity both in the cold and at room temperature. A biphasic first-order progress curve can be resolved from their data (1,2). Both steps of this curve were shown to be dependent on the half power of the enzyme concentration and are accompanied by a decrease in the sedimentation coefficient from 8.6 to 3.0S, followed by a slower decrease to 1.7S. The data are consistent with the binding of a minimum of two FDP per molecule of enzyme with a geometric average dissociation constant of 63μM. This thesis will report efforts to elucidate the mechanism of this inactivation, with the hope that the results would lead to a clearer insight concerning protein structure and function. Reports of other cold labile enzymes are now appearing with increasing frequency and motivated the experimenter to evaluate the correct mechanism of this temperature inactivation phenomenon.

RESULTS

I. Presentation of Previous Information

1. Examination of the Experimental Data

The FDP-enhanced inactivation of yeast pyruvate kinase has been observed at several temperatures between 0° and 23° (3). Biphasic curves were obtained at all temperatures but a cursory examination suggested different mechanisms at the two temperatures. The data were plotted as log specific activity versus time for various protein concentrations (see Figures 1 and 2). Close inspection reveals that the loss of activity at 0° is much faster initially than at 23° but at longer times the slope of the curve does not decrease as fast as the 23° case.

The biphasic curves for both 0° and 23° cases were separated into a fast and a slow inactivation process. The slopes of each of these were treated independently as first order processes. At zero degrees extrapolation of the slow inactivation steps to zero time gave different intercepts for each protein concentration. In comparison, curves for the 23° case show that the slow steps extrapolate to the same value at zero time. The qualitative behavior of the curves can be used as one criterion for examining the rate equations. In section VI.2 an empirical analysis of the slopes for both 0° and 23° is presented and is shown to be consistent with the experimental data.

Figure 1. Effect of protein Concentration on the FDP-Enhanced Inactivation of Yeast Pyruvate Kinase at 0° in 0.1 M Tris-HCl, pH 7.5

FDP concentration in all cases was 1.26×10^{-3} M. (Data from the PhD Thesis of R.T. Kuczenski, Michigan State University, 1970)

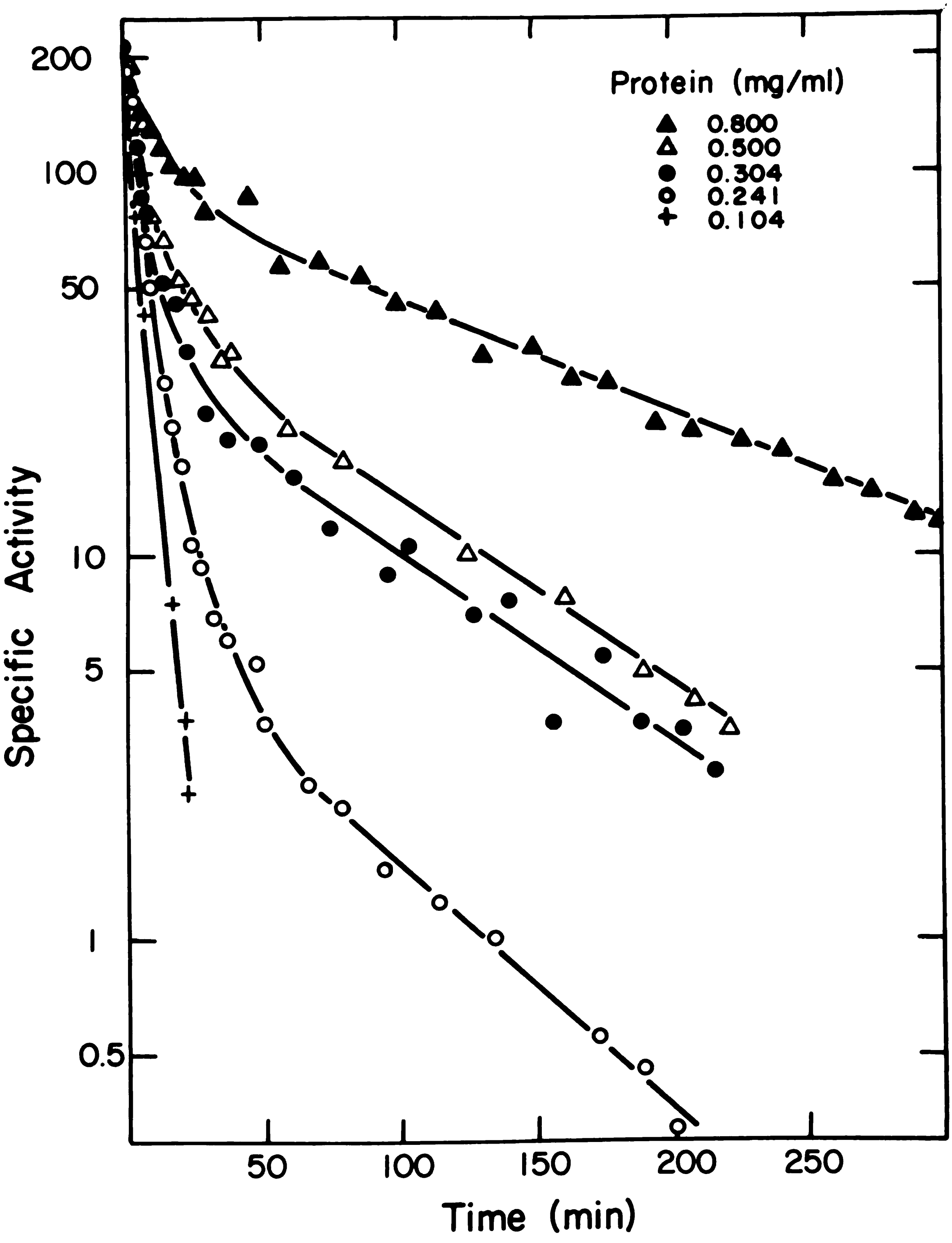
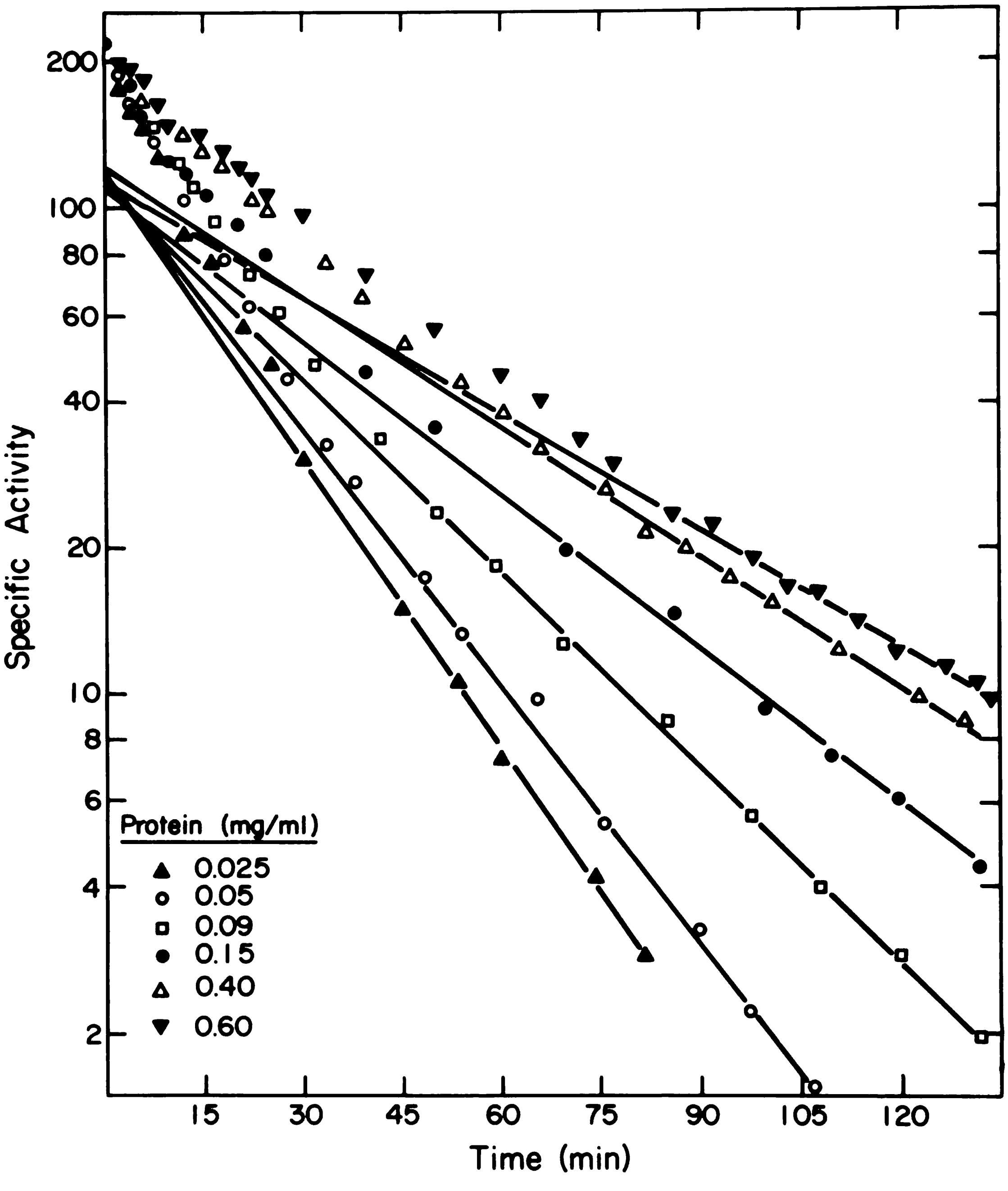

Protein (mg/ml)
▲ 0.800
△ 0.500
● 0.304
○ 0.241
+ 0.104
Specific Activity
200
100
50
10
5
1
0.5
50
100
150
200
250
Time (min)

Figure 2. Effect of Protein Concentration on the FDP-Enhanced Inactivation of Yeast Pyruvate Kinase at 23° in 0.1 M Tris-HCl, pH 7.5

FDP concentration in all cases was 2.68 mM. (Data from the PhD Thesis of R.T. Kuczenski, Michigan State University, 1970)


Specific Activity
200
100
80
60
40
20
10
8
6
4
2
15
30
45
60
75
90
105
120
Time (min)
Protein (mg/ml)
▲ 0.025
○ 0.05
□ 0.09
● 0.15
△ 0.40
▼ 0.60

2. Analysis of the reaction order

A reaction of the n^{th} order $(n>1)$ with respect to a single reactant has the rate law

$$dx/dt = k(c_o-x)^n \tag{1.1}$$

which upon integration yields

$$1/(n-1)\{1/(c_o-x)^{n-1}-1/c_o^{n-1}\} = kt \tag{1.2}$$

from which the order of the reaction can be deduced. From the integrated rate equation it can be seen that for a first order reaction

$$t_{1/2}= \ln(2/k) \tag{1.3}$$

while for reactions of order n with $n>1$

$$t_{1/2}= 1/(k(n-1))$$

From equation (1.1) the half life may be defined for all values of n as

$$t_{1/2} = f(n,k)/a^{n-1} \tag{1.5}$$

where f is some function of n and k, and a is defined as the initial concentration of the reactant. In log form equation (1.5) yields

$$\log t_{1/2} = \log f - (n-1)\log a$$

A log-log plot of $t_{1/2}$ versus a should yield a straight line with slope $(1-n)$. Therefore, the dependence of $t_{1/2}$ on the initial concentration determines the reaction order directly. This type of analysis was presented by Kuczenski and Suelter (2) in order to evalualte the order in protein concentration for the inactivation process at 0°. Since the two linear portions of the biphasic curve were treated as first order processes, the half lives determined from the slope at short times plotted against tetramer concentration gave a value of 0.47 for the order with respect to protein concentration for this portion of the curve. Similarly, the slope for the long time inactivation portion of the biphasic curve was designated as rate k_3. From a log-log plot of $t_{1/2}$ versus initial protein concentration a straight line was obtained which yielded a value of $n = 0.48$. Thus the rapid and slow steps of the inactivation involving dissociation of the protein into subunits are consistent with the half power dependence on protein concentration.

The mechanism to be developed must be consistent with the following criteria: (1) two different biphasic curves, one for 0° and one for 23°; (2) a rate expression that includes a half power protein concentration dependence; (3) a model involving a tetrameric protein dissociating into four independent subunits.

II. Presentation of the Theoretical Model

A schematic mechanism of the inactivation process consistent with the experimental data is presented as the following:

$$\begin{aligned} T &\xrightleftharpoons[k_{-1}]{k_1} 2D^* \\ D^* &\xrightarrow{k_2} D \\ D &\xrightarrow{k_3} 2M \end{aligned} \tag{2.1}$$

In this model T represents the concentration of active tetramer, D^* represents the concentration of a short lived and unstable form of D, D represents the concentration of dimer, and M represents the concentration of inactive monomer. It is assumed that an equilibrium is maintained between active tetramer T and dimer D^*. D^* represents an unstable form of D which irreversibly converts to a stable and active D.

The rate equations that are consistent with the model presented are:

$$d(T)/dt = -k_1(T) + k_{-1}(D^*)^2 \tag{2.2}$$

$$d(D^*)/dt = 2k_1(T) - 2k_{-1}(D^*)^2 - k_2(D^*) \tag{2.3}$$

$$d(D)/dt = k_2(D^*) - k_3(D) \tag{2.4}$$

$$d(M)/dt = 2k_3(D) \tag{2.5}$$

When the differential equations describing a reaction mechanism cannot be described by a simple one term rate equation, the mathematical problem of integrating the rate equations can become quite complex. No general method of solving such problems can be given, since usually each reaction mechanism is a special case. For this reason, in order to provide the most simple and yet explicit expressions from the integrated rate equations assumptions must be made. One of these simplifying assumptions is the steady state approximation. Very often intermediates are present in very small concentrations. In such a situation the rate of change of the concentration of the intermediates with time is much smaller than the corresponding quantities for the reactants and products. The intermediate is then said to be in steady state and its time derivative can be set equal to zero. The result is a vast simplification of the rate equation. Therfore , it will be assumed that changes in steady state concentration of D^* is zero on the basis that it is a short-lived intermediate which rapidly converts to the more stable form, D. Further assumptions concerning the mathematical treatment of the integrated rate equations will be developed and each assumption will be explained in detail.

From the steady state assumption that $d(D^*)/dt = 0$, equation (2.3) can be solved for (D^*) as a function of (T).

$$d(D^*)/dt = 2k_1(T)-2k_{-1}(D^*)^2-k_2(D^*) = 0$$

$$(D^*) = \frac{-k_2+(k_2^2 + 16k_1k_{-1}(T)^{1/2})}{4k_{-1}}$$

$$(D^*) = \frac{k_2}{4k_{-1}}[(1 + 16k_1k_{-1}(T)/k_2^2)^{1/2} - 1] \qquad (2.6)$$

From equation (2.1)

$$d(T)/dt = -k_1(T) + k_{-1}(D^*)^2$$

and after substitution for (D^*) into this equation

$$d(T)/dt = \frac{k_2^2}{8k_{-1}}[1 - (1 + 16k_1k_{-1}(T)/k_2^2)^{1/2}] \qquad (2.7)$$

Equation (2.7) can be integrated exactly after applying the boundary condition that at t = 0, T = T°, to give the following expression:

$$(1 + 16k_1k_{-1}(T)/k_2^2)^{1/2} - (1 + 16k_1k_{-1}(T°)/k_2^2)^{1/2}$$
$$+ \ln\left[\frac{(1 + 16k_1k_{-1}(T°)/k_2^2)^{1/2}}{(1 + 16k_1k_{-1}(T)/k_2^2)^{1/2}}\right] = -k_1t \qquad (2.8)$$

Equation (2.8) gives the rate of decay of the concentration of T as a function of the initial protein concentration T°, the rate constants k_1, k_{-1}, and k_2, and the independent variable t. In order to solve for an explicit relationship for T with respect to time, equation (2.8) must be converted to a linear equation in T. Before this is accomplished two cases will be treated in which equation (2.8) can be simplified into a form which will provide a comparison to the experimental data and the kinetic mechanism.

The first case will be a treatment for short times or for those times in which the following relationship will hold:

$$16k_1k_{-1}(T)/k_2^2 >> 1$$

or $$4(k_1k_{-1}/k_2^2)^{1/2}(T)^{1/2} >> 1$$

With this assumption, equation (2.8) can be simplified to

$$\frac{4(k_1k_{-1})^{1/2}}{k_2}\,[T^{1/2} - T^{\circ 1/2}] + \ln\frac{T^{1/2}}{T^{\circ 1/2}} = -k_1 t$$

then
$$\frac{4T^{\circ 1/2}}{k_2K_{eq.}^{1/2}}\,[1 - (T/T^{\circ})^{1/2}] - \ln\frac{(T/T^{\circ})^{1/2}}{k_1} = t$$

from which follows
$$(T/T^{\circ})^{1/2} + \frac{k_2\ln(T/T^{\circ})^{1/2}}{4(k_1k_{-1})^{1/2}T^{\circ 1/2}} = 1 - \frac{k_2K_{eq.}^{1/2}t}{4\,T^{\circ 1/2}}$$

$$\ln\left[(T/T^{\circ})^{1/2}\left\{1 + \frac{\ln(T/T^{\circ})^{1/2}}{\frac{4(k_1k_{-1})^{1/2}}{k_2}\,T^{\circ 1/2}(T/T^{\circ})^{1/2}}\right\}\right] = \ln\left[1 - \frac{k_2K_{eq.}^{1/2}\,t}{4T^{\circ 1/2}}\right]$$

If it is assumed that
$$|\ln(T/T^{\circ})^{1/2}| < \frac{4(k_1k_{-1})^{1/2}\,T^{\circ 1/2}(T/T^{\circ})^{1/2}}{k_2} \qquad (2.11)$$

then the above can be further simplified by expanding the natural log term in (2.11) to give

$$\ln(T/T^{\circ})^{1/2}[1 + \frac{1}{C} - \frac{1}{2C^2}\ln(T/T^{\circ})^{1/2} + \frac{1}{3C^2}(\ln^2[T/T^{\circ}]^{1/2}) = \ln\left[1 - \frac{k_2K_{eq.}^{1/2}\,t}{4T^{\circ 1/2}}\right]$$

where
$$C = \frac{4(k_1k_{-1})^{1/2}T^{1/2}}{k_2}$$

If the latter term in equation (2.11) is greater than one, then

$$1/C \rightarrow 0 \quad \text{for } 4(k_1k_{-1})^{1/2}T^{1/2} >> 1$$

and
$$\ln(T/T^{\circ}) = \left[1 - \frac{k_2\,K_{eq.}^{1/2}\,t}{4T^{\circ 1/2}}\right]$$

For t small, this last term can be expanded about $k_2K_{eq.}^{1/2}t/4T^{\circ 1/2}$ and after dropping terms t^2, t^3,....t^n, the following equation is obtained

$$T = T^{\circ}\ \mathrm{EXP}\left[-\left(\frac{k_2^2\,k_1}{4k_{-1}T^{\circ}}\right) t\right] \qquad (2.13)$$

If lnT were plotted versus t, a curve with slope equal to $-[(k_2^2k_1)/4k_{-1}T°]^{1/2}$ and intercept of lnT° will result. This shows that the theoretical model is consistent with the experimental data since the slope is a function of half power protein concentration. However, this is an approximation and should apply only to very short times.

For the second case, an approximate evaluation will be made for longer times. Equation (2.9) is rewritten to give

$$(16k_1k_{-1}T)/k_2^2 << 1 \tag{2.14}$$

Setting $(16k_1k_{-1}T)/k_2^2 = a$ and $(16k_1k_{-1}T°)/k_2^2 = a°$ in equation (2.8), it follows

$$(1+a)^{1/2} - (1+a°)^{1/2} + \ln[\frac{(1+a)^{1/2} - 1}{(1+a°)^{1/2}-1}] = -k_1t$$

Consistent with the long time approximation that $a << 1$, terms of $(1+a)^{1/2}$ can be expanded to give

$$(1+a)^{1/2} = 1 + a/2 - a^2/8 + a^3/16 \;......$$

Dropping terms of magnitude greater than a will give the following

$$\ln T = A - k_1t \tag{2.15}$$

where

$$A = (1+a°)^{1/2} + \ln[\frac{(1+a°)^{1/2} - 1}{(8k_1k_{-1}/\,k_2^2\,)}]$$

Equation (2.15) gives the long time approximation for the decay of T as a function of t. Plotting ln T versus t will give an intercept equal to A and a slope of $-k_1$.

Treating equation (2.8) in this fashion makes it possible to give a qualitative explanation of the kinetic model. It is apparent that equations (2.13) and (2.15) predict a biphasic curve which describes the decay of T as a function of t. At short times this curve should give an intercept of ln T° and an initial slope a function of $T°^{1/2}$. Increasing T° should decrease this initial slope. The approximate slope of the curve at longer times is not a function of T° but of the initial rate constant. The intercept, however, is a function of T°. Therefore, at longer times changing T° should only change the intercept and not affect the slope.

These theoretical conclusions are consistent with the experimental data. Figures 1 and 2 are semi-log plots of the decay in activity of T at different initial protein concentrations. Inspection of the graphs shows a biphasic curve for each T° with an initial slope which decreases for decreasing T° and the slope at longer times is also a function of T°. Furthermore, the initial intercept changes with decreasing T°. Some revision of the model is needed since two different cases are being treated, namely, 0° and the 23° cases. The model should be consistent with both cases and therefore a new presentation in terms of activity rather than concentration will be considered.

III. Concept of Activity as Applied to Both 0° and 23° Cases

Initial attempts to curvefit the data to equation (2.8) ended in failure since the predicted decay was more rapid than that observed, i.e. the integrated rate equation did not duplicate the experimental curves since the calculated values described a decay rate for T that was much too rapid at longer times. The observed rate of decay was much slower. The difference between the observed and calculated rates were much too great to be neglected. On the basis of this experience, the addition of an integrated rate expression for the decay of dimer concentration as a function of time was investigated.

Kuczenski and Suelter (1,2,3) have shown with the aid of ultracentrifugation data that at 0°, T is fully active, D is less than 50% active, and M is non-active. It has been assumed that D^* is not active being only a transition intermediate which rapidly undergoes a conformational change to give D, a quasi-stable molecule. However, whether or not D^* is active or non-active is not crucial to the argument since the concentration is assumed to be low and unchanging. At 23°, however, the only change is that D is 50% active whereas at 0° it is less than 50% active.

The specific activity of an enzyme is defined as micromoles of product formed per minute per milligram of enzyme and theoretically should differ from the concentration of the enzyme by a constant. On this basis consider the following model.

$$A = C([T] + N[D]) \tag{3.1}$$

A is the activity, C is a constant, [T] is the concentration of tetramer at any time, [D] is the concentration of the dimer at any time, and N is the activity of the dimer compared to the tetramer. This model is consistent with both the 0° and 23° cases since the only change is the fractional number assigned to N for each case. At 23° N has a value of 1/4 whereas for 0°, N has a value of less than 1/4 but greater than 0°.

If T' is defined as the concentration of active protein at any time, then its value is A/C and equation (3.1) can be rewritten as

$$T' = A/C = [T] + N[D] \tag{3.2}$$

This form of equation (3.1) gives a direct relationship between the concentration of active protein and the concentration of tetramer and dimer at any time. It is still dependent on activity but only in such a way that it is a function of the concentration of active protein.

At this point it is necessary to determine both a linear solution in T for the integrated rate equation (2.8) and an equation that will describe the decay of D as a function of time. The solution for an explicit expression for the decay of T will be evaluated by an approximation procedure. However, the solution for D will be somewhat more difficult since the differential rate equation (2.4) must be solved. Equation (2.4) is an ordinary differential equation with two dependent variables D and D^*. Its solution will be evaluated by two different methods: 1) an approximate solution involving a series expansion for D in terms of t; and 2) an exact solution of the differential equation.

IV. Equation Development for T and D

In order to obtain a linear solution of equation (2.8) in terms of T, either the log term or the square root term in which T appears must be represented independent of the function T. Rewriting equation (2.8) as

$$(1+a)^{1/2}-(1+a^\circ)^{1/2}+\ln\left[\frac{(1+a)^{1/2}-1}{(1+a^\circ)^{1/2}-1}\right] = -k_1t \qquad (4.1)$$

where $a = 16k_1k_{-1}T/k_2^2$ and $a^\circ = 16k_1k_{-1}T^\circ/k_2^2$

or

$$(1+a)^{1/2}-1 = e^{(b-k_1t)} \qquad (4.2)$$

where

$$b = (1+a^\circ)^{1/2}-(1+a)^{1/2}+\ln[(1+a^\circ)^{1/2}-1] \qquad (4.3)$$

In (4.3) b is a function of T°, a constant, and of T, the time dependent variable. In order to arrive at an explicit relationship for T in terms of t, b must be independent of T. It is assumed that at short times $(1+a^\circ)^{1/2}$ is approximately identical to $(1+a)^{1/2}$. However, at longer times the error made by this assumption becomes quite large and can no longer be neglected. Therefore, an error function defined as the difference between the two terms $(1+a^\circ)^{1/2}$ and $(1+a)^{1/2}$ was constructed in order to give an approximate linear solution in T. This assumption is correct since the natural log term is more important than the square root term at longer times or

$$|\ln[(1+a)^{1/2}-1]| > (1+a)^{1/2} \quad \text{for t large} \tag{4.4}$$

Assuming that the error can be treated as a function of time, the following equation showing dependence on time where ε is a small value can be obtained.

$$(1+a^\circ)^{1/2}-(1+a)^{1/2} = \varepsilon t \tag{4.5}$$

and $(1+a) = [(1+a^\circ)^{1/2}-\varepsilon t]^2$

for $\varepsilon<<1$

then $(1+a)^{1/2} \simeq (1+a^\circ)[1-2\ t/(1+a^\circ)^{1/2}]$

or $T = \frac{(1+a^\circ)}{\zeta}[1-2\varepsilon t/(1+a^\circ)^{1/2}] - 1/\zeta, \quad \zeta = 16k_1k_{-1}/k_2^2$

and $T = T^\circ[1-2\varepsilon t/(1+a^\circ)^{1/2}]$

If the term $2\varepsilon/(1+a^\circ)^{1/2}$ is small then the above can be simplified as

$$T = T^\circ \text{EXP}[-2\varepsilon t/(1+a^\circ)^{1/2}] \tag{4.6}$$

It is interesting to note that equation (4.6) has a form comparable to equation (2.12) derived for the short time approximation.

Substituting equation (4.5) into equation (4.1) and solving for T the following equation is obtained

$$T = (1/\zeta)[(\text{EXP}\{\varepsilon T+\ln[(1+a^\circ)^{1/2}-1]-k_1t\}+1)^2-1] \tag{4.7}$$

If $(1+a^\circ)^{1/2}-1$ is given the symbol $\sigma^\circ-1$ then equation (4.7) can be rewritten as $T = (1/\zeta)[\{(\sigma^\circ-1)\text{EXP}(-[k_1-\varepsilon]t)+1\}^2-1]$ (4.8)

It is evident that ε should be less than k_1 in order to obtain the appropriate decay of T. To document this, ε was evaluated by a treatment which also gave a solution for D as a function of time.

The solution for D was obtained by two methods outlined previously, and ε should be obtained from this analysis. The first method for the solution of D will be a series solution in terms of t. From equation

(2.6) $D^{*} = (k_2/4k_{-1})(\sigma - 1)$

where σ is the symbol for $(1+a)^{1/2}$

and letting $\psi = k_2 D^{*} = (k_2^2/4k_{-1})(\sigma - 1)$ (4.9)

then the differential equations (2.2) and (2.4) will become

$$dT/dt = -k_1 T + k_{-1} D^{*2} = -(1/2)\psi \tag{4.10}$$

$$dD/dt = \psi - k_3 D \tag{4.11}$$

The differential equation for ψ from equation (4.9) will be

$$d\psi/dt = \frac{k_2^2\ (16k_1k_{-1})}{8k_{-1}\sigma k_2^2}\ dT/dt \tag{4.12}$$

but from equation (4.9) $\sigma = (4k_{-1}/k_2^2)\psi + 1$ which when substituted into equation (4.12) gives

$$d\psi/dt = \frac{2k_{-1}}{(4k_{-1}/k_2^2)\psi + 1}\ dT/dt \tag{4.13}$$

then $dT/dt = (1/2k_1)[(4k_{-1}/k_2^2)\psi + 1]d\psi/dt = -\psi/2$ (4.14)

and now $d\psi/dt + k_1/\ [(4k_{-1}/k_2^2) + 1/\psi] = 0$ (4.15)

Equation (4.15) is an ordinary differential equation which can be solved explicitly for ψ to give

$$(4k_{-1}/k_2^2)\psi + \ln\psi = -k_1 t + \text{constant} \tag{4.16}$$

Applying the boundary condition that at $t = 0$, $\psi = \psi°$, equation (4.16)

becomes $(4k_{-1}/k_2^2)[\psi - \psi°] + \ln(\psi/\psi°) = -k_1 t$

From equation (4.9) $\psi° = (k_2^2/4k_{-1})[\sigma° - 1]$

At long times $|\ln\psi/\psi°| >> (4k_{-1}/k_2^2)\psi°(\psi/\psi°)$

and the $\lim\limits_{\psi\to 0}|\psi| \to -\infty$

then using this approximation equation (4.17) becomes

$$\ln\psi/\psi^\circ = (4k_{-1}/k_2^2)\psi^\circ - k_1t$$

where it is assumed that

$$|(4k_{-1}/k_2^2)\psi^\circ - k_1t| >> (4k_{-1}/k_2^2)\psi$$

Therefore, at long times and using the previous approximations

$$\psi = \psi^\circ \mathrm{EXP}[(4k_{-1}/k_2^2)\psi^\circ]\mathrm{EXP}[-k_1t] \qquad (4.18)$$

At shorter times, consider $\psi/\psi^\circ = 1-\delta$, and $\delta = 1-(\psi/\psi^\circ) = (\psi^\circ-\psi)/\psi^\circ$ where δ can have a maximum value of 0 at $t = 0$, and of 1 at $t = \infty$. Introducing δ into equation (4.17) will give

$$(4k_{-1}/k_2^2)\psi^\circ-(4k_{-1}/k_2^2)\psi^\circ\delta+\ln(1-\delta) = (4k_{-1}/k_2^2)\psi^\circ-k_1t$$

or

$$(4k_{-1}/k_2^2)\delta-\ln(1-\delta) = k_1t \qquad (4.19)$$

Expanding $\ln(1-\delta)$ for δ small

$$(4k_{-1}/k_2^2)\delta+\delta[1+\delta/2+\delta^2/3+\delta^3/4+....] = k_1t \qquad (4.20)$$

or

$$\delta(1+4k_{-1}\psi^\circ/k_2^2) +\delta^2/2 +\delta^3/3 +\delta^4/4 +...+ = k_1t$$

Letting $\phi = [1+(4k_{-1}/k_2^2)\psi^\circ]$ then δ can be evaluated by the following expansion in terms of t.

$$\delta = k_1t/\phi + bt^2 + ct^3 +....+ \qquad (4.21)$$

$$\delta = k_1^2t^2/\phi^2 + 2bk_1t^3/\ ^3 +....+ \qquad (4.22)$$

$$\delta = k_1^3t^3/\phi^3 +....+ \qquad (4.23)$$

Solving for the coefficients b and c gives

$$b = -k_1^2/2\phi^3 \qquad (4.24)$$

$$c = -k_1^3/3\phi^4 + k_1^3/2\phi^5$$

Substituting these values for b and c into equation (4.21) gives

$$\delta = 1-\mathrm{EXP}[-k_1t/\phi] \qquad (4.25)$$

then

$$\ln(1-\delta) = -k_1t/\phi \qquad (4.26)$$

Substituting equation (4.26) into equation (4.19) gives

$$\frac{4k_{-1}}{k_2^2}\,\delta = k_1t(1-1/\phi) = k_1t(4k_{-1}\psi^\circ/\phi k_2^2 \qquad (4.27)$$

but from the expansion of equation (4.26)

$$\delta = k_1t/\phi - k_1^2t^2/2\phi^2 + [k_1^3t^3/6\phi^3][-4k_{-1}\psi^\circ/k_2^2] \qquad (4.28)$$

Letting $Y^\circ = 1 + (4k_{-1}/k_2^2)\psi^\circ = \sigma^\circ$ and substituting this expression into equation (4.20) gives

$$\delta + \delta^2/2Y^\circ + \delta^3/3Y^\circ + \delta^4/4Y^\circ + \ldots. + = k_1t/Y^\circ \qquad (4.29)$$

Expanding terms according to a Taylor series expansion about t=0 for δ and higher magnitudes, then

$$\delta = k_1t/Y^\circ + ak_1^2t^2/Y^{\circ 3} + bk_1^3t^3/Y^{\circ 5} + ck_1^4t^4/Y^{\circ 7} \qquad (4.30)$$

$$\delta^2 = k_1^2t^2/Y^{\circ 2} + 2ak_1^3t^3/Y^{\circ 4} + 2bk_1^4t^4 + a^2k_1^4t^4/Y^{\circ 6} \qquad (4.31)$$

$$\delta^3 = k_1^3t^3/Y^{\circ 3} + 3ak_1^4t^4/Y^{\circ 5} \qquad (4.32)$$

$$\delta^4 = k_1^4t^4/Y^{\circ 4} \qquad (4.33)$$

Substituting these expressions back into equation (4.29) and solving for the coefficients a, b, and c resulted in the following values

$$a = -1/2 \qquad (4.34)$$

$$b = (3-2Y^\circ)/6$$

$$c = -(15-14Y^\circ+6Y^{\circ 2})/24$$

therefore,

$$\delta = k_1t/Y^\circ - k_1^2t^2/2Y^{\circ 3} + k_1^3t^3(3-2Y^\circ)/Y^{\circ 5} - k_1^4t^4(15-14Y^\circ+6Y^{\circ 2})/24Y^{\circ 7} \qquad (4.35)$$

But from equation (4.26) $\delta = 1-\mathrm{EXP}(-k_1t/Y^\circ)$ and thus

$$\delta = 1-\mathrm{EXP}(-k_1t/Y^\circ) - \text{equation (4.35)} \qquad (4.36)$$

or

$$\delta = 1-\mathrm{EXP}(-k_1t/Y^\circ) + k_1^2t^2(Y^\circ-1)/2Y^{\circ 3} - k_1^3t^3(Y^{\circ 2}+2Y^\circ-3)/6Y^{\circ 5} + k_1^4t^4(Y^{\circ 3}-6Y^{\circ 2}+14Y^\circ-15)/24Y^{\circ 7} \qquad (4.37)$$

The expressions for ψ and ψ° can be substituted into equation (4.37) using the relation $\psi/\psi° = 1-\delta$.

$$\psi/\psi° = \mathrm{EXP}(-k_1t/Y°)+k_1^2t^2(1-Y°)/2Y°^3[1-k_1t(3-Y°)/3Y°^3 +k_1^2t^2(15-5Y°+Y°^2)/12Y°^4] \qquad (4.38)$$

The differential equation for D from equation (2.4) is

$$dD/dt = k_2D^* - k_3D \quad \text{and} \quad \psi = k_2D^*$$

Therefore, $$dD/dt + k_3D = \psi \qquad (4.39)$$

The differential equation (4.39) can now be solved using the expression for $\psi/\psi°$ obtained from equation (4.38). After substituting equation (4.38) into equation (4.39), solving the differential for D, and applying the boundary condition that at t=0, D=0, the following series solution for D is obtained after some manipulation.

$$\begin{aligned} D = {} & (\psi°/(k_3-k_1/Y°))[\mathrm{EXP}(-k_1t/Y°)-\mathrm{EXP}(-k_3t)] \\ & +[\psi°k_1^2(1-Y°)/6Y°^3]t^3\{1- \tfrac{1}{4}k_3t[1+k_1(3-Y°)/Y°^2k_3] + \\ & \frac{k_3^2t^2}{20}[1+ \frac{k_1(3-Y°)}{Y°^2k_3} + \frac{k_1^2(15-5Y°+Y°^2)}{Y°^4k_3^2}]\} \end{aligned} \qquad (4.40)$$

Equation (4.40) is valid for

$$t^2 << \left| \frac{k_1^2(1-Y°)}{2Y°^3} \right|^{-1}$$

or

$$t << \left| \frac{2Y°^3}{k_1^2(1-Y°)} \right|^{1/2} \qquad (4.41)$$

It can be shown that equation (4.41) is valid for approximately 75% of the entire time interval since the introduction of the constants for Y° and k_1 obtained from the next section resulted in a value of 150 minutes for t. Furthermore, introduction of the constants Y° and k_1 into equation (4.40) substantiates truncating the series at powers of t^2 and higher terms since the coefficients are small (10^{-8} and larger). Therefore, equation (4.40) can be simplified into the following

equation
$$D = \frac{\psi°}{k_3-k_1/Y°}[\ \mathrm{EXP}(-k_1t/Y°) - \mathrm{EXP}(-k_3t)\ ] \qquad (4.41)$$

Equation (4.41) will be utilized in order to solve for ε. Much of this work for the solution of the differential equation (4.39) seems unnecessary since equation (2.4) can be solved exactly for D. However, this will become clear when a solution for ε is evaluated.

From equation (4.8) the value of ψ can be obtained.

$$\psi = k_2^2[(1+a)^{1/2}-1]/4k_{-1}$$

or
$$\psi = k_2^2[(\sigma°-1)\mathrm{EXP}(-(k_1-\varepsilon)t)]/4k_{-1} \qquad (4.42)$$

Substituting this expression into equation (2.4) gives

$$dD/dt + k_3D = \psi = k_2^2[(\sigma°-1)\mathrm{EXP}(-(k_1-\varepsilon)t)]/4k_{-1} \qquad (4.43)$$

Solving this differential equation and applying the boundary condition that at t=0, D=0, gives the following expression

$$D = \frac{(k_2^2/4k_{-1})(\sigma°-1)}{k_3-(k_1-\varepsilon)}[\ \mathrm{EXP}(-(k_1-\varepsilon)t) - \mathrm{EXP}(-k_3t)](4.44)$$

Substituting the values for Y° and $\sigma°$ into equation (4.41) gives another expression for D.

$$D = \frac{k_2^2/4k_{-1}(\sigma°-1)}{k_3-k_1/\sigma°}[\ \mathrm{EXP}(-k_1t/\sigma°) - \mathrm{EXP}(-k_3t)\ ](4.45)$$

A solution for ε can be obtained by equating these last two expressions for D.
$$\varepsilon = k_1(1-1/\sigma°) \qquad (4.46)$$

As previously indicated ε indeed has a value much smaller than k_1. The coefficient $(1-1/\sigma°)$ makes k_1 smaller which substantiates the previous assumptions for ε. Substitution of the expression for ε into equation (4.8) gives

$$T = (1/\zeta)[\{(\sigma°-1)\mathrm{EXP}(-k_1t/\sigma°)+1\}^2-1] \qquad (4.47)$$

The decay of T as indicated by (4.47) is a function of k_1 and T° This is in agreement with the short time approximation derived in equation (4.12) which also indicates that the decay of T is dictated by the rate constant k_1 and $T^{\circ^{-1/2}}$.

Equations (4.45) and (4.47) can now be substituted into the theoretical model presented in equation (4.2) to give

$$T' = (1/\zeta)[\{(\sigma^\circ-1)\mathrm{EXP}(-k_1t/\sigma^\circ)+1\}^2-1] + \frac{N(k_2^2/4k_{-1})(\sigma^\circ-1)}{(k_3-k_1/\sigma^\circ)}[\mathrm{EXP}(-k_1t/\sigma^\circ)-\mathrm{EXP}(-k_3t)] \qquad (4.48)$$

where N is a fractional number having a maximum values of 1/4 for the 23° case and between 0 and 1/4 for the 0° case. However, equation (4.48) must be developed further since calculated parameters obtained for k_1/σ° and k_3 indicated that these two constants are fairly close in magnitude. Therefore, the expression for D becomes indeterminant as k_3 approaches the value of k_1/σ°. Taking the limit of equation (4.45) as the value of k_3 approaches k_1/σ° gives

$$\lim_{k_3\to k_1/\sigma^\circ} \frac{(k_2^2/4k_{-1})(\sigma^\circ-1)}{(k_3-k_1/\sigma^\circ)}[\mathrm{EXP}(-k_1t/\sigma^\circ)-\mathrm{EXP}(-k_3t)] \qquad (4.49)$$

and applying L'Hospital's rule (4.49) becomes equivalent to

$$\text{limit } (4.49) = (k_2^2/4k_{-1})(\sigma^\circ-1)t\mathrm{EXP}(-k_3t) \qquad (4.50)$$

and the expression for D becomes

$$D = (k_2^2/4k_{-1})(\sigma^\circ-1)\ t\ \mathrm{EXP}(-k_3t) \qquad (4.51)$$

and substitution of (4.51) into (4.2) gives

$$T' = (1/\zeta)[\{(\sigma^\circ-1)\mathrm{EXP}(-k_1t/\sigma^\circ)+1\}^2-1] + N(k_2^2/4k_{-1})t\ \mathrm{EXP}(-k_3t) \qquad (4.52)$$

Equation (4.52) should give a close approximation to the experimental biphasic curve. In order to test for its validity a general curve-fitting and equation-solving program was utilized.

V. Application of the Theoretical Rate Equation to a Curve-Fitting and Equation-Solving Routine.

1. Application to the 0° case

The difficulty that was encountered in curve-fitting the data with respect to equation (4.52) resulted because of the non-linear nature of the equation with respect to time and to the parameters. Curve-fitting to the experimental data was accomplished by a routine developed by Nicely and Dye (4) which handles problems which are either linear or non-linear in the adjustable parameters. In order for the routine to handle non-linear problems it was necessary to proceed from a set of initial estimates of the parameters. It was also necessary that these estimates be carefully chosen in order that convergence to the "correct" set of parameters be made. Therefore, it was important to begin with an approximation of equation (4.52) which would have only one adjustable parameter to content with. This is evident since equation (4.52) has a maximum of five adjustable parameters and because of the non-linear nature of these parameters it would be impossible to make absolute "guesses" and have the program converge.

For the initial parameter estimation the short time approximation (2.13) was utilized in the curve-fitting routine. The parameter that was adjusted was the following

$$T = T^{\circ}\ \mathrm{EXP}(-U(1)/2T^{\circ 1/2}) \quad \text{and} \quad U(1)=[k_2^2 k_1/k_{-1}]^{1/2} \qquad (5.1)$$

U(I) signifies a parameter in the routine, where I is the number of the parameter to be found. As indicated previously, equation (2.13) did not give a good fit to the data but its main purpose at this point was to provide for an initial estimate of the rate constants. The long time approximation (2.15) was treated similarly. Rewriting equation

(2.15) $$\ln T = A - k_1 t \tag{5.2}$$

or $$T = e^A e^{-k_1 t} \tag{5.3}$$

Setting $U(1)=e^A$ and $U(2)=k_1$ results in an equation with two adjustable parameters.

$$T = U(1)e^{-U(2)t} \tag{5.4}$$

Equations (5.1) and (5.4) are exponential equations and it was quite easy to make initial guesses for the parameters and obtain convergence to the true value. This would not be the case if a more complex equation were used and more than two parameters needed to be adjusted.

Using the values of the parameters obtained in equations (5.1) and (5.4) and making appropriate substitutions, equation (5.5) was then fitted to the experimental data.

$$T = U(1)[(U(2)EXP(-U(3)t)+1)^2-1] \tag{5.5}$$

where $$U(1) = 1/\zeta = [16k_1k_{-1}/k_2^2]^{-1}$$

$$U(2) = (\sigma°-1) = (1+16k_1k_{-1}T°/k_2^2)^{1/2}-1$$

$$U(3) = k_1/\sigma°$$

Since the initial estimates were in the range of the true values, convergence was obtained. Nevertheless, equation (5.5) gave a much better fit to the data than either of the long time or short time approximations. However, (5.5) did not give as good a resolution as expected since the decay rate was too rapid at long times. Apparently, this was due to the lack of the D term which would have compensated for this rapid decay rate at longer times. This assumption was substantiated since the fit at shorter times appeared to be quite good. Therefore, the theoretical model presented in (4.2) indicated correctly that the total activity would not only be a function of T but also of D. The D term apparently accounts for the activity at long times. This could be predicted qualitatively since at long times the reservoir to total T would be depleted and the activity seen at this time would be due primarily to D.

The maximum number of parameters that could be adjusted in the complete equation (4.52) is three. Any more than this number resulted in incomplete convergence by the curve-fitting routine. Apparently, several of the parameters in (4.52) are correlated and thus are linearly dependent. When this happens the result is that the parameters become strongly "coupled" and a large change in one parameter is compensated for by appropriate changes in the others. This is frequently the case when the parameters are non-linear. With this consideration (4.52) can be rewritten as follows.

$$T' = (1/\zeta)[\{(\sigma^\circ-1)e^{-U(1)t}+1\}^2-1] + U(2)te^{-U(3)t} \qquad (5.6)$$

where

$$U(1) = k_1/\sigma^\circ$$

$$U(2) = N(k_2^2/4k_{-1})(\sigma^\circ-1)$$

$$U(3) = k_3$$

It has been assumed that the best values of $1/\zeta$ and $(\sigma^\circ-1)$ have been selected from (5.6). For the 0° case, equation (5.6) provided a good fit to the experimental data. The parameters presented in equation (5.6) were correlated to some extent but reasonable values for each parameter were obtained. Four data sets each at a different T° were evaluated in this manner and the results for the kinetic rate constants and N are tabulated in Table I. Plots of calculated and experimental T' versus time for four different data sets are presented in Figures 3,4,5, and 6. By inspection, it is apparent that equation (5.6) does give a reasonably good approximation to the inactivation process and moreover, substantiates the kinetic model.

An inspection of Table I reveals that the collection of kinetic rate constants k_1k_{-1}/k_2^2 remains constant. The parameter k_1 also remains reasonably constant whereas, k_3 increases more than twofold. The parameter N also varies although it should remain constant. It appears that N and k_3 compensate each other and are thus linearly dependent. A better resolution of these two parameters will be discussed in section VI.2.

2. Application to the 23° case

The same procedure for the initial estimates of the parameters as discussed for the 0° data was utilized for the 23° case. Equations (5.1) and (5.4) gave reasonably good approximations to the parameters and these were substituted into equation (5.5). Surprisingly, adjustment of the parameters in equation (5.5) gave a very good fit to the experimental data. This indicated that the D term was not necessary. Nevertheless, the complete equation (5.6) was then fitted to the data which resulted in as good a fit to the experimental data as the partial equation (5.5). These results are presented in Figures 7 through12. An inspection of these computer plots indicates a reasonably good fit to the experimental data.

The results of the curve-fitting for 23° have been presented in Table II. An inspection of the results again reveals that the collection of rate constants k_1k_{-1}/k_2^2 remains constant. However, k_1, k_3, and N increase more than twofold which indicates that these parameters are correlated or linearly dependent. Better resolution for these parameters will be discussed in section VI.2.

The value of N in the theoretical model was assumed to have a maximum value of 1/4 for the 23° case since studies by Kuczenski and

TABLE I

Kinetic Parameters for 0° Data Evaluated by a Curve-Fitting Routine[a]

(T°) (mg/ml)	$(\sigma^\circ-1)$[b]	(k_1k_{-1}/k_2^2) (mg^{-1})
.800 ± .040	.26883 ± .0299	.04765 ± .00241
.500 ± .020	.21809 ± .0152	.06046 ± .00324
.304 ± .015	.13389 ± .0128	.05874 ± .00519
.241 ± .012	.11723 ± .0446	.06437 ± .00883

k_1 $(\text{min.})^{-1}$	k_3 $(\text{min.})^{-1}$
.0701 ± .00564	.01576 ± .00114
.1123 ± .00933	.02419 ± .00484
.1798 ± .00786	.03386 ± .00390
.1559 ± .01460	.03865 ± .00133

N
.09357 ± .0165
.04175 ± .0125
.02646 ± .0055
.02310 ± .0077

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[a] Nicely and Dye (4)

[b] $(\sigma^\circ-1) = (1+16k_1k_{-1}T^\circ/k_2^2)^{1/2}-1$

Figures 3,4,5, and 6. Computer printouts of calculated and experimental values for the concentration of active protein versus time at 0°. The initial protein concentrations were 0.800, 0.500, 0.304, and 0.241 mg/ml indicated at the bottom of each graph.

```
ACROSS IS TIME, TIME AT THE LEFT =  0.       ,TIME AT THE RIGHT =   .317F+03,TIME INCREMENT =   .320F+01
VERTICAL IS THE TIME DEPENDENT OBSERVABLE,TOP =   .800E+00, BOTTOM =   .199E-01, INCREMENT =   .159F-01
X MEANS AN EXPERIMENTAL POINT
O MEANS A CALCULATED POINT
= MEANS AN EXPERIMENTAL AND CALCULATED POINT ARE IN THE SAME DELTA X BY DELTA Y

         I----5----5----5----5----5----5----5----5----5----5----5----5----5----5----5----5----5----5----5----5I
         IX                                                                                                   I
         I                                             A COMPARISON OF THE FINAL RESULTS                      I
         I                                             NUMBER     TIME EXPERIMENTAL CALCULATED      RESIDUAL  I
         IO                                                 1   0.0000       .80000     .76800    -.03200000  I
         5                                                  2   2.0000       .73100     .69552    -.03548101  5
         IX                                                 3   5.0000       .53100     .60573     .07472837  I
         I                                                  4   9.0000       .48300     .51385     .03085350  I
         IO                                                 5  13.0000       .43400     .44583     .01182717  I
         I                                                  6  17.0000       .40400     .39526    -.00874225  I
         5                                                  7  21.0000       .37200     .35751    -.01448624  5
         I                                                  8  25.0000       .36800     .32921    -.03879011  I
         I                                                  9  29.0000       .30300     .30785     .00485292  I
         I                                                 10  46.0000       .33100     .25956    -.07143754  I
         I O                                               11  56.0000       .21600     .24531     .02930588  I
         5                                                 12  71.0000       .22400     .22847     .00447459  5
         I                                                 13  84.0000       .20300     .21398     .01098313  I
         I                                                 14  99.0000       .17200     .19595     .02395052  I
         I X                                               15 114.0000       .16100     .17682     .01582488  I
         I  O                                              16 131.0000       .12400     .15485     .03084988  I
         5                                                 17 149.0000       .13200     .13240     .00039991  5
         I  X                                              18 163.0000       .11000     .11609     .00609378  I
         I                                                 19 177.0000       .10600     .10108    -.00492459  I
         I                                                 20 195.0000       .08400     .08384    -.00016252  I
         I    =                                            21 208.0000       .08000     .07286    -.00714279  I
         5                                                 22 226.0000       .07400     .05961    -.01439039  5
         I     X                                           23 242.0000       .07000     .04960    -.02039548  I
         I     O                                           24 260.0000       .05800     .04013    -.01786761  I
         I      X                                          25 274.0000       .05500     .03392    -.02107934  I
         I      OX                                         26 290.0000       .04100     .02790    -.01309912  I
         5                                                 27 306.0000       .04500     .02288    -.02212042  5
         I       O      X                                  28 317.0000       .02650     .01993    -.00656990  I
         I         O                                                                                          I
         I         X                                                                                          I
         I                                                                                                    I
         5              O                                                                                     5
         I                 O                                                                                  I
         I                      O                                                                             I
         I                 X    X   O                                                                         I
         I                          X   O                                                                     I
         5                                                                                                    5
         I                              X    O                                                                I
         I                                   X    O                                                           I
         I                                              =                                                     I
         I                                        X         O                                                 I
         5                                                  X    =                                            5
         I                                                            =                                       I
         I                                                                =     X    X                        I
         I                                                                      O          X   X              I
         I                                                                           O     O        X    X    I
         5                                                                                     O    O    O   =5
         I----5----5----5----5----5----5----5----5----5----5----5----5----5----5----5----5----5----5----5----5I
END ************************* THIS IS THE DATA FOR THE T=0.8 MG/ML
END OF INPUT
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ACROSS IS TIME, TIME AT THE LEFT =  0.        ,TIME AT THE RIGHT =   .222E+03,TIME INCREMENT =   .224E+01
VERTICAL IS THE TIME DEPENDENT OBSERVABLE,TOP =   .500E+00, BOTTOM =   .416E-02, INCREMENT =   .101E-01
X MEANS AN EXPERIMENTAL POINT
O MEANS A CALCULATED POINT
= MEANS AN EXPERIMENTAL AND CALCULATED POINT ARE IN THE SAME DELTA X BY DELTA Y

          I----5----5----5----5----5----5----5----5----5----5----5----5----5----5----5----5----5----5----5----5I
          IX                                                                                                   I
          I                                                                                                    I
          IO                                             A COMPARISON OF THE FINAL RESULTS                     I
          IX                                             NUMBER     TIME EXPERIMENTAL CALCULATED     RESIDUAL  I
          5                                                   1   0.0000       .50000     .48000   -.02000000  5
          IO                                                  2    .5000       .47200     .45834   -.01366154  I
          IX                                                  3   2.0000       .44400     .40025   -.04375079  I
          I                                                   4   5.0000       .30950     .30958    .00007776  I
          I                                                   5  10.0000       .18400     .21121    .02721456  I
          5                                                   6  14.0000       .15700     .16277    .00577096  5
          IO                                                  7  19.0000       .12450     .12477    .00027009  I
          I                                                   8  24.0000       .11000     .10193   -.00806518  I
          I                                                   9  30.0000       .10000     .08582   -.01418205  I
          I                                                  10  34.0000       .07750     .07900    .00150351  I
          5                                                  11  37.0000       .07900     .07514   -.00386042  5
          I                                                  12  58.0000       .05040     .05942    .00901793  I
          I                                                  13  80.0000       .04130     .04674    .00543823  I
          I                                                  14 125.0000       .02340     .02445    .00105276  I
          I                                                  15 161.0000       .01790     .01318   -.00471746  I
          5  =                                               16 190.0000       .01150     .00771   -.00378595  5
          I                                                  17 209.0000       .00970     .00536   -.00434101  I
          I                                                  18 222.0000       .00800     .00416   -.00384351  I
          I                                                                                                    I
          I                                                                                                    I
          5                                                                                                    5
          I                                                                                                    I
          I                                                                                                    I
          I                                                                                                    I
          I                                                                                                    I
          5    O                                                                                               5
          I                                                                                                    I
          I                                                                                                    I
          I    X                                                                                               I
          I                                                                                                    I
          5      =                                                                                             5
          I                                                                                                    I
          I                                                                                                    I
          I                                                                                                    I
          I        =                                                                                           I
          5          X                                                                                         5
          I          O  X                                                                                      I
          I             O                                                                                      I
          I               ==                                                                                   I
          I                                                                                                    I
          5                         O                                                                          5
          I                         X         O                                                                I
          I                                   X                                                                I
          I                                                      O                                             I
          I                                                      X               X                             I
          5                                                                      O            =        =     = 5
          I----5----5----5----5----5----5----5----5----5----5----5----5----5----5----5----5----5----5----5----5I
END ********************* THIS IS THE DATA FOR T=0.500 MG./ML.
END OF INPUT
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ACROSS IS TIME, TIME AT THE LEFT =  0.        ,TIME AT THE RIGHT =   .216E+03,TIME INCREMENT =   .216E+01
VERTICAL IS THE TIME DEPENDENT OBSERVABLE,TOP =   .304E+00, BOTTOM =   .390E-03, INCREMENT =   .620E-02
X MEANS AN EXPERIMENTAL POINT
O MEANS A CALCULATED POINT
= MEANS AN EXPERIMENTAL AND CALCULATED POINT ARE IN THE SAME DELTA X BY DELTA Y

         I----5----5----5----5----5----5----5----5----5----5----5----5----5----5----5----5----5----5----5----5I
         I                                                                                                    I
         IX                                            A COMPARISON OF THE FINAL RESULTS                      I
         IO                                            NUMBER     TIME EXPERIMENTAL CALCULATED      RESIDUAL  I
         I                                                  1   0.0000       .30400     .29184    -.01216000  I
         5                                                  2   2.0000       .21100     .21389     .00289333  5
         I                                                  3   5.0000       .13600     .13888     .00288302  I
         I                                                  4   8.0000       .09300     .09483     .00183412  I
         I                                                  5  13.0000       .06000     .05775    -.00224512  I
         I                                                  6  18.0000       .05400     .04232    -.01168314  I
         5                                                  7  22.0000       .04000     .03667    -.00332873  5
         I                                                  8  29.0000       .02700     .03220     .00519550  I
         I                                                  9  36.0000       .02340     .02974     .00634450  I
         I                                                 10  47.0000       .02490     .02610     .00120030  I
         I                                                 11  61.0000       .01860     .02098     .00237537  I
         5                                                 12  74.0000       .01360     .01637     .00277334  5
         IO                                                13  96.0000       .01030     .01008    -.00021693  I
         IX                                                14 103.0000       .01230     .00854    -.00376475  I
         I                                                 15 127.0000       .00810     .00467    -.00343081  I
         I                                                 16 140.0000       .00880     .00331    -.00548572  I
         5                                                 17 157.0000       .00420     .00209    -.00210993  5
         I                                                 18 175.0000       .00630     .00127    -.00503353  I
         I                                                 19 189.0000       .00420     .00085    -.00334860  I
         I                                                 20 204.0000       .00410     .00055    -.00354701  I
         I                                                 21 216.0000       .00310     .00039    -.00271000  I
         5                                                                                                    5
         I                                                                                                    I
         I                                                                                                    I
         I  O                                                                                                 I
         I  X                                                                                                 I
         5                                                                                                    5
         I                                                                                                    I
         I                                                                                                    I
         I                                                                                                    I
         I                                                                                                    I
         5   O                                                                                                5
         I   X                                                                                                I
         I                                                                                                    I
         I                                                                                                    I
         I                                                                                                    I
         5                                                                                                    5
         I     =                                                                                              I
         I        X                                                                                           I
         I                                                                                                    I
         I        O X                                                                                         I
         5          O  O                                                                                      5
         I             X  O    O                                                                              I
         I                X    X     O                                                                        I
         I                           X     =                                                                  I
         I                                            =  =          X     X                                   I
         5                                                          O     O      =        =     =      =    = 5
         I----5----5----5----5----5----5----5----5----5----5----5----5----5----5----5----5----5----5----5----5I
END ************************  THIS IS THE DATA FOR T=.304 MG/ML.
END OF INPUT
```

```
ACROSS IS TIME, TIME AT THE LEFT =  0.       ,TIME AT THE RIGHT =   .201E+03,TIME INCREMENT =    .203E+01
VERTICAL IS THE TIME DEPENDENT OBSERVABLE,TOP =   .241E+00, BOTTOM =   .139E-04, INCREMENT =   .492E-02
X MEANS AN EXPERIMENTAL POINT
O MEANS A CALCULATED POINT
= MEANS AN EXPERIMENTAL AND CALCULATED POINT ARE IN THE SAME DELTA X BY DELTA Y

         I----5----5----5----5----5----5----5----5----5----5----5----5----5----5----5----5----5----5----5----5I
         IX                                                                                                   I
         I                                                                                                    I
         IO                                            A COMPARISON OF THE FINAL RESULTS                      I
         I                                             NUMBER      TIME EXPERIMENTAL CALCULATED     RESIDUAL  I
         5                                                  1    0.0000       .24100     .23136   -.00964000  5
         IX                                                 2    1.0000       .21660     .20002   -.01658046  I
         I                                                  3    3.0000       .17110     .14995   -.02115131  I
         I                                                  4    7.0000       .07450     .08515    .01065490  I
         I                                                  5    9.0000       .05630     .06448    .00818128  I
         5O                                                 6   13.0000       .03170     .03735    .00565171  5
         I                                                  7   16.0000       .02450     .02507    .00056704  I
         I                                                  8   20.0000       .01920     .01501   -.00418575  I
         I                                                  9   23.0000       .01210     .01042   -.00167834  I
         I                                                 10   27.0000       .01040     .00663   -.00377002  I
         5                                                 11   31.0000       .00770     .00443   -.00326700  5
         I X                                               12   36.0000       .00670     .00291   -.00379035  I
         I                                                 13   47.0000       .00590     .00156   -.00433780  I
         I                                                 14   56.0000       .00330     .00114   -.00215853  I
         I                                                 15   66.0000       .00280     .00087   -.00193490  I
         5 O                                               16   79.0000       .00250     .00061   -.00188576  5
         I                                                 17   94.0000       .00170     .00041   -.00129261  I
         I                                                 18  114.0000       .00140     .00023   -.00117213  I
         I                                                 19  134.0000       .00110     .00012   -.00097636  I
         I                                                 20  172.0000       .00060     .00004   -.00056346  I
         5                                                 21  190.0000       .00050     .00002   -.00047987  5
         I                                                 22  201.0000       .00040     .00001   -.00038608  I
         I                                                                                                    I
         I                                                                                                    I
         I                                                                                                    I
         I                                                                                                    I
         5                                                                                                    5
         I                                                                                                    I
         I                                                                                                    I
         I   O                                                                                                I
         I                                                                                                    I
         5   X                                                                                                5
         I                                                                                                    I
         I    O                                                                                               I
         I                                                                                                    I
         I    X                                                                                               I
         5                                                                                                    5
         I                                                                                                    I
         I                                                                                                    I
         I      O                                                                                             I
         I      X                                                                                             I
         5       O                                                                                            5
         I       X                                                                                            I
         I         =                                                                                          I
         I           = X                                                                                      I
         I             O X X     X                                                                            I
         5               O O     O   =    =     =       =         =        =                  =        =     =5
         I----5----5----5----5----5----5----5----5----5----5----5----5----5----5----5----5----5----5----5----5I
END ************************ THIS IS THE DATA FOR T=.241MG/ML
END OF INPUT
```

TABLE II

Kinetic Parameters for 23° Data Evaluated by a Curve-Fitting Routine[a]

(T°) (mg/ml)	$(\sigma^\circ-1)$	(k_1k_{-1}/k_2^2) (mg^{-1})	k_1 (min^{-1})	k_1[b] (min^{-1})	k_3 (min^{-1})	N
.600 ± .030	.25795 ± .00292	.06067 ± .00429	.0431 ± .0040	.0315 ± .0016	.02715 ± .0027	.1114 ± .0194
.400 ± .020	.17644 ± .00205	.06000 ± .00439	.0606 ± .0016	.0336 ± .0042	.03010 ± .0049	.1080 ± .0070
.150 ± .007	.07316 ± .00352	.06320 ± .00397	.0435 ± .0014	.0440 ± .0072	.03424 ± .0063	.3701 ± .0160
.090 ± .005	.05054 ± .00066	.07200 ± .00363	.0783 ± .0037	.0452 ± .0027	.06519 ± .0028	.3355 ± .0112
.050 ± .003	.02665 ± .00060	.06751 ± .00346	.0803 ± .0013	.0516 ± .0032	.06576 ± .0068	.5716 ± .0199
.025 ± .001	.01191 ± .00019	.05990 ± .00303	.0840 ± .0035	.0636 ± .0028	.05827 ± .0032	.3712 ± .0376

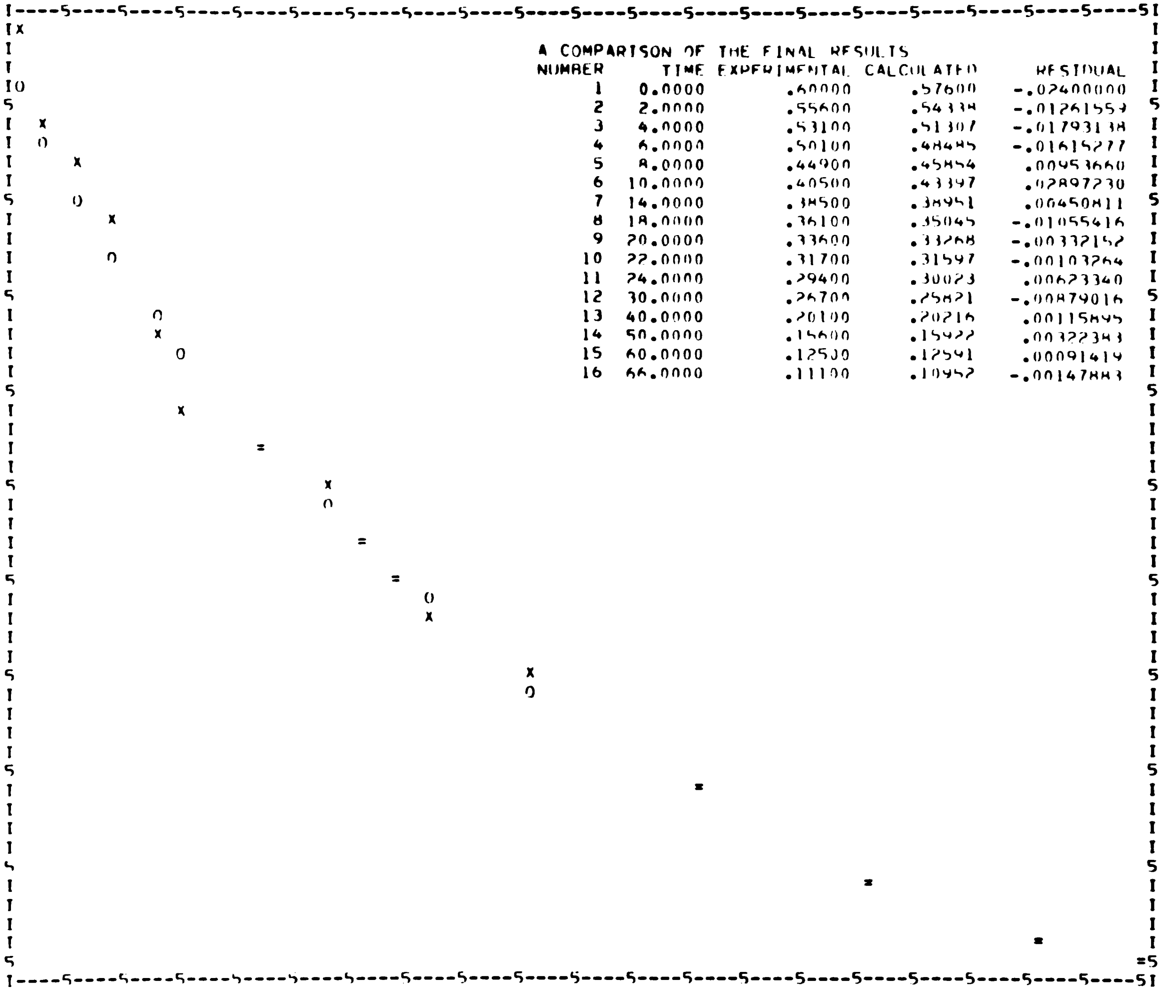
---

[a] Nicely and Dye (4)

[b] calculated by means of equation (4.5)

Figures 7 through 12. Computer printouts of the calculated and experimental values for the contration of active protein versus time at 23°. The initial protein concentrations were 0.600, 0.400, 0.150 0.090, 0.050, and 0.025 mg/ml indicated at the bottom of each graph.

```
ACROSS IS TIME, TIME AT THE LEFT =  0.        .TIME AT THE RIGHT =   .660E+02.TIME INCREMENT =   .667E+00
VERTICAL IS THE TIME DEPENDENT OBSERVABLE.TOP =   .600E+00, BOTTOM =   .110E+00, INCREMENT =   .100F-01
X MEANS AN EXPERIMENTAL POINT
O MEANS A CALCULATED POINT
= MEANS AN EXPERIMENTAL AND CALCULATED POINT ARE IN THE SAME DELTA X BY DELTA Y
```



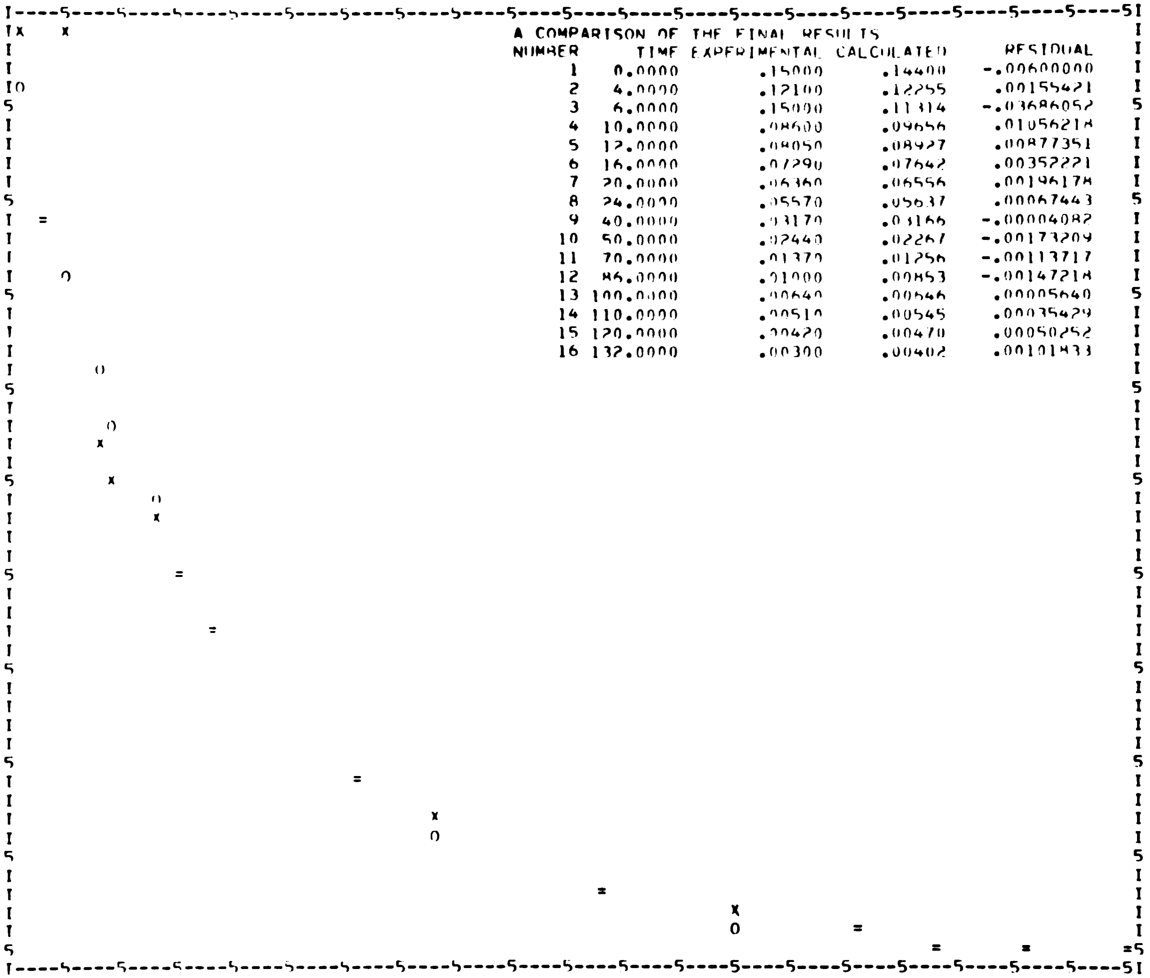
```
A COMPARISON OF THE FINAL RESULTS
NUMBER      TIME EXPERIMENTAL CALCULATED      RESIDUAL
     1    0.0000        .60000     .57600   -.02400000
     2    2.0000        .55600     .54338   -.01261559
     3    4.0000        .53100     .51307   -.01793138
     4    6.0000        .50100     .48485   -.01615277
     5    8.0000        .44900     .45854    .00953660
     6   10.0000        .40500     .43397    .02897230
     7   14.0000        .38500     .38951    .00450811
     8   18.0000        .36100     .35045   -.01055416
     9   20.0000        .33600     .33268   -.00332152
    10   22.0000        .31700     .31597   -.00103264
    11   24.0000        .29400     .30023    .00623340
    12   30.0000        .26700     .25821   -.00879016
    13   40.0000        .20100     .20216    .00115895
    14   50.0000        .15600     .15922    .00322343
    15   60.0000        .12500     .12591    .00091419
    16   66.0000        .11100     .10952   -.00147883
```

```
END ************************* 23 DEGREE DATA CURVE FITTING FOR 0.60 MG./ML.
END OF INPUT
```

```
ACROSS IS TIME, TIME AT THE LEFT =  0.        .TIME AT THE RIGHT =   .600E+02.TIME INCREMENT =   .606E+00
VERTICAL IS THE TIME DEPENDENT OBSERVABLE.TOP =   .400E+00, BOTTOM =   .637E-01, INCREMENT =   .686E-02
X MEANS AN EXPERIMENTAL POINT
O MEANS A CALCULATED POINT
= MEANS AN EXPERIMENTAL AND CALCULATED POINT ARE IN THE SAME DELTA X BY DELTA Y

         I----5----5----5----5----5----5----5----5----5----5----5----5----5----5----5----5----5----5----5----5I
         I                                                                                                    I
         IX                                           A COMPARISON OF THE FINAL RESULTS                       I
         I                                            NUMBER     TIME EXPERIMENTAL CALCULATED    RESIDUAL     I
         IO                                                 1   0.0000       .40000     .38400   -.01600000   I
         5                                                  2   3.0000       .34800     .33848   -.00951954   5
         I                                                  3   6.0000       .30600     .30010   -.00590460   I
         I                                                  4  12.0000       .26100     .23971   -.02128792   I
         I                                                  5  15.0000       .24000     .21582   -.02418069   I
         I    X                                             6  18.0000       .22400     .19517   -.02883188   I
         5    O                                             7  22.0000       .19300     .17175   -.02125381   5
         I                                                  8  24.0000       .18300     .16151   -.02149269   I
         I                                                  9  34.0000       .14300     .12125   -.02174664   I
         I                                                 10  39.0000       .12200     .10618   -.01582419   I
         I                                                 11  46.0000       .09700     .08894   -.00805901   I
         5         X                                       12  55.0000       .08100     .07161   -.00939158   5
         I         O                                       13  60.0000       .07000     .06372   -.00628037   I
         I                                                                                                    I
         I                                                                                                    I
         I                                                                                                    I
         5                                                                                                    5
         I                                                                                                    I
         I                   X                                                                                I
         I                                                                                                    I
         I                                                                                                    I
         5                   O    X                                                                           5
         I                                                                                                    I
         I                             X                                                                      I
         I                        O                                                                           I
         I                                                                                                    I
         5                                                                                                    5
         I                             O                                                                      I
         I                                    X                                                               I
         I                                       X                                                            I
         I                                                                                                    I
         5                                    O                                                               5
         I                                       O                                                            I
         I                                                                                                    I
         I                                                                                                    I
         I                                                        X                                           I
         5                                                                                                    5
         I                                                                                                    I
         I                                                        O       X                                   I
         I                                                                                                    I
         I                                                                O                                   I
         5                                                                                                    5
         I                                                                           X                        I
         I                                                                           O                        I
         I                                                                                          X         I
         I                                                                                          O         I
         5                                                                                                   =5
         I----5----5----5----5----5----5----5----5----5----5----5----5----5----5----5----5----5----5----5----5I
END ************************* 23 DEGREE DATA CURVE FITTING FOR 0.4 MG./ML.
END OF INPUT
```

```
ACROSS IS TIME, TIME AT THE LEFT =  0.        ,TIME AT THE RIGHT =   .132E+03,TIME INCREMENT =   .133E+01
VERTICAL IS THE TIME DEPENDENT OBSERVABLE,TOP =   .150E+00, BOTTOM =   .300E-02, INCREMENT =   .300E-02
X MEANS AN EXPERIMENTAL POINT
O MEANS A CALCULATED POINT
= MEANS AN EXPERIMENTAL AND CALCULATED POINT ARE IN THE SAME DELTA X BY DELTA Y
```



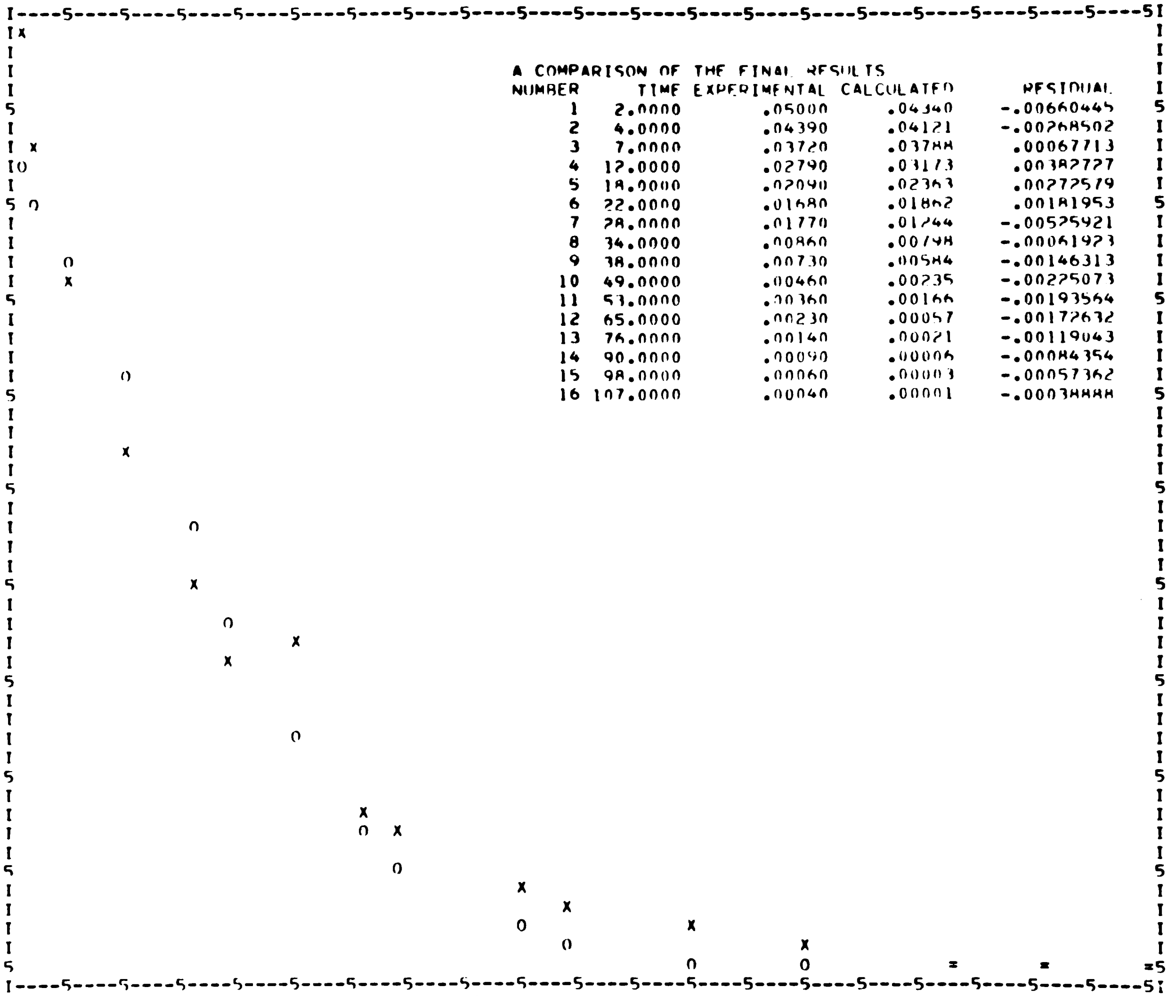
```
A COMPARISON OF THE FINAL RESULTS
NUMBER      TIME EXPERIMENTAL CALCULATED       RESIDUAL
     1    0.0000       .15000     .14400    -.00600000
     2    4.0000       .12100     .12255     .00155421
     3    6.0000       .15000     .11314    -.03686052
     4   10.0000       .08600     .09656     .01056218
     5   12.0000       .08050     .08927     .00877351
     6   16.0000       .07290     .07642     .00352221
     7   20.0000       .06360     .06556     .00196178
     8   24.0000       .05570     .05637     .00067443
     9   40.0000       .03170     .03166    -.00004082
    10   50.0000       .02440     .02267    -.00173209
    11   70.0000       .01370     .01256    -.00113717
    12   86.0000       .01000     .00853    -.00147218
    13  100.0000       .00640     .00646     .00005640
    14  110.0000       .00510     .00545     .00035429
    15  120.0000       .00420     .00470     .00050252
    16  132.0000       .00300     .00402     .00101833
```

```
END ************************ 23 DEGREE DATA CURVE FITTING FOR 0.15 MG./ML.
END OF INPUT
```

```
ACROSS IS TIME, TIME AT THE LEFT =    .300E+01,TIME AT THE RIGHT =   .132E+03,TIME INCREMENT =   .130E+01
VERTICAL IS THE TIME DEPENDENT OBSERVABLE,TOP =   .900E-01, BOTTOM =   .116E-03, INCREMENT =   .183E-02
X MEANS AN EXPERIMENTAL POINT
O MEANS A CALCULATED POINT
= MEANS AN EXPERIMENTAL AND CALCULATED POINT ARE IN THE SAME DELTA X BY DELTA Y

          I----5----5----5----5----5----5----5----5----5----5----5----5----5----5----5----5----5----5----5----5I
          IX                                                                                                   I
          I                                            A COMPARISON OF THE FINAL RESULTS                       I
          I                                            NUMBER     TIME EXPERIMENTAL CALCULATED      RESIDUAL   I
          I                                                 1   3.0000       .09000     .08020    -.00980269   I
          5                                                 2   8.0000       .07560     .06902    -.00658017   5
          I                                                 3  11.0000       .06380     .06235    -.00145499   I
          IO                                                4  14.0000       .05660     .05591    -.00069385   I
          I                                                 5  17.0000       .04860     .04982     .00121859   I
          I   X                                             6  22.0000       .03780     .04063     .00282865   I
          5                                                 7  26.0000       .03190     .03421     .00230683   5
          I                                                 8  32.0000       .02470     .02611     .00140597   I
          I                                                 9  42.0000       .01720     .01622    -.00098380   I
          I   O                                            10  50.0000       .01220     .01088    -.00131753   I
          I                                                11  59.0000       .00950     .00685    -.00265379   I
          5                                                12  69.0000       .00650     .00403    -.00246854   5
          I      X                                         13  88.0000       .00440     .00143    -.00297275   I
          I      O                                         14  98.0000       .00290     .00082    -.00208449   I
          I                                                15 108.0000       .00210     .00046    -.00163738   I
          I                                                16 120.0000       .00150     .00023    -.00126759   I
          5        =                                       17 132.0000       .00100     .00012    -.00088411   5
          I                                                                                                    I
          I                                                                                                    I
          I          O                                                                                         I
          I          X                                                                                         I
          5                                                                                                    5
          I                                                                                                    I
          I                                                                                                    I
          I              O                                                                                     I
          I                                                                                                    I
          5              X                                                                                     5
          I                                                                                                    I
          I                 O                                                                                  I
          I                 X                                                                                  I
          I                                                                                                    I
          5                                                                                                    5
          I                      O                                                                             I
          I                      X                                                                             I
          I                                                                                                    I
          I                                                                                                    I
          5                                                                                                    5
          I                             X                                                                      I
          I                             O                                                                      I
          I                                                                                                    I
          I                                    X                                                               I
          5                                    O     X                                                         5
          I                                                                                                    I
          I                                          O       X                                                 I
          I                                                  O              X                                  I
          I                                                                        X       X                   I
          5                                                                 O      O       O        =         =5
          I----5----5----5----5----5----5----5----5----5----5----5----5----5----5----5----5----5----5----5----5I
END ************************ 23 DEGREE DATA CURVE FITTING FOR 0.09 MG./ML.
END OF INPUT
```

```
ACROSS IS TIME. TIME AT THE LEFT =   .200E+01.TIME AT THE RIGHT =   .107E+03.TIME INCREMENT =   .106E+01
VERTICAL IS THE TIME DEPENDENT OBSERVABLE.TOP =   .500E-01, BOTTOM =   .111E-04, INCREMENT =   .102E-02
X MEANS AN EXPERIMENTAL POINT
O MEANS A CALCULATED POINT
= MEANS AN EXPERIMENTAL AND CALCULATED POINT ARE IN THE SAME DELTA X BY DELTA Y
```



```
A COMPARISON OF THE FINAL RESULTS
NUMBER      TIME EXPERIMENTAL CALCULATED       RESIDUAL
     1    2.0000       .05000     .04340    -.00660445
     2    4.0000       .04390     .04121    -.00268502
     3    7.0000       .03720     .03788     .00067713
     4   12.0000       .02790     .03173     .00382727
     5   18.0000       .02090     .02363     .00272579
     6   22.0000       .01680     .01862     .00181953
     7   28.0000       .01770     .01244    -.00525921
     8   34.0000       .00860     .00798    -.00061923
     9   38.0000       .00730     .00584    -.00146313
    10   49.0000       .00460     .00235    -.00225073
    11   53.0000       .00360     .00166    -.00193564
    12   65.0000       .00230     .00057    -.00172632
    13   76.0000       .00140     .00021    -.00119043
    14   90.0000       .00090     .00006    -.00084354
    15   98.0000       .00060     .00003    -.00057362
    16  107.0000       .00040     .00001    -.00038888
END ************************ 23 DEGREE DATA CURVE FITTING FOR 0.05 MG./ML.
END OF INPUT
```

```
ACROSS IS TIME, TIME AT THE LEFT =  0.       ,TIME AT THE RIGHT =   .820E+02,TIME INCREMENT =   .828E+00
VERTICAL IS THE TIME DEPENDENT OBSERVABLE,TOP =   .250E-01, BOTTOM =   .271E-03, INCREMENT =   .505E-03
X MEANS AN EXPERIMENTAL POINT
O MEANS A CALCULATED POINT
= MEANS AN EXPERIMENTAL AND CALCULATED POINT ARE IN THE SAME DELTA X BY DELTA Y

         I----5----5----5----5----5----5----5----5----5----5----5----5----5----5----5----5----5----5----5----5I
         IX                                                                                                   I
         I                                           A COMPARISON OF THE FINAL RESULTS                        I
         IO                                          NUMBER     TIME EXPERIMENTAL CALCULATED F    RESIDUAL    I
         I                                                1   0.0000       .02500     .02400    -.00100000    I
         5                                                2   2.0000       .02020     .02094     .00074147    5
         I                                                3   4.0000       .01800     .01831     .00031380    I
         I                                                4   6.0000       .01670     .01605    -.00064874    I
         I                                                5   8.0000       .01460     .01410    -.00050127    I
         I                                                6  12.0000       .01010     .01095     .00084589    I
         5  O                                             7  16.0000       .00880     .00857    -.00023394    5
         I  X                                             8  21.0000       .00660     .00637    -.00022956    I
         I                                                9  24.0000       .00550     .00536    -.00013988    I
         I                                               10  30.0000       .00350     .00383     .00033232    I
         I                                               11  45.0000       .00170     .00173     .00003006    I
         5    =                                          12  53.0000       .00120     .00115    -.00004915    5
         I                                               13  60.0000       .00080     .00081     .00000978    I
         I                                               14  74.0000       .00050     .00040    -.00009635    I
         I       X                                       15  82.0000       .00030     .00027    -.00002861    I
         I       O                                                                                            I
         5                                                                                                    5
         I                                                                                                    I
         I         X                                                                                          I
         I         O                                                                                          I
         I                                                                                                    I
         5                                                                                                    5
         I                                                                                                    I
         I                                                                                                    I
         I                                                                                                    I
         I              O                                                                                     I
         5                                                                                                    5
         I              X                                                                                     I
         I                                                                                                    I
         I                                                                                                    I
         I                   =                                                                                I
         5                                                                                                    5
         I                                                                                                    I
         I                                                                                                    I
         I                         =                                                                          I
         I                                                                                                    I
         5                            =                                                                       5
         I                                                                                                    I
         I                                                                                                    I
         I                                    O                                                               I
         I                                    X                                                               I
         5                                                                                                    5
         I                                                                                                    I
         I                                                                                                    I
         I                                                      =                                             I
         I                                                               =        =                           I
         5                                                                                         =         =5
         I----5----5----5----5----5----5----5----5----5----5----5----5----5----5----5----5----5----5----5----5I
END ************************ 23 DEGREE DATA CURVE FITTING FOR 0.025 MG./ML.
END OF INPUT
```

Suelter (1,2,3) with the aid of ultracentrifugation data showed that a dimer was present at this temperature and had a maximum activity of 50% the tetramer activity. The average value of N obtained from adjustment of the parameters in equation (5.6) was observed to be 1/3 whereas, the theoretical value is 1/4.

High concentrations of FDP, with a net charge near four at pH 7.5 markedly increases the ionic strength of the solution over the contribution of the buffer. Therefore, an ionic strength effect, arising from the highly charged FDP molecule itself, is considered to have an effect on the dimer population by apparently stabilizing the dimer (see Figure 13). Therefore, at 23° the dimer concentration is strongly influenced by the ionic strength of the medium. A decreasing value of the slope as a function of the FDP concentration was observed in the linear portion of the curves presented in Figure 13. Previously, it was determined that these slopes were dictated by the concentration of the dimer. Thus, the slope of the "slow step" is markedly decreased by high FDP whereas, the fast step is relatively unaffected by FDP.

The inactivation process as a function of the FDP concentration was evaluated by the curve-fitting routine using equation (5.6). Plots of calculated and experimental T' versus time for three different FDP concentrations (T° at a constant level of 0.250 mg/ml) are presented in Figures 14, 15, and 16. The calculated parameters k_1, k_3, and N are presented in Table III. A cursory inspection of the table reveals that k_1 remains relatively constant over the entire range. Coupled with this is the trend for k_3 to decrease for increasing FDP. The decreasing value for k_3 predicts an increase in the D population for increasing FDP. Since k_3 appears in the exponential term in equation (5.6) the D

Figure 13. Stabilizing Effect of FDP Concentrations Above 5.36 mM for Yeast Pyruvate Kinase at 23°

Enzyme was incubated in 0.1 M Tris-HCl, pH 7.5, at 0.25 mg/ml. (Data from the PhD Thesis of R.T. Kuczenski, Michigan State University, 1970)

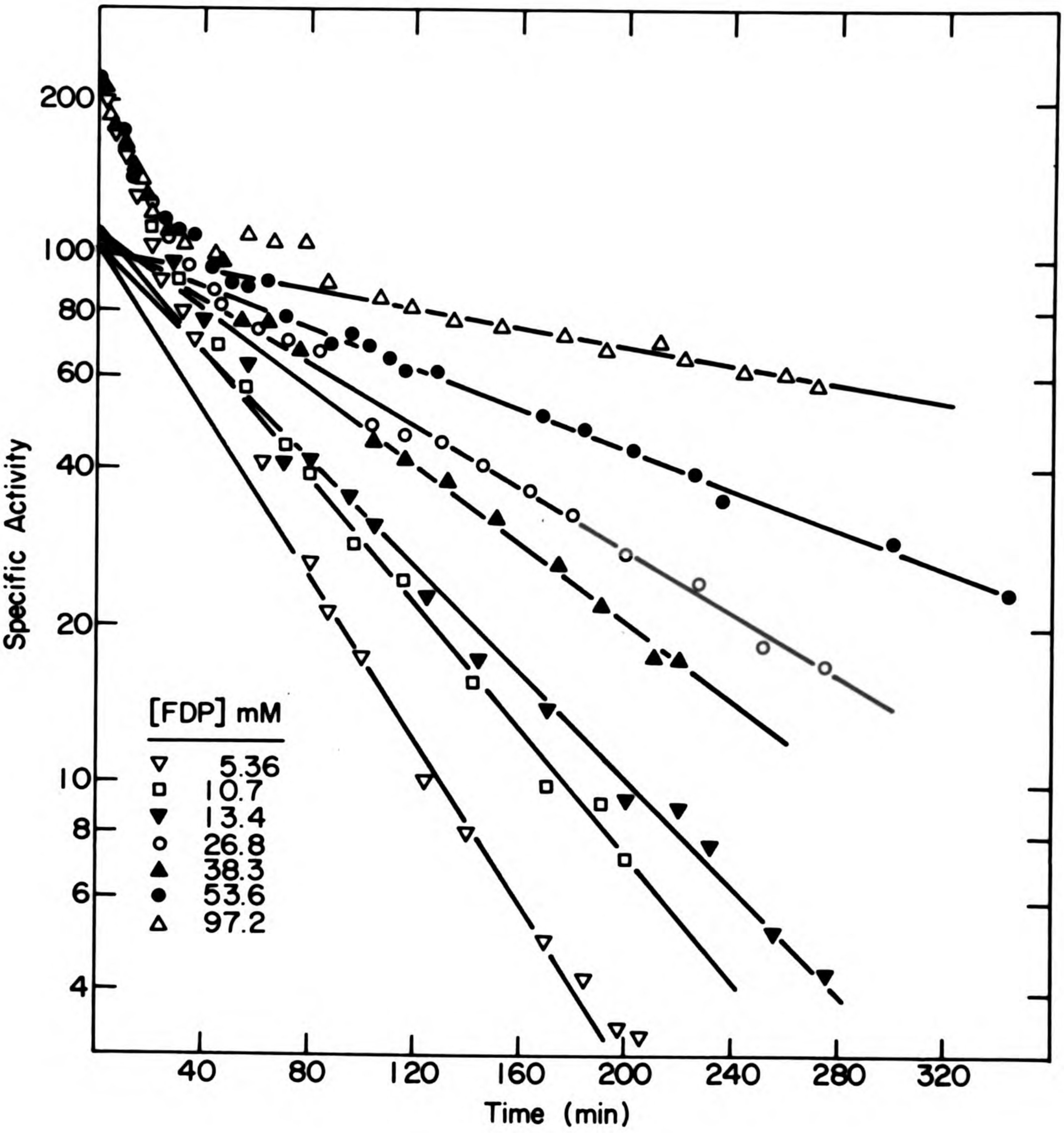

Specific Activity
200
100
80
60
40
20
10
8
6
4
40
80
120
160
200
240
280
320
Time (min)
[FDP] mM
▽ 5.36
□ 10.7
▼ 13.4
○ 26.8
▲ 38.3
● 53.6
△ 97.2

TABLE III

Kinetic Parameters for 23° Data Evaluated by a Curve-Fitting Routine[a] at Varying Concentrations of FDP[b]

FDP (mM)	k_3 (min.$^{-1}$)	k_1[c] (min.$^{-1}$)	N[c]
5.36 ± 0.27	.02237 ± .00535	.04424 ± .00629	.07059 ± .0092
10.7 ± 0.53	.03403 ± .00558	.01814 ± .00202	.42671 ± .0045
13.4 ± 0.67	.02989 ± .00416	.01591 ± .00175	.49387 ± .0037
26.8 ± 1.34	.01263 ± .00029	.03972 ± .00278	.10603 ± .0062
38.3 ± 1.61	.01627 ± .00147	.04187 ± .00445	.14150 ± .0014
97.2 ± 4.86	.00856 ± .00063	.03868 ± .00365	.12274 ± .0100

---

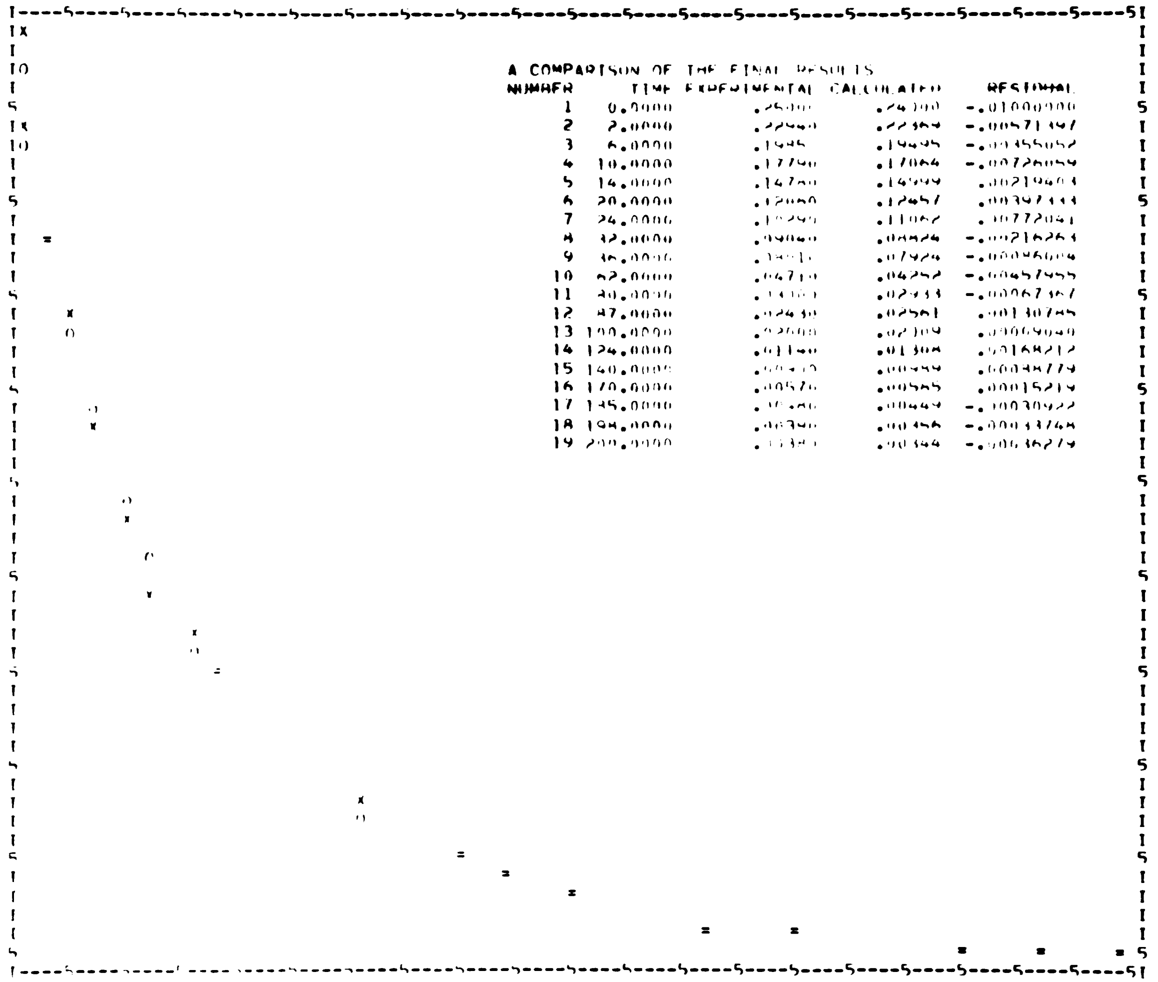
[a] Nicely and Dye (4)

[b] T° concentration remained constant at 0.250 mg/ml

[c] These parameters were calculated by using the average value of $(\sigma^\circ-1)$ obtained at 23° previously. This gave a value of 1.1204 ± .0024 mg/ml for σ°.

Figures 14, 15, and 16. Computer printouts of the calculated and experimental values for the concentration of active protein versus time at 23°. The initial protein concentration was 0.250 mg/ml at FDP concentrations of 5.36, 10.7, and 26.8 indicated at the bottom of each graph.

```
ACROSS IS TIME. TIME AT THE LEFT =  0.        .TIME AT THE RIGHT =   .200E+03.TIME INCREMENT =   .202E+01
VERTICAL IS THE TIME DEPENDENT OBSERVABLE.TOP =   .250E+00. BOTTOM =   .344E-02. INCREMENT =   .503E-02
X MEANS AN EXPERIMENTAL POINT
O MEANS A CALCULATED POINT
= MEANS AN EXPERIMENTAL AND CALCULATED POINT ARE IN THE SAME DELTA X BY DELTA Y
```



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A COMPARISON OF THE FINAL RESULTS
NUMBER      TIME EXPERIMENTAL CALCULATED     RESIDUAL
    1    0.0000       .25000      .24000  -.01000000
    2    2.0000       .22940      .22369  -.00571397
    3    6.0000       .1985       .19495  -.00355052
    4   10.0000       .17790      .17064  -.00726059
    5   14.0000       .14780      .14999   .00219603
    6   20.0000       .12060      .12457   .00397333
    7   24.0000       .10290      .11062   .00772041
    8   32.0000       .09040      .08824  -.00216263
    9   36.0000       .08010      .07924  -.00086004
   10   52.0000       .04710      .04252  -.00457955
   11   80.0000       .03300      .02933  -.00367367
   12   87.0000       .02430      .02561   .00130785
   13  100.0000       .02040      .02109   .00069040
   14  124.0000       .01140      .01308   .00168212
   15  140.0000       .00950      .00999   .00048779
   16  170.0000       .00570      .00585   .00015219
   17  185.0000       .00480      .00449  -.00030922
   18  194.0000       .00390      .00346  -.00043748
   19  200.0000       .00380      .00344  -.00036279
```

```
END ************************** 2ND ORDER ....IONIC STRENGTH EFFECT I=.250  EDP =53.6
```

```
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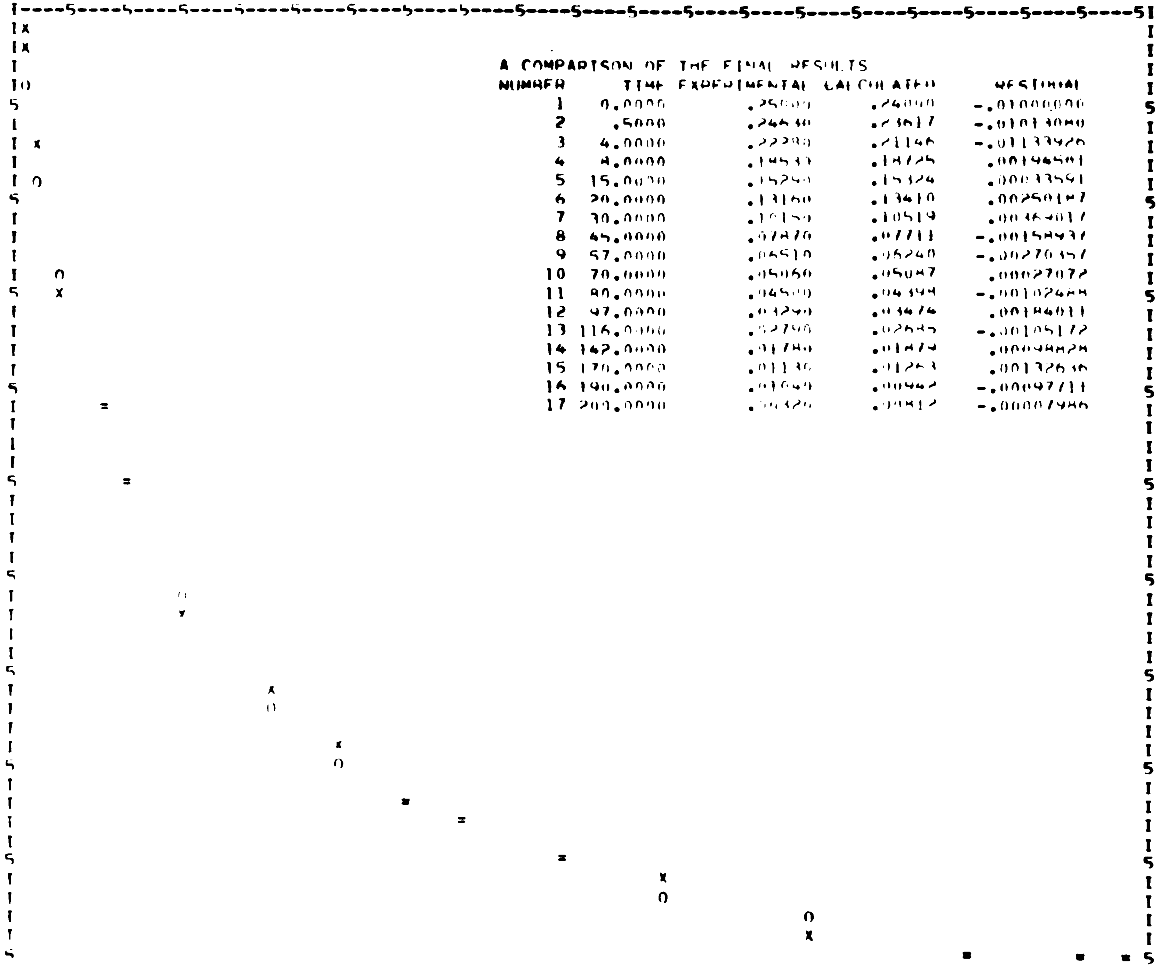
VERTICAL IS THE TIME DEPENDENT OBSERVABLE.TOP =   .250E+00. BOTTOM =   .812E-02. INCREMENT =   .494E-02
X MEANS AN EXPERIMENTAL POINT
O MEANS A CALCULATED POINT
= MEANS AN EXPERIMENTAL AND CALCULATED POINT ARE IN THE SAME DELTA X BY DELTA Y
```



```
A COMPARISON OF THE FINAL RESULTS
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     1    0.0000        .25000      .24000    -.01000000
     2     .5000        .24630      .23617    -.01013080
     3    4.0000        .22280      .21146    -.01133926
     4    8.0000        .18530      .18725     .00194501
     5   15.0000        .15290      .15324     .00033591
     6   20.0000        .13160      .13410     .00250187
     7   30.0000        .10150      .10519     .00369017
     8   45.0000        .07870      .07711    -.00158937
     9   57.0000        .06510      .06240    -.00270357
    10   70.0000        .05060      .05087     .00027072
    11   80.0000        .04500      .04398    -.00102488
    12   97.0000        .03290      .03474     .00184011
    13  116.0000        .02790      .02685    -.00105172
    14  142.0000        .01780      .01879     .00098828
    15  170.0000        .01130      .01263     .00132636
    16  190.0000        .01040      .00942    -.00097711
    17  200.0000        .00820      .00812    -.00007986
```

```
END ************************ 23 DEGREE...IONIC STRENGTH EFFECT I=.250 FDP=10.7
```

```
ACROSS IS TIME. TIME AT THE LEFT =  0.         .TIME AT THE RIGHT =   .276E+03.TIME INCREMENT =   .279E+01
VERTICAL IS THE TIME DEPENDENT OBSERVABLE.TOP =   .250E+00. BOTTOM =   .168E-01. INCREMENT =   .476E-02
X MEANS AN EXPERIMENTAL POINT
O MEANS A CALCULATED POINT
= MEANS AN EXPERIMENTAL AND CALCULATED POINT ARE IN THE SAME DELTA X BY DELTA Y
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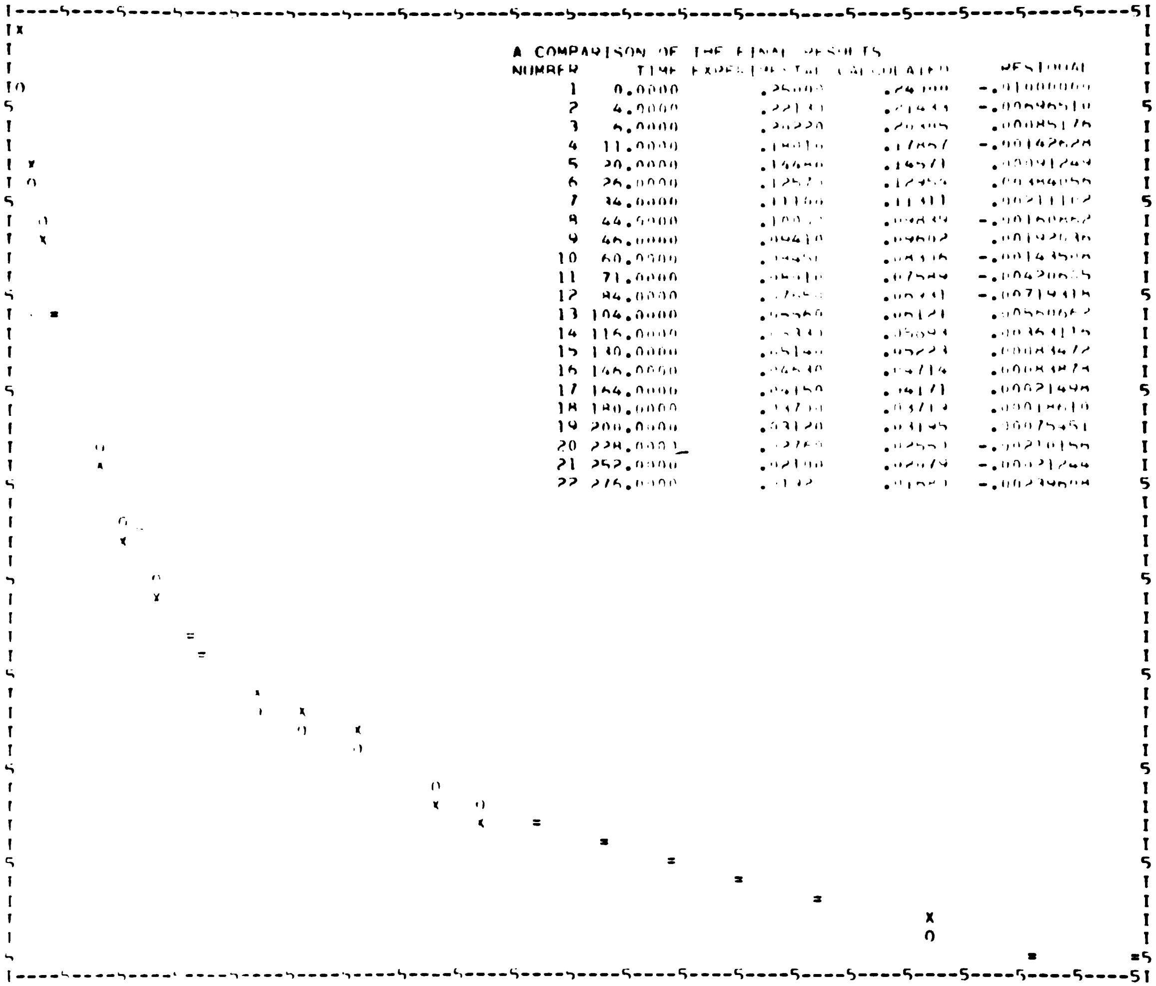


```
A COMPARISON OF THE FINAL RESULTS
NUMBER      TIME EXPERIMENTAL CALCULATED     RESIDUAL
   1    0.0000      .25000      .24000    -.01000000
   2    4.0000      .22140      .21443    -.00696510
   3    6.0000      .20220      .20305     .00085176
   4   11.0000      .18010      .17867    -.00142628
   5   20.0000      .14480      .14571     .00091249
   6   26.0000      .12570      .12954     .00384055
   7   34.0000      .11100      .11311     .00211102
   8   44.0000      .10000      .09839    -.00160862
   9   46.0000      .09410      .09602     .00192036
  10   60.0000      .08460      .08316    -.00143506
  11   71.0000      .08010      .07589    -.00420655
  12   84.0000      .07660      .06941    -.00719418
  13  104.0000      .05560      .06121     .00560662
  14  116.0000      .05330      .05694     .00364115
  15  130.0000      .05140      .05223     .00083472
  16  146.0000      .04630      .04714     .00083874
  17  164.0000      .04150      .04171     .00021498
  18  180.0000      .03700      .03719     .00018610
  19  200.0000      .03120      .03195     .00075451
  20  228.0000      .02760      .02551    -.00210155
  21  252.0000      .02100      .02079    -.00021244
  22  276.0000      .01820      .01581    -.00239604
```

```
END ************************ 25 DEGREE...IONIC STRENGTH EFFECT I=.250 FOR =26.8
```

population is much more sensitive to small changes in k_3 than in N. Therefore, the overall trend is for k_1 and k_3 to strongly control the T and D populations at any given FDP concentration.

VI. Calculations Based on the Kinetic Model

1. Slope Calculations

The slope of the semi-log plot of T versus time was developed from the theoretical model based on equations (4.47) and (4.48) from which the qualitative behavior of the curves at 0° and 23° was deduced. Differentiating equation (4.48) with respect to t gives

$$dT'/dt = -(2/\zeta)(\sigma^\circ-1)(k_1/\sigma^\circ)[(\sigma^\circ-1)\mathrm{EXP}(-k_1t/\sigma^\circ)+1] + N(\sigma^\circ-1)\mathrm{EXP}(-k_3t)[1-k_3t] \qquad (6.1)$$

Dividing through equation (6.1) by equation (4.48) and simplifying

$$\frac{d\ln T'}{dt} = \frac{-(2/\zeta)(k_1/\sigma^\circ)[(\sigma^\circ-1)\mathrm{EXP}(-k_1t/\sigma^\circ)+1]\mathrm{EXP}(k_3t) + N(1-k_3t)}{(1/\zeta)\mathrm{EXP}(k_3t)\mathrm{EXP}(-k_1t/\sigma^\circ)[(\sigma^\circ-1)\mathrm{EXP}(-k_1t/\sigma^\circ)+2]+Nt} \qquad (6.2)$$

Equation (6.2) gives the slope of the semi-log plot of T versus t. A qualitative interpretation of the slope of the biphasic curve presented in Figure 1 can be obtained from equation (6.2) by considering approximations at both long and short times. At short times the $N(1-k_3)$ and Nt terms may be considered small and dropped. With this assumption equation (6.2) takes the form of

$$d\ln T'/dt = \frac{-2k_1/\sigma^\circ[(\sigma^\circ-1)\mathrm{EXP}(-k_1t/\sigma^\circ)+1]}{\mathrm{EXP}(-k_1t/\sigma^\circ)[\frac{(\sigma^\circ-1)}{2}\mathrm{EXP}(-k_1t/\sigma^\circ)+1]} \qquad (6.3)$$

If $(\sigma^\circ-1)\mathrm{EXP}(-k_1t/\sigma^\circ)$ term is considered small and not too different from $\frac{(\sigma^\circ-1)}{2}\mathrm{EXP}(-k_1t/\sigma^\circ)$ equation (6.3) becomes

$$d\ln T'/dt = -(2k_1/\sigma^\circ)\mathrm{EXP}(k_1t/\sigma^\circ) \qquad (6.4)$$

At extremely short times $\mathrm{EXP}(k_1t/\sigma^\circ)$ is approximately unity and (6.4) is equal to $-(2k_1/\sigma^\circ)$.

Therefore, $$d\ln T'/dt = -2k_1/(1+16k_1k_{-1}T°/k_2^2)^{1/2} \quad (6.6)$$

The slope of the biphasic curve at short times is a function only of the rate constants and $T°^{1/2}$. The slope should change with T° and this is verified by the experimental curve. If a plot is made of the initial slopes ($-d\ln T'/dt$) versus $(1+T°)^{1/2}$ a straight line is obtained with a slope of $2k_1$ and a zero intercept.

At longer times $EXP(-k_1t/\sigma°)$ approaches a very small value and can be neglected and equation (6.2) can be rewritten as

$$d\ln T'/dt = \frac{-(2/\zeta)(k_1/\sigma°)EXP(k_3t)+N(1-k_3t)}{(1/\zeta)(EXP(k_3t)/EXP(k_1t/\sigma°) + Nt} \quad (6.7)$$

From the data obtained in Table I, $(1/\zeta)$ is approximately equal to unity. Also, if k_3 is assumed to be not too different from $(k_1/\sigma°)$ equation (6.7) can be approximated as

$$d\ln T'/dt = \frac{-2k_1EXP(k_3t)}{\sigma°Nt} - k_3 + 1/t \quad (6.8)$$

Taking the limit of $EXP(k_3t)/t$ as t approaches large values and using L'Hospital's rule to evaluate this limit, equation (6.8) can be simplified even further. Also, $1/t$ is considered small for t large.

$$d\ln T'/dt = -k_3[EXP(k_3t)(2k_1)/N\sigma° + 1] \quad (6.9)$$

The slope of the biphasic curve at long times is a function of all the rate constants, the theoretical constant N, and of $T°^{1/2}$. Since k_3 appears in the exponential term it is apparent that it should dictate the slope at long times to a much greater extent than k_1, k_{-1}, or k_2. This appears to be the case since at longer times the reservoir of total T has been depleted to the extent that dimer dictates the activity and k_3 is the rate constant which determines the decay of D.

A large k_3 would predict a faster decay for D and hence a smaller a smaller population of D. The opposite case would be evident for a small k_3. A large value for N which is evident for the 23° case should limit the decay and give a small negative value for the slope. This would reflect in a more rapid decay process overall for 23°. Comparison to the 0° case gives a predicted decay that is much slower at longer times. This is true since N at this temperature is very small and would give a large negative value for the slope. Thus, at longer times the controlling factor for the decay process as a function of temperature is largely N and k_3. The preceding analysis can be summarized in the following way, assuming that N, k_1, and k_3 are functions of temperature only.

$$N_{23^\circ} > N_{0^\circ}$$

$$(k_3)_{23^\circ} > (k_3)_{0^\circ}$$

$$(k_1)_{23^\circ} < (k_1)_{0^\circ}$$

$$(\text{slope})_{23^\circ} < (\text{slope})_{0^\circ}$$

2. Reduction in the number of parameters

Sometimes in the estimation of parameters in nonlinear cases certain parameters are almost linearly dependent. If two parameters are correlated then both parameters cannot be simultaneously estimated. One could simply set one at a nominal value and then estimate the other, recognizing the dependence of the two. Beck (5) previously examined the basic differential equations that relate the correlating parameters and found the exact relation that could be used to replace the two dependent parameters. His studies have been utilized in this section in order to correct the apparent correlation between N and k_3.

Theoretically, if a set of parameters β_1, β_2, ... β_p cannot be found simultaneously, i.e., they are linearly dependent, the following equation represents this dependency.

$$A_1 \frac{\partial \eta}{\partial \beta_1} + A_2 \frac{\partial \eta}{\partial \beta_2} + \ldots + A_p \frac{\partial \eta}{\partial \beta_p} = 0 \qquad (6.10)$$

A_1, A_2, ...A_p are constants, β_1, β_2, ...β_p are parameters and η is the dependent variable.

The insights into parameter estimation can be gained through the study of sensitivity coefficients. Sensitivity coefficients are defined as

$$S^Y_{\beta_i} = \beta_i \frac{\partial Y}{\partial \beta} \qquad (6.11)$$

where β_i are the parameters, and Y is the dependent variable. Sensitivity coefficients, especially in the study of heat transfer effects, is largely due to Beck (5). A plot of sensitivity coefficients versus the independent variable will show conclusively whether two or more parameters may be correlated on the basis that the curves are linearly dependent, i.e., each curve can be superimposed on another linearly dependent curve. In this manner sensitivity coefficients have been applied to the mathematical model developed in this thesis. The following sensitivity coefficients were plotted versus the independent variable t.

$$k_1 \frac{\partial T}{\partial k_1}, \quad k_3 \frac{\partial T}{\partial k_3}, \text{ and } N \frac{\partial T}{\partial N}$$

As a result N and k_3 appeared to be strongly correlated whereas k_1 and k_3 were only slightly correlated. Inspection of Table IV reveals that the sensitivities for k_3 can be multiplied by an appropriate constant and thereby closely duplicate the values of the sensitivities for N. Therefore, N and k_3 are sufficiently correlated that as a close approximation the following differential equation can be written.

$$A_1 \frac{\partial T}{\partial k_3} + A_2 \frac{\partial T}{\partial N} = 0 \qquad (6.12)$$

A_1 and A_2 are arbitrary constants. In order for the constants to be

TABLE IV

Sensitivity Coefficients Evaluated at 23° for the Protein Concentration of 0.6 mg/ml

t (min.)	$k_3 \frac{\partial T}{\partial k_3}$	$N \frac{\partial T}{\partial N}$	$k_1 \frac{\partial T}{\partial k_1}$
0	0	0	0
2	-.0024	.0929	-.0008
4	-.0158	.2315	-.0776
8	-.0567	.4157	-.1322
15	-.1651	.6452	-.1883
20	-.2564	.7502	-.2072
30	-.4405	.8611	-.2134
45	-.6610	.8610	-.1851
57	-.7670	.7888	-.1528
70	-.8145	.6821	-.1188
80	-.8118	.5948	-.0958
97	-.7546	.4560	-.0644
116	-.6454	.3262	-.0499
142	-.4791	.1978	-.0201
170	-.3243	.1118	-.0092
190	-.2343	.0723	-.0052
200	-.1980	.0495	-.0039

independent of the units of the problem, (6.12) can be written as

$$A_1 k_3 \frac{\partial T}{\partial k_3} + A_2 \frac{\partial T}{\partial N} = 0 \qquad (6.13)$$

where now A_1 and A_2 are dimensionless constants. In (6.12) $T = T(k_3,N,t)$ but a new relation is sought to replace the two dependent parameters k_3 and N which would make $T = T(\alpha(k_3,N),t)$. Differentiation of T with repect to the new parameter α according to the chain rule resulted in

$$A_1 k_3 \frac{\partial T}{\partial \alpha} \frac{\partial \alpha}{\partial k_3} + A_2 N \frac{\partial T}{\partial \alpha} \frac{\partial \alpha}{\partial N} = 0$$

or

$$\frac{\partial T}{\partial \alpha}[A_1 k_3 \frac{\partial \alpha}{\partial k_3} + A_2 N \frac{\partial \alpha}{\partial N}] = 0$$

Since $\frac{\partial T}{\partial \alpha} \neq 0$

$$A_1 k_3 \frac{\partial \alpha}{\partial k_3} + A_2 N \frac{\partial \alpha}{\partial N} = 0 \qquad (6.14)$$

A solution of (6.14) is $\alpha = N k_3^{-A_1/A_2}$. (6.15)

From an analysis of the sensitivity coefficients $k_3 \frac{\partial T}{\partial k_3}$ and $N \frac{\partial T}{\partial N}$ the ratio $-A_1/A_2$ was determined to be approximately 2.3445 at long times, i.e. it is only at longer times that N and k_3 appear to be strongly correlated. Unfortunately, N and k_3 do not appear as a group in the rate equation making a substitution of equation (6.15) impossible. However, (6.15) can be solved for N in terms of α and k_3 to give

$$N = \frac{\alpha}{k_3^{-A_1/A_2}} \qquad (6.16)$$

Assuming a constant value of 1/4 for N at 23° and an average value for k_3, α was determined to be 3.519×10^{-5}. In the complete time interval the ratio $-A_1/A_2$ is approximately unity and an adequate approximation to (6.16) is

$$N = \frac{3.519 \times 10^{-5}}{k_3} \qquad (6.17)$$

which can now be substituted into the rate equation as a replacement for the parameter N. The result is an equation which has only two adjustable parameters k_1 and k_3 which appear to be only slightly correlated. After substitution of (6.17) into (5.6) the following equation is evident.

$$T' = (1/\zeta)[((\sigma^\circ-1)\mathrm{EXP}(-k_1t)+1)^2-1] + \frac{3.519\mathrm{x}10^{-5}\ \xi t\mathrm{EXP}(-k_3t)}{k_3} \qquad (6.18)$$

where

$$\zeta = 16k_1k_{-1}/k_2^2$$

$$\xi = (k_2^2/4k_{-1})(\sigma^\circ-1)$$

Estimated values for k_1 and k_3 at a constant value of N are presented in Table V. Curve-fitting to the data resulted in a good fit and the correlation between k_1 and k_3 appeared to be small.

3. Half-life calculation

Calculation of the half life for the decay in activity of the protein can be treated in a similar procedure applied to equation (6.2). If for both the 0° and the 23° cases the D term can be neglected since it is only necessary at long times then substituting the relation that at $t_{1/2}$, $T = T^\circ/2$ into (4.46) the following equation results, where

$$t_{1/2} = \frac{(1+T^\circ)^{1/2}}{k_1}\ln[\frac{(1+T^\circ)^{1/2}-1}{(1+T^\circ/2)^{1/2}-1}] \qquad (6.19)$$

$(1/\zeta)$ is considered to be approximately unity. Plotting $t_{1/2}$ versus $(1+T^\circ)^{1/2}\ln[\frac{(1+T^\circ)^{1/2}-1}{(1+T^\circ/2)^{1/2}-1}]$ resulted in a straight line with a slope equal to $1/k_1$ and a zero intercept. Figure 17 presents this plot.

Values for k_1 were calculated for 0° and 23° and are 7.16×10^{-3} $\mathrm{min.}^{-1}$ and 6.45×10^{-3} $\mathrm{min.}^{-1}$ respectively. These values are a full order of magnitude smaller than those presented in Tables I, II, and III. This must be considered since the equation is only an approximation. As the value of T° approaches zero, $t_{1/2}$ approaches zero whereas, (6.19) states that $t_{1/2}$ approaches negative infinity. Therefore, it is impossible to maintain a zero concentration of protein and still calculate a half life. However, the important consideration is that $t_{1/2}$ approaches zero as T° approaches zero. An inspection of Figure 17 reveals that the half life curve at 23° has an extrapolated intercept which is greater

TABLE V

Kinetic Parameters Evaluated at 23° at a Constant Value of N[1]

T° (mg/ml)	k_1 (min.$^{-1}$)	k_3 (min.$^{-1}$)
.600 ± .030	.055 ± .0026	.0137 ± .0042
.400 ± .020	.064 ± .0031	.0129 ± .0033
.150 ± .007	.074 ± .0111	.0211 ± .0191
.090 ± .005	.070 ± .0141	.0615 ± .0124
.050 ± .003	.087 ± .0122	.0222 ± .0153
.025 ± .001	.134 ± .0108	.0217 ± .0101

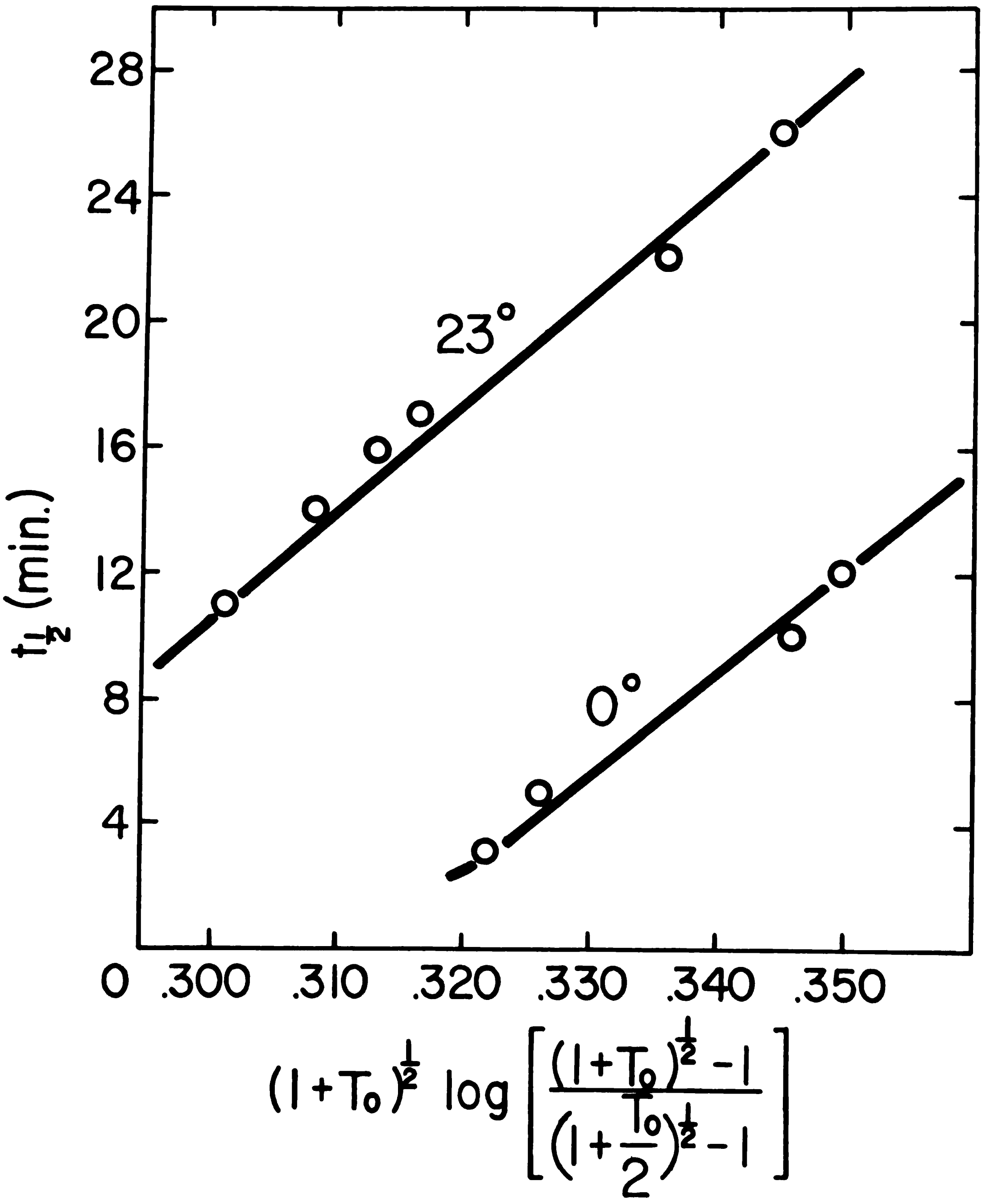
FDP (mM)	k_1 (min.$^{-1}$)	k_3 (min.$^{-1}$)
5.36 ± .268	.0672 ± .0015	.0138 ± .00015
10.7 ± .530	.0594 ± .0022	.0109 ± .00073
13.4 ± .670	.0578 ± .0020	.0103 ± .0054
26.8 ± 1.34	.0517 ± .0018	.0075 ± .0023
38.3 ± 1.61	.0472 ± .0021	.0084 ± .00043
53.6 ± 2.68	.0457 ± .0017	.0065 ± .00102
97.2 ± 4.86	.0465 ± .0030	.0052 ± .00015

---

[1] N = 0.25 at 23°

Figure 17. Effect of Half Life as a Function of the Initial Protein Concentration for the FDP-enhanced Inactivation of Yeast Pyruvate Kinase at 0° and 23°.

FDP concentration in all cases was 1.26 mM.


28
24
20
16
12
8
4
0
.300
.310
.320
.330
.340
.350
$t_{\frac{1}{2}}$ (min.)
23°
0°
$(1+T_0)^{\frac{1}{2}} \log\left[\frac{(1+T_0)^{\frac{1}{2}}-1}{(1+\frac{T_0}{2})^{\frac{1}{2}}-1}\right]$

than that for 0°. Apparently, this suggests that the enzyme is more stable at 23° than at 0°.

DISCUSSION

From sedimentation and diffusion data (1,2,3) a weight average molecular weight near 167,000 was determined for native yeast pyruvate kinase. The molecular weight of the subunit is near 42,000 and it has been concluded that yeast pyruvate kinase, like muscle enzyme is composed of four subunits and that these subunits are very similar in size. Yeast pyruvate kinase exhibits an instability enhanced by the allosteric activator FDP. This instability is inversely proportional to the protein concentration and involves a protein dissociation to lower molecular weight species.

On the basis of fluorescence data, FDP promotes a major conformational transition of yeast pyruvate kinase (3). This relationship has been observed in the presence and absence of Mg^{2+}. The FDP activation as a regulatory mechanism for glycolysis suggests that there are sites on the protein surface to which FDP binds. In addition, there are specific sites to which Mg^{2+} binds. A possible explanation of the stabilization by FDP and Mg^{2+} is that the binding of FDP induces a partial conformational change to a highly unstable form of the enzyme, which in the presence of Mg^{2+}, would be completed to the fully active, stabilized conformer. Therefore, it can be implied that the effects of temperature and ionic strength produced by FDP are consistent with binding sites and similar conformational changes in both activation and inactivation. On this basis, an analysis of the inactivation mechanism could reveal information concerning the structural changes involved.

The sedimentation data for yeast pyruvate kinase in the presence and absence of FDP at 23° and the inverse dependence of both steps of the process of inactivation on protein concentration are consistent with a mechanism involving two consecutive dissociations of the native tetramer to inactive monomers. The kinetic model for this inactivation presented in equation (2.1) is as follows:

$$T \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} 2D^*$$

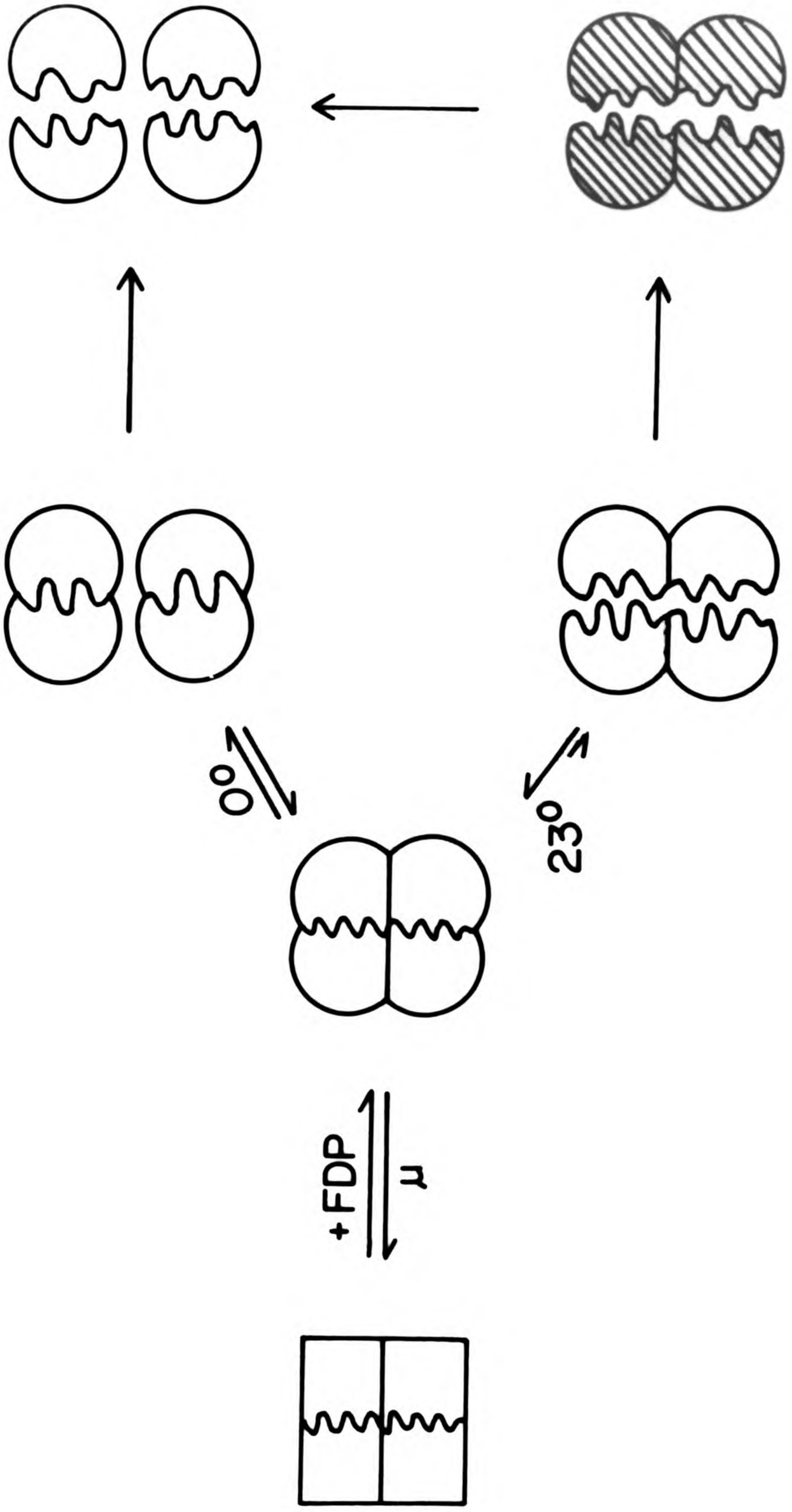
$$D^* \xrightarrow{k_2} D$$

$$D \xrightarrow{k_3} 2M$$

D^* represents unstable dimer which is an intermediate in the inactivation process. D represents a more stable dimer which at 23° has 50% the activity of the tetramer. D subsequently dissociates to give two monomers. The biphasic inactivation curve obtained at 0° and 23°, both in the presence of FDP, suggests that similar mechanisms of inactivation are operative in both cases.

It has been suggested by Kuczenski and Suelter (1,2,3) that alternate pathways from tetramer to monomer exist at 0° and 23°. This is based on the FDP-enhanced inactivation and the effects of FDP on the course of inactivation. The overall inactivation scheme presented in Figure 18 indicates two different dimers are present at the two temperatures. From his studies Kuczenski indicated that the extent of dimer formation at 23° is independent of FDP and protein concentration whereas, at 0° it is dependent on both FDP and protein concentration. The evaluation of rate equations from the model in (2.1) shows that at both temperatures dimer formation is dependent on protein concentration. Therefore, some inconsistency in the existence of different dimers have been presented. Unfortunately, the kinetic model cannot and does not

Figure 18. Proposed Mechanism of FDP-enhanced Inactivation of Yeast Pyruvate Kinase at 0° and 23°.


0°
23°
+FDP
μ

differentiate between the two dimers and consequently, the rate equations remain unbiased. However, the complete rate equation (5.6) which predicts the inactivation of tetramer as a function of time and initial protein concentration has been shown to be adequate at both temperatures. If the dimers are different they cannot be proven as such by the kinetic treatment outlined in this thesis. Evidently, further experimentation is necessary.

Successful application of the theoretical model to the experimental data can be attributed to the assumption that the total activity of the protein is a function of both the tetramer and dimer concentrations or

$$T' = A/C = [\ (T) + N(D)\ ]$$

where N represents a theoretical percentage of D that contributes to the overall activity. From this model the following rate equation which adequately predicts the inactivation of pyruvate kinase at 0° and 23° has been evaluated:

$$T' = (1/\zeta)[\{(\sigma^\circ-1)\mathrm{EXP}(-k_1 t/\sigma^\circ)+1\}^2-1] + N(k_2^2/4k_{-1})(\sigma^\circ-1)t\mathrm{EXP}(-k_3 t)$$

It is reasonable to assume that this rate equation adequately predicts the inactivation at intermediate temperatures providing that the correct choices for k_1/σ°, $(\sigma^\circ-1)$, and N have been made.

The dependence of the dimer concentration as a function of the ionic strength has also been established. Increasing the ionic strength at 23° favors an increase in the dimer population. This effect is largely due to a decreasing value in k_3 as the FDP concentration is increased.

Furthermore, an increase in temperature favors an increase in the dimer population. However, this effect is less sensitive to a large increase in temperature compared to a similar rise in FDP concentration. This is deduced by a comparison of the values for k_1 and k_3 as a function of temperature. k_1 decreases for a rise in temperature (whereas, it increases for increasing FDP) which favors a subsequent decrease in the D population. This is coupled with a still larger decrease in k_3. Overall, the effect is the net rise of D as the temperature increases.

Unfortunately, no values for the equilibrium constant nor the rate constants k_{-1} and k_2 were given since the ratio of k_1/k_{-1} cannot be calculated. There individual parameters are correlated and convergence cannot be obtained by the curve-fitting routine. However, the value of (k_1k_{-1}/k_2^2) appears to be constant over the temperature range from 0° to 23° which implies that the ratio of k_{-1}/k_2^2 undergoes a change opposite to that for k_1. Therefore, k_2 and k_{-1} increase for increasing temperature and this favors a concomittant rise in the D population. Furthermore, this increasing value of k_{-1} as a function of temperature favors a decreasing value for the equilibrium constant.

Low temperature instability of proteins indicates that associations between apolar groups are important in these proteins, since hydrophobic bonding would be expected to be significantly weakened at low temperatures (6,7). Although thermodynamic analyses of the interaction of apolar groups with water indicate a large unfavorable entropy loss as the temperature is lowered, which may be attributed to a major change in the structuring of water around the apolar groups in some manner analagous to the "iceberg" concept of Frank and Evans (8). Brandts (9) has suggested that the favorable free energy change involved in removing an apolar residue from an aqueous medium is entirely entropic and due to the concomitant "melting" of the "clathrate" water structure around the residue. Hence, the optimum temperature for stability of a protein should increase as the hydrophobicity of the protein increases. Therefore, the effects of temperature on the stability of yeast pyruvate kinase implicate water structure, and specifically hydrophobic bonding in the association between subunits of the enzyme.

In conclusion, it may be interesting to examine other reports of cold-labile enzymes now appearing with increasing frequency (for a partial review, see Jarabak *et*. *al*. (10)) with respect to the effect of temperature on their structure and kinetic properties. It was not until the early 1960's that other reports of cold labile enzymes began to appear with the number since increasing steadily.

In 1960, Shukuya and Schwert (11) reported that dilute solutions of glutamate decarboxylase from *E. coli* were more stable at 20° than at 0°. Addition of bovine serum albumin protected against the inactivation, while pyridoxal phosphate, a required cofactor, not only protected but also reversed the inactivation. Similarly, dilute solutions of D(-)β-hydroxybutyric acid dehydrogenase were highly unstable at 0° (12). A partial restoration of the activity could be obtained by rewarming. The stability of the enzyme increased at increased protein concentrations as did the reactivation, suggesting an equilibrium which the authors considered might model a dissociated enzyme species. An N_2-fixing enzyme from *Clostridium pasteurionum* (13) exhibited a maximum stability at 22°. The activation was biphasic and was again inversely proportional to the protein concentration. Partial reactivation could be accomplished by rewarming.

It could be worthwhile to approach the problem of these cold labile enzymes in the same manner as the evaluation of the kinetic processes for the inactivation of pyruvate kinase. If the kinetic analysis works favorably for these cases then a general kinetic model could be constructed which may apply to any cold-labile process.

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