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# KINETIC STUDIES ON THE REACTION CATALYSED BY THREONINE DEHYDRATASE FROM *RHODOPSEUDOMONAS SPHEROIDES*

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SUMMARY

A highly purified preparation of biosynthetic threonine dehydratase (L-threonine hydro-lyase (deaminating), EC 4.2.1.16) from *Rhodopseudomonas spheroides*, has been used for a kinetic study of its reaction mechanism in the absence and presence of modifiers. At pH 7.4 and in the absence of modifiers, the initial velocity data gave curvilinear double reciprocal plots and could be fitted to a rate equation that describes the addition of threonine to the enzyme at two interacting sites which catalyse substrate hydrolysis at different rates. The initial velocity data obtained in the presence of lower, but not higher, concentrations of isoleucine fitted well to the same equation. The inhibition of the reaction increased as a sigmoidal function of the isoleucine concentration and with threonine at a concentration of 40 mM, the data were consistent with a rate equation which is the ratio of second order polynomials.

Enzyme activity increased as a hyperbolic function of the concentration of valine which was also capable of causing curvilinear double reciprocal plots to become linear. The latter effect was also observed with allothreonine that can act as a linear competitive inhibitor. Neither valine nor isoleucine affected the maximum velocity of the reaction and the inhibition by isoleucine could be reversed completely by valine. Attention has also been drawn to some of the inherent difficulties associated with the analysis of kinetic data for allosteric enzymes.

## INTRODUCTION

Over the past ten years, there has been obtained a considerable amount of qualitative information relating to the effects of substrates and modifiers on the kinetics of reactions catalysed by regulatory enzymes. But as yet, no detailed description has been given of the catalytic mechanism for any one such enzyme. Many

Abbreviation: TES, *N*-tris(hydroxymethyl)methyl-2-aminoethanesulphonic acid.

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attempts have been made to explain the kinetic data for regulatory enzymes in terms of the hypotheses that are based on the binding of ligands[1–3], while only a few investigators[4–9] have endeavoured to utilize the kinetic approach which has been applied so successfully to the elucidation of reaction mechanisms of the classical-type enzymes[10,11]. A number of difficulties are associated with the analysis of kinetic data which give rise to curvilinear double reciprocal plots[7,11]. However, previous kinetic investigations in this laboratory on nucleoside diphosphatase (EC 3.6.1.6) have resulted in definitive conclusions being reached about the catalytic mechanism of this allosteric enzyme[7,9]. Therefore, it was of interest to extend these studies to another regulatory enzyme which exhibits kinetic characteristics similar to those of nucleoside diphosphatase.

The enzyme chosen for the present study was biosynthetic threonine dehydratase (L-threonine hydro-lyase (deaminating), EC 4.2.1.16) from *Rhodopseudomonas spheroides*. This enzyme has been obtained as a stable, purified preparation[12], and can be considered to catalyse a single substrate reaction under conditions where pyridoxal phosphate is present at near-saturating concentrations. The aim of the present work was to obtain accurate and extensive initial velocity kinetic data for the reaction, both in the absence and presence of modifiers, and to analyse the resulting data by statistical curve fitting procedures. The results obtained allow a number of conclusions to be reached about the mechanism of the reaction in the absence and presence of valine and allothreonine. However, because of the complexity of the reaction kinetics, it has not been possible to arrive at definitive conclusions about the reaction mechanism in the presence of isoleucine.

## MATERIALS AND METHODS

### *Materials*

Reagents and the preparation of threonine dehydratase from cell-free extracts of *R. spheroides* were as previously described[12]. The enzyme had an activity of 1900 $\mu$moles/h per mg of protein at pH 8.0, with the exception of that used in the experiments described in Fig. 1, where the activity was 200 $\mu$moles/h per mg of protein, and was stored at −15 °C in 0.05 M *N*,*N*-bis(2-hydroxyethyl)glycine(Bicine)–NaOH buffer (pH 8.0) containing 0.5 M KCl, 5.0 mM allothreonine, 20 $\mu$M pyridoxal phosphate, 0.1 mM dithiothreitol and 0.1 mM EDTA.

### *Methods*

#### *Measurement of initial velocities*

Unless otherwise indicated, threonine dehydratase activity was determined[12] in reaction mixtures which, in a total volume of 1.0 ml, contained: 0.1 M *N*-tris(hydroxymethyl)methyl-2-aminoethane sulphonic acid (TES)–NaOH buffer (pH 7.4), 0.1 M KCl, 2.0 $\mu$M pyridoxal phosphate and 0.4–1.0 $\mu$g of purified enzyme as well as threonine and other components at concentrations that are given in the legends to the figures. KCl was included so as to minimize the loss of activity that occurs at low threonine concentrations in the absence of this compound[12]. Care was taken to ensure that, under all conditions investigated and at all substrate concentrations, true initial steady-state velocities were measured[12], and it was demonstrated that there was a linear relationship between these velocities and the enzyme concentration up

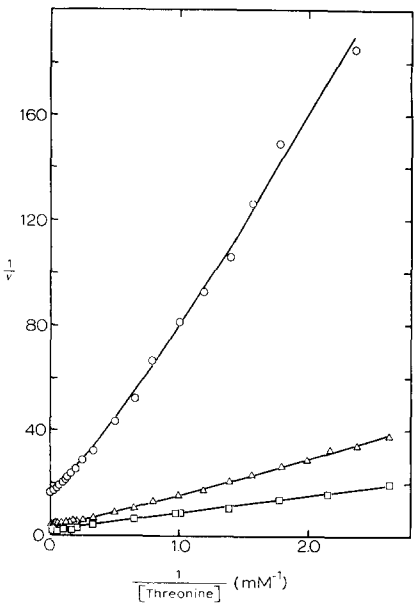



Fig. 1. Effect of the concentration of threonine on the initial velocity of the reaction at different pH values. The pH (and compositon) of buffers were: ○, pH 7.4 (0.1 M TES–NaOH); △, pH 8.0 (0.1 M *N*-ethylmorpholine–HCl); □, pH 9.0 (0.1 M *N*-ethylmorpholine–HCl). Plots of product formation as a function of time were linear for all substrate concentrations at each of the three pH values investigated. Data obtained at pH 7.4 and pH 8.0 were fitted to Eqn 3 while those obtained at pH 9.0 were fitted to Eqn 1. Velocity is expressed as $\mu$moles/h per $\mu$g of protein.

to a concentration of 2.5 $\mu$g/ml. In the presence of high concentrations of isoleucine, where a relatively long time (0–10 min) elapses before the inhibited steady-state velocity is achieved[12], the steady-state inhibited velocity was measured. It has previously been shown that the steady-state velocity observed in the presence of isoleucine is proportional to the enzyme concentration[12]. It should be mentioned that the greatest amount of allothreonine introduced into reaction mixtures with the enzyme gave a final concentration of 10 $\mu$M which would cause a maximum increase in its activity of only 2% with threonine at a concentration of 0.5 mM[12].

*Analysis of data*

The kinetic data were plotted graphically in double reciprocal form to determine the patterns of the plots. Analyses were then made by using the appropriate computer program[7,13,14] in conjunction with an IBM 360 computer. Data giving linear double reciprocal plots were fitted to Eqn 1 as the variance of the experimental velocities was not constant, but varied in proportion to the velocity value (see Results). Data that gave parabolic or curvilinear double reciprocal plots were fitted to Eqns 2 or 3, respectively, where b, c and d are combinations of rate constants. Data for which the initial velocity varied as a hyperbolic function of the modifier (M) concentration were fitted to Eqn 4. Data conforming to linear competitive inhibition were fitted to Eqn 5.

$$\log v = \log \left[ \frac{VA}{K_a + A} \right] \quad (1)$$

$$v = \frac{VA^2}{A^2 + bA + c} \tag{2}$$

$$v = \frac{V(A^2 + dA)}{A^2 + bA + c} \tag{3}$$

$$v = V' \left[ \frac{1 + \frac{M}{K_N}}{1 + \frac{M}{K_D}} \right] \tag{4}$$

$$v = \frac{VA}{K_a \left[ 1 + \frac{I}{K_i} \right] + A} \tag{5}$$

All programs for fitting the data to Eqns 2–5 were modified to allow for the inclusion of weighting factors equal to $1/v^2$ because of the aforementioned finding in relation to the variance of the initial velocity values. Unless otherwise stated, the values of the kinetic parameters obtained from the analyses were used to draw the curves illustrated in the figures. Weighted mean values of the kinetic constants, and their standard errors, were calculated as described[15].

## RESULTS

### *Effect of pyridoxal phosphate and pH on initial velocities*

Preliminary experiments at pH 7.4 showed that, over a 40-fold range of threonine concentrations from 0.5–20 mM, maximum activity of threonine dehydratase was obtained in the presence of 2.0 $\mu$M pyridoxal phosphate. Therefore, this concentration of pyridoxal phosphate was used in all subsequent experiments.

The shape of double reciprocal plots of velocity as a function of threonine concentration was found to vary with pH (Fig. 1). Thus, while the plots are linear at pH 9.0. they are curvilinear at both pH 7.4 and pH 8.0. In this respect, threonine dehydratase from *R. spheroides* appears similar to the same enzyme from *Escherichia coli*[16] but differs from the enzymes from *Saccharomyces cerevisiae*[17] and *Salmonella typhimurium*[6,18]. All further kinetic studies were performed at pH 7.4 as it was of interest to investigate the mechanism of the threonine dehydratase reaction under conditions where the double reciprocal plots were non-linear.

### *Kinetic studies in the absence of modifiers*

It has been pointed out previously[7] that accurate analysis of kinetic data which give rise to curvilinear double reciprocal plots, requires that a large number of initial velocities be determined over a wide range of substrate concentrations. Therefore, in the following experiments 20–24 different threonine concentrations, within the range from 0.4–80 mM, have been used. Double reciprocal plots of the initial velocity as a function of the threonine concentration were found to be curvilinear (Fig. 2A). When the initial velocity data were fitted to Eqn 3 on the assumption that the variance of the individual velocity values was constant, there was a marked deviation of the experimental points from the curve of best fit at lower substrate concentrations (Fig. 2B). Moreover, the standard errors of the values for the kinetic parameters were relatively high. Further investigations showed that the

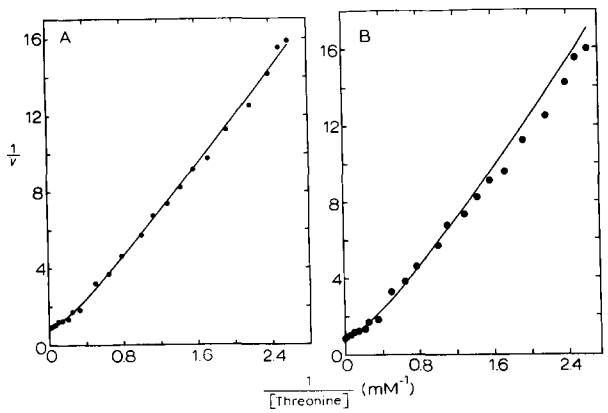



Fig. 2. Effect of the concentration of threonine on the initial velocity of the reaction. Data were analysed by making weighted (A) and unweighted (B) fits to Eqn 3. The weighting factor used was $1/v^2$. Velocity is expressed as $\mu$mole/h per $\mu$g of protein.

TABLE I

STANDARD DEVIATIONS OF MEAN VELOCITY VALUES AT SIX DIFFERENT SUBSTRATE CONCENTRATIONS

| *Threonine concn (mM)* | *Initial velocity ($\mu$mole/h per $\mu$g protein)* | *Standard deviation* | *Standard deviation (%)* | *Number of determinations* |
|---|---|---|---|---|
| 0.50 | 0.084 | 0.004 | 4.8 | 10 |
| 0.84 | 0.130 | 0.005 | 3.8 | 9 |
| 1.26 | 0.205 | 0.008 | 3.9 | 9 |
| 2.0 | 0.352 | 0.020 | 5.7 | 10 |
| 6.0 | 0.660 | 0.026 | 3.9 | 9 |
| 20.0 | 0.937 | 0.046 | 4.9 | 10 |

TABLE II

VALUES FOR THE APPARENT KINETIC PARAMETERS OF THE REACTION IN THE ABSENCE AND PRESENCE OF ISOLEUCINE

Values obtained in the absence of isoleucine are the weighted means of those obtained by fitting to Eqn 3 two sets of initial velocity data including that of Fig. 2. Values obtained at each concentration of isoleucine are weighted means of those obtained by fitting to Eqn 3 three sets of initial velocity data, including the data shown in Fig. 4. $K_m$ is defined as the concentration of substrate which gives half maximal velocity and was calculated from the expression:

$$K_m = \frac{b}{2} - d + \sqrt{\left(\frac{b}{2} - d\right)^2 + c}$$

| *Isoleucine concn ($\mu$M)* | *V ($\mu$moles/h per $\mu$g)* | *Apparent kinetic parameter* | | | |
|---|---|---|---|---|---|
| | | *b (mM)* | *c (mM²)* | *d (mM)* | *$K_m$ (mM)* |
| None | 1.07 ± 0.05 | 4.18 ± 0.38 | 21.3 ± 6.0 | 3.0 ± 1.0 | 3.76 ± 0.21 |
| 10 | 1.14 ± 0.05 | 3.35 ± 0.39 | 13.7 ± 1.8 | 0.6 ± 0.1 | 4.82 ± 0.21 |
| 20 | 1.11 ± 0.06 | 1.06 ± 0.47 | 31.3 ± 1.9 | 0.60 ± 0.09 | 5.54 ± 0.20 |
| 30 | 1.08 ± 0.05 | −2.66 ± 0.64 | 62.4 ± 3.3 | 0.64 ± 0.10 | 6.26 ± 0.20 |

TABLE III

COMPARISON OF THE VALUES FOR THE KINETIC PARAMETERS OF THE REACTION AT DIFFERENT pH VALUES

Values were obtained by fitting the data obtained at pH 8.0 and pH 9.0 (Fig. 1) to Eqns 3 and 1, respectively.

| *Kinetic parameter* | *pH 8.0* | *pH 9.0* |
|---|---|---|
| $v$ ($\mu$mole/h per $\mu$g) | 0.300 ± 0.005 | 0.434 ± 0.006 |
| $b$ (mM) | 3.6 ± 0.3 | |
| $c$ (mM$^2$) | 2.6 ± 2.0 | |
| $d$ (mM) | 0.57 ± 0.49 | |
| $K_m$ (mM) | 3.26 ± 0.14 | 2.75 ± 0.07 |

standard deviation of the mean velocity values at six different threonine concentrations was not constant, but rather varied proportionately to the magnitude of the values (Table I). Similar results have been reported by Schramm and Morrison[7] from their studies on nucleoside diphosphatase. Because of the above finding, a weighted fit of the data to Eqn 3 was made using $1/v^2$ as the weighting factor. The results illustrated in Fig. 2A, as well as the relatively low standard errors associated with the values for the kinetic parameters (Table II) indicate that the data give a good fit to Eqn 3. On the other hand, a weighted fit of the data to Eqn 2 showed that the experimental points at both high and low substrate concentrations deviated considerably from the theoretical curve.

For comparative purposes, the data obtained at pH 8.0 (Fig. 1) were also fitted to Eqn 3. Although the experimental points appeared to fit well to the theoretical curve, the standard errors of the values for $c$ and $d$ are high (Table III). Table III also lists the values for $K_m$ and the maximum velocity as determined by fitting the data obtained at pH 9.0 to Eqn 1.

*Inhibition of the reaction by allothreonine*

Allothreonine, which is a stereoisomer of threonine, is not a substrate for, but acts as an inhibitor of, threonine dehydratase[12,19,20]. Kinetic studies showed that the non-linear double reciprocal plots obtained in the absence of allothreonine become linear in the presence of this compound and that the inhibition appears to be of the competitive type (Fig. 3A, Table IV). More detailed investigations of the inhibition were made over a range of higher threonine concentrations (5–80 mM) such that, in the absence of modifiers, plots of $1/v$ against 1/[threonine] appear linear. In the presence of four fixed concentrations of allothreonine, a family of straight lines was observed (Fig. 3B). The data were fitted to Eqn 1, and the resulting lines of best fit were found to intersect at the ordinate. Moreover, replots of the slopes of the lines as a function of the allothreonine concentration are linear, indicating that, at relatively high concentrations of threonine, allothreonine functions as a linear competitive inhibitor. This was confirmed by fitting the data to Eqn 5, and the results indicate that quite a good fit was obtained (Fig. 3B). The values obtained for the maximum velocity, $K_a$ and $K_i$ were 0.88 ± 0.01 $\mu$mole/h per $\mu$g of protein, 4.2 ± 0.2 mM and 0.58 ± 0.03 mM, respectively. The results suggest either that one molecule of allothreonine is involved in the inhibition or that multiple molecules react in

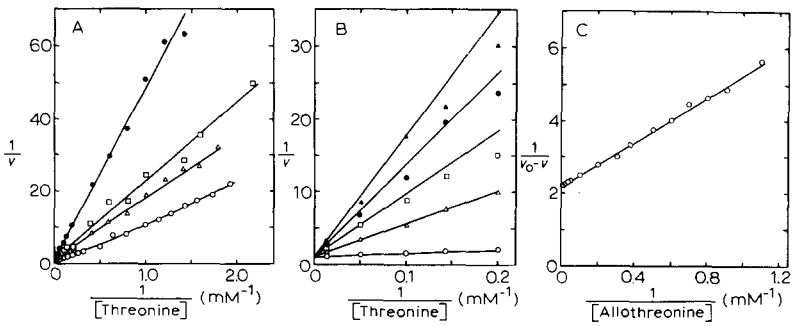



Fig. 3. Inhibition of the reaction by allothreonine. (A) Threonine varied over a wide range of concentrations with allothreonine at concentrations of: ○, none; △, 1.0 mM; □, 2.0 mM; ●, 5.0 mM. Data obtained in the absence and presence of allothreonine were fitted to Eqns 3 and 1, respectively. (B) Threonine varied over a range of higher concentrations with allothreonine at concentrations of: ○, none; △, 5.0 mM; □, 10.0 mM; ●, 15.0 mM; ▲, 20.0 mM. The data were fitted to Eqn 5. (C) Threonine fixed at a concentration of 5.0 mM. $v_0$ and $v$ represent initial velocities in the absence and presence of allothreonine, respectively. The data were fitted to Eqn 1. Velocity is expressed as μmoles/h per μg of protein.

TABLE IV

VALUES OF THE APPARENT KINETIC CONSTANTS OBTAINED IN THE PRESENCE OF ALLOTHREONINE

Values were obtained by fitting the data of Fig. 3A to Eqn 1.

| *Allothreonine concn (mM)* | *Kinetic parameter* | |
|---|---|---|
| | *V (μmoles/h per μg)* | *Apparent $K_m$ (mM)* |
| 1.0 | 1.00 ± 0.05 | 17 ± 1 |
| 2.0 | 0.87 ± 0.07 | 19 ± 2 |
| 5.0 | 1.16 ± 0.08 | 56 ± 4 |

an independent fashion. Additional evidence in support of this conclusion comes from measurements of the initial velocity as a function of the allothreonine concentration. Thus plots of the reciprocal of $(v_0 - v)$, where $v_0$ and $v$ represent the velocity in the absence and presence of allothreonine, respectively, against the reciprocal of the allothreonine concentration are linear (Fig. 3C) and the data give a good fit to Eqn 1.

*Inhibition of the reaction by isoleucine*

Isoleucine causes marked inhibition of the reaction catalysed by biosynthetic threonine dehydratase from many sources, including *R. spheroides*[17,20–25]. In an endeavour to determine if the kinetic data obtained in the presence of isoleucine could be described by an equation of the same general form as Eqn 3, the inhibition of the reaction by three different concentrations of the inhibitor was studied with about twenty concentrations of threonine over the same wide range as used for the initial velocity experiments (Fig. 2). From the results illustrated in Fig. 4, it is apparent that, in contrast to allothreonine which causes plots of $1/v$ against 1/[threonine] to become linear (Fig. 3A), isoleucine increases the curvature of such plots.

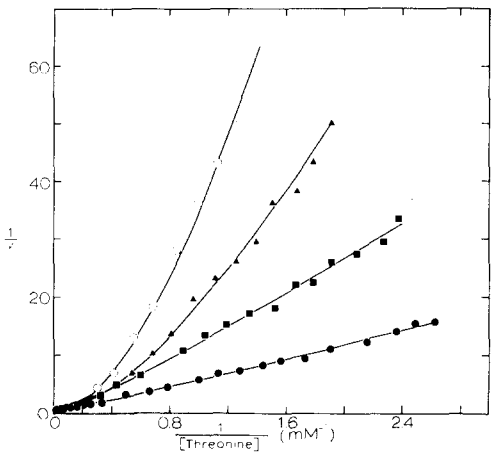



Fig. 4. Inhibition of the reaction by isoleucine. The concentrations of isoleucine were: ●, none; ■, 10 $\mu$M; ▲, 20 $\mu$M; ○, 30 $\mu$M. The velocities obtained in the presence of isoleucine are the initial steady-state velocities, and were determined as described under Methods. Data were fitted to Eqn 3 and velocity is expressed as $\mu$moles/h per $\mu$g of protein.

The results also show that, for each set of data, there is good agreement between the theoretical and experimental points while the data of Table II demonstrate the relatively low standard errors associated with the values for the various kinetic parameters. However, it should be noted that with isoleucine at a concentration of 30 $\mu$M, the value for $b$ is negative. Although the meaning of this constant can change in accordance with the details of the mechanism giving rise to Eqn 3, it must always be positive as it represents a combination of rate constants. Hence it must be concluded that Eqn 3 does not adequately describe the kinetics of the reaction in the presence of 30 $\mu$M isoleucine. It is conceivable that these data could give a good fit to the more complex equation

$$v = V\frac{(A^3 + bA^2 + cA)}{A^3 + dA^2 + eA + f} \tag{6}$$

where $b$, $c$, $d$, $e$ and $f$ represent combinations of rate constants. But from studies with theoretical data generated from Eqn 6 and into which random scatter has been introduced, it has been found that satisfactory analysis is obtained only when the accuracy of the initial velocities is at least 1% and this degree of accuracy cannot be achieved for the threonine dehydratase reaction. Therefore, tests of the above postulate have been precluded.

In spite of the aforementioned analytical difficulties, the data of Fig. 4 suggest that isoleucine does not affect the maximum velocity of the reaction and this suggestion is confirmed by the results obtained when the concentrations of threonine and isoleucine are varied in constant ratio (Fig. 5A). Thus, it may be concluded that both enzyme–threonine and enzyme–threonine–isoleucine complexes give rise to products at the same rate. By contrast, the maximum velocity of the reaction decreased from 0.76 $\pm$ 0.01 $\mu$mole/h per $\mu$g protein in the absence of allothreonine to 0.165 $\pm$ 0.005 $\mu$mole/h per $\mu$g protein when threonine and allothreonine were

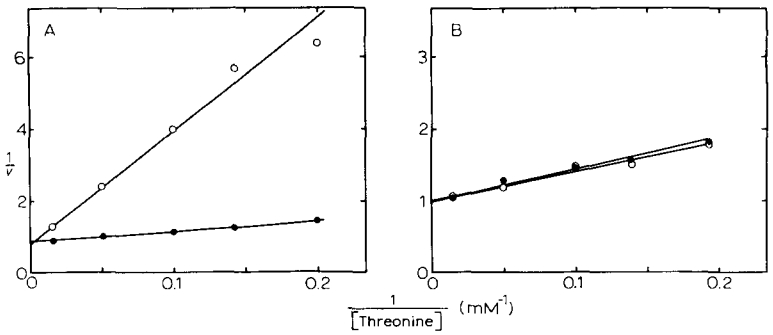



Fig. 5. Effect of isoleucine and valine on the maximum velocity of the reaction. (A) velocities were determined in the absence of isoleucine (●) and with threonine and isoleucine varied in a constant ratio of 72:1 (○). (B) velocities were determined in the absence of valine (●) and with threonine and valine varied in a constant ratio of 75:1. Each set of data was fitted to Eqn 1 and velocities are expressed as μmoles/h per μg of protein.

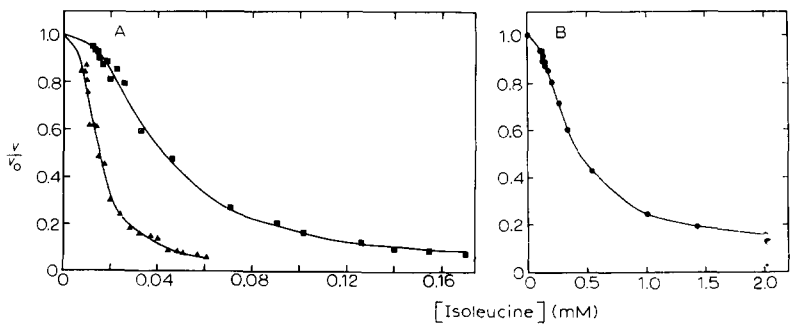



Fig. 6. Inhibition of the reaction by isoleucine at fixed concentrations of threonine. The concentrations of threonine were: ▲, 1.0 mM, ■, 5.0 mM; ●, 40.0 mM. In each case, the initial steady-state inhibited velocity was measured as described under Methods. The data obtained at 40.0 mM threonine (B) were fitted to Eqn 7. Velocity is expressed as μmoles/h per μg of protein.

varied in a constant ratio of 2:1. Such a result would be expected for a compound that behaves as a linear competitive inhibitor.

Fig. 6 illustrates the results obtained when the initial velocities were determined as a function of the isoleucine concentration at a fixed concentration of threonine. At all concentrations of threonine, the plots were sigmoidal and all sets of data gave curved plots of $1/(v_0 - v)$ against the reciprocal of the concentration of isoleucine where $v_0$ and $v$ represent velocities in the absence and presence of the inhibitor. The data for each fixed concentration of threonine were fitted to Eqn 7, where $V'$, $b$, $c$, $d$ and $e$ are combinations of rate constants, as well as functions of the threonine concentration, and $I$ represents the isoleucine concentration.

$$v = \frac{V'(I^2 + bI + c)}{I^2 + dI + e} \tag{7}$$

This equation is the simplest kinetic equation which can yield plots of the same general shape as those shown in Fig. 6, and for the analysis, a computer program, which allows for the inclusion of weighting factors equal to $1/v^2$, was developed. The data

obtained at 40 mM threonine fitted well to this equation giving the following values for the kinetic parameters: $V'$, 0.082 ± 0.001 μmole/h per μg protein; $b$, 1.60 ± 0.07 mM; $c$, 1.00 ± 0.05 mM²; $d$, 0.093 ± 0.007 mM; $e$, 0.081 ± 0.005 mM². However, attempts to fit the data obtained at 5 mM and 1 mM threonine were not successful as the residual sums of squares failed to converge to minimum values. It appears, therefore, that these data are not in accord with Eqn 7.

*Activation of the reaction by valine*

At lower concentrations, valine functions as an activator of threonine dehydratase[16,17,21,24] and in the presence of a sufficiently high concentration of this modifier (0.2 mM), linear double reciprocal plots of velocity as a function of substrate concentration are obtained (Fig. 7). From a fit of these data to Eqn 1, the maximum velocity and apparent $K_m$ values were determined to be 0.82 ± 0.02 μmole/h per μg protein and 3.7 ± 0.2 mM, respectively. Fig. 7 also indicates that valine has no significant effect on the maximum velocity of the reaction. As a further check on this point, velocities were determined over a limited range of higher threonine concentrations in the absence of valine and with the concentrations of threonine and valine varied in constant ratio. The results (Fig. 5B) show that, under the chosen

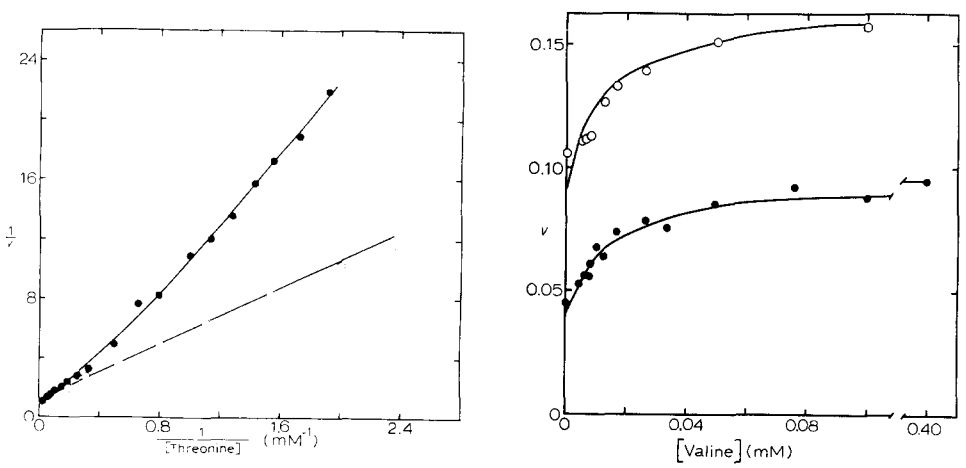



Fig. 7. Activation of the reaction by valine. The concentrations of valine were: ●, none; ○, 0.2 mM. Data obtained in the absence and presence of valine were fitted to Eqns 3 and 1, respectively. Velocity is expressed as μmoles/h per μg of protein.

Fig. 8. Effect of the concentration of valine on the initial velocity of the reaction at different fixed concentrations of threonine. The concentrations of threonine were: ●, 0.4 mM; ○, 0.7 mM. The data were fitted to Eqn 4. Velocity is expressed as μmoles/h per μg of protein.

experimental conditions, both double reciprocal plots are linear. Further, it is clear that valine causes little activation at higher threonine concentrations and has no effect on the maximum velocity. More detailed investigations of the activation by valine at two fixed concentrations of threonine gave the results illustrated in Fig. 8 which show that each set of experimental data gives a good fit to Eqn 4. Analysis of the data yielded the values for $V'$, $K_N$ and $K_D$ which are recorded in Table V. From studies of the activation by valine, it may be concluded that (a) only a single

TABLE V

VALUES OF THE APPARENT KINETIC CONSTANTS ASSOCIATED WITH THE ACTIVATION OF THE REACTION BY VALINE AT FIXED CONCENTRATIONS OF THREONINE

Values of the apparent kinetic constants were obtained by fitting the data of Fig. 8 to Eqn 4.

| *Threonine concn (mM)* | *Apparent kinetic parameter* | | |
|---|---|---|---|
| | *V' (μmole/h per μg* | *$K_N$ (mM)* | *$K_D$ (mM)* |
| 0.4 | 0.041 ± 0.003 | 0.006 ± 0.002 | 0.015 ± 0.003 |
| 0.7 | 0.094 ± 0.006 | 0.008 ± 0.002 | 0.013 ± 0.003 |

molecule of the modifier undergoes reaction with the enzyme or alternatively, multiple molecules react in an independent manner, (b) there is formed an enzyme–threonine–valine complex which gives rise to products at the same rate as does the enzyme–threonine complex.

At higher concentrations, valine was found to cause inhibition of the reaction as reported previously by Datta[21].

*Activation of the reaction by valine in the presence of isoleucine*

While evidence has been obtained for the partial reversal by valine of the inhibition of threonine dehydratase by isoleucine[18–21,24,25] it has not been established that the inhibition can be reversed completely by valine. Towards this end, the velocity of the reaction has been measured as a function of the concentration of valine at three fixed concentrations of threonine and isoleucine. The kinetics of the activation by valine at 5.0 mM threonine and 0.4 mM isoleucine are shown in Fig. 9. It will be noted that this modifier causes a considerable increase in the reaction velocity which is not observed when isoleucine is absent. The data gave a good fit to Eqn 4 (Fig. 9A) and plots of $1/(v - v_0)$ against 1/[valine] where $v$ and $v_0$ represent

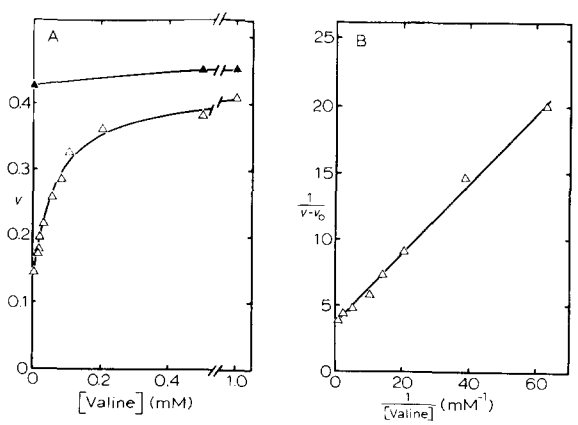



Fig. 9. Effect of the concentration of valine on the initial velocity of the reaction at a fixed concentration of threonine in the absence and presence of isoleucine. The concentration of threonine was 5.0 mM while the concentrations of isoleucine were: ▲, none; △, 0.4 mM. Data obtained in the presence of 0.4 mM isoleucine were fitted to Eqn 4 (A) or Eqn 1 (B). $v$ and $v_0$ represent initial velocities in the presence and absence of valine, respectively. Velocity is expressed as μmoles/h per μg of protein.

TABLE VI

VALUES OF THE APPARENT KINETIC CONSTANTS FOR THE ACTIVATION OF THE REACTION BY VALINE IN THE PRESENCE OF ISOLEUCINE

Values were obtained by fitting the data to Eqn 4, and the maximum velocity in the presence of saturating concentrations of valine ($V_m$) was calculated from the equation:

$$V_m = V' \frac{K_D}{K_N}$$

Velocity is expressed as μmole/h per μg of protein.

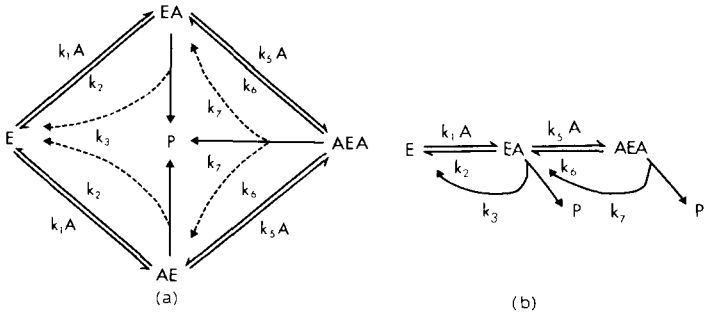
| *Threonine concentration (mM)* | *Isoleucine concentration (mM)* | *Velocity in absence of modifiers* | *Velocity in presence of isoleucine (V')* | *$K_N$ (mM)* | *$K_D$ (mM)* | *Maximum velocity ($V_m$)* |
|---|---|---|---|---|---|---|
| 5.0 | 0.025 | 0.45 | 0.260±0.008 | 0.018±0.003 | 0.031±0.005 | 0.45±0.07 |
| 5.0 | 0.04 | 0.45 | 0.137±0.008 | 0.021±0.004 | 0.064±0.010 | 0.42±0.09 |
| 40.0 | 0.5 | 0.67 | 0.142±0.02 | 0.36 ±0.12 | 1.66 ±0.43 | 0.66±0.20 |

the velocity in the presence and absence of valine, respectively, are linear (Fig. 9B). Similar results were obtained under two other sets of conditions and the values determined for the various kinetic parameters are recorded in Table VI. These values were substituted into the relationship, $V_m = V'(K_D/K_N)$, which is derived from Eqn 4 for the condition that $M$ approaches infinity, to calculate the apparent maximum velocity of the reaction at a fixed concentration of threonine in the presence of a saturating concentration of valine. Since the calculated maximum velocity values are similar to those obtained in the presence of threonine alone, it may be concluded that valine can reverse completely the inhibition by isoleucine.

In contrast to the above finding, no activation of the reaction by valine was observed in the presence of allothreonine.

## DISCUSSION

The purpose of the present investigation was to obtain extensive, accurate kinetic data for the reaction catalysed by threonine dehydratase in the absence and presence of modifiers and to determine if these data could be fitted to rate equations that describe mechanisms involving the interaction of two molecules of substrate and/or modifier with the enzyme. To simplify the study, all initial velocities were measured at a fixed concentration of pyridoxal phosphate which was sufficiently high so as to convert the free enzyme completely into an enzyme–pyridoxal phosphate complex. The results show that while some sets of kinetic data are consistent with the relatively simple mechanisms considered, others are not. But the latter finding does not necessarily invalidate the concept that the kinetic effects observed with allosteric enzymes can be explained in terms of classical steady-state kinetic theory. It is possible that the rate equations for the various reactions are, in fact, of a complexity which precludes their use at the present time. An important finding which arises from the work is that the fitting of experimental data to rate equations which are the ratio of cubic polynomials requires data of an accuracy that cannot be achieved with the available analytical techniques. In spite of these difficulties, it has been possible to



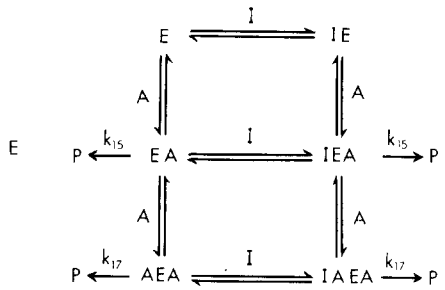

Scheme I

reach a limited number of conclusions about the mechanisms of the threonine dehydratase reaction and these are enumerated below.

As the initial velocity data of Fig. 2 give a good fit to Eqn 3, it may be concluded that they are in accord with the idea that at pH 7.4 there is an interdependent addition of two molecules of threonine to the enzyme which occurs either *via* a random, rapid equilibrium (Scheme Ia) or ordered, steady-state (Scheme Ib) mechanism involving product formation from both binary (*EA*, *AE*) and ternary (*AEA*) enzyme–substrate complexes. Mechanisms of these types have been discussed by Schramm and Morrison[7] who have derived the initial rate equations and shown them to have the same form as Eqn 3. The conclusion about the mechanism does not preclude the possibility that more than two molecules of substrate can react with threonine dehydratase, but it does imply that only two kinetically-distinct enzyme–substrate complexes are formed. Since the data did not fit to Eqn 2 the possibility that only ternary (*AEA*) complexes give rise to products can be eliminated. In this respect, threonine dehydratase from *R. spheroides* appears to differ from the same enzyme from *Bacillus subtilis*, where the initial velocity data obtained in the presence of isoleucine were found to be consistent with Eqn 2[25]. At pH 9.0, the reaction conforms to Michaelis–Menten kinetics and hence, under these conditions, independent binding of substrate must occur.

The initial velocity data obtained in the presence of isoleucine could also be fitted to Eqn 3. Therefore it might be concluded that the random, rapid equilibrium mechanism is applicable so that the form of the rate equation with substrate as the variable reactant does not change when allowance is made for the formation of enzyme–inhibitor complexes. However, the negative value for b with isoleucine at a concentration of 30 $\mu$M (Table II) suggests that the correct rate equation may, in fact, be more complex and approximates to Eqn 3 at lower isoleucine concentrations. The kinetics for the reaction with respect to substrate can become more complex when an inhibitor is present. Thus, if a single inhibitor molecule were to react with each enzyme species of Scheme Ib to give the mechanism illustrated in Scheme II and if the reactions were to occur under steady-state conditions, then the initial rate equation becomes the ratio of 4th power polynomials in substrate concentration (Eqn 8), where $a, b \cdots\cdots i$ represent combinations of rate constants and the inhibitor concentration.

$$v = \frac{aA^4 + bA^3 + cA^2 + dA}{eA^4 + fA^3 + gA^2 + hA + i} \tag{8}$$




Scheme II

In the absence of inhibitor, Eqn 8 would reduce to Eqn 3, and with inhibitor as the variable reactant would take the general form

$$v = \frac{jI^3 + kI^2 + lI + m}{nI^3 + oI^2 + pI + q} \tag{9}$$

where $j$, $k \cdots\cdots q$ are each a function of certain rate constants as well as the substrate concentration. Consequently, it follows that the results shown in Fig. 6 do not necessarily mean that multiple molecules of isoleucine undergo interdependent reactions with threonine dehydratase. Eqns 8 and 9 would yield curves of the same general form as those shown in Figs 4 and 6, respectively, but as mentioned above, it has not been possible to fit the data to equations in which the variable reactant is raised to the third or fourth power.

The data of Fig. 4 could be interpreted to indicate that isoleucine competes with threonine at the catalytic sites on the enzyme. However, as the maximum velocity of the reaction is not decreased by isoleucine at an infinite concentration, it follows that (a) threonine and isoleucine combine at separate, distinct sites on the enzyme so that both amino acids can be present at the same time, (b) the rate of product formation from enzyme–threonine and enzyme–threonine–isoleucine complexes is the same. Such conclusions are in accord with the proposals of Scheme II and the reports of studies on the enzymes from other sources[6,19,20], but they are at variance with the suggestion that enzyme complexes containing isoleucine are inactive[25,26]. It appears probable that isoleucine causes inhibition of threonine dehydratases by virtue of its ability to reduce the binding of threonine.

The kinetic effects of isoleucine and valine are similar in that, at saturating concentrations, neither compound affects the maximum velocity of the reaction (Fig. 5). But in contrast to isoleucine, valine functions as an activator and eliminates the interaction between substrate binding sites (Fig. 7). Further, if multiple molecules of valine interact with the enzyme, they must do so in an independent manner. These results are consistent with the postulate that valine and threonine combine at separate sites on the enzyme and the question then arises as to whether valine and isoleucine react at the same or different[19,25] modifier sites. The facts that these two amino acids exhibit structural similarities and that the inhibition by isoleucine can be reversed completely by valine (Table VI) might appear to argue in favor of their combination with a single type of modifier site. However, kinetic experiments cannot distinguish between the two possibilities and such information must come from binding studies. These were not attempted as the enzyme preparation was not pure.

While allothreonine competes with threonine for the active site(s) on the enzyme, this may not be the only interaction between allothreonine and the enzyme because this substrate analogue also causes non-linear initial velocity plots to become linear (Fig. 3A). Therefore it is possible that allothreonine can also combine at a modifier site and eliminate the interdependent reaction of multiple molecules of substrates with the enzyme. This conclusion is consistent with the explanation offered for the activation of the enzyme by relatively low concentrations of allothreonine[12].

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