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THREONINE DEHYDRATASE OF *BACILLUS LICHENIFORMIS*

I. PURIFICATION AND PROPERTIES

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SUMMARY

Threonine dehydratase (threonine hydro-lyase (deaminating), EC 4.2.1.16) has been purified about 50-fold from extracts of *Bacillus licheniformis* by heat treatments, ammonium sulfate fractionation, and gel filtration. The purified enzyme exhibits properties similar to the enzyme in crude extracts.

The enzyme is stabilized in 1 M potassium phosphate at pH 8.0 by pyridoxal phosphate and L-isoleucine against denaturation at 0° and against heat inactivation. L-Threonine protects the enzyme against inactivation by dilution.

L-Threonine and L-serine can serve as substrates, but threonine is deaminated 5–8 times more rapidly than the other substrate. The pH optimum of the reaction is between 9.0 and 9.5.

The rate of reaction at increasing substrate concentration shows a slightly sigmoidal curve with an apparent K_m of $3 \cdot 10^{-3}$ M. L-Isoleucine inhibits the enzyme to about 50% at a threonine to isoleucine ratio of 1000:1. This inhibition is pH dependent and is overcome in part by L-α-aminobutyrate, although by itself L-α-aminobutyrate does not show any activating effect.

The treatment of the enzyme with $HgCl_2$ results in partial inactivation, but the remaining enzyme activity is completely insensitive to the inhibition by L-isoleucine. The extent of inactivation of the enzyme by urea is dependent on the urea concentration and the temperature.

INTRODUCTION

The pyridoxal phosphate requiring enzyme threonine dehydratase (or threonine deaminase, threonine hydro-lyase (deaminating), EC 4.2.1.16) catalyses the dehydration and deaminination of L-threonine to α-ketobutyrate, ammonia and water. UMBARGER[1] made the important discovery that L-isoleucine, which is the end product

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of the biosynthetic sequence, regulates the activity of the enzyme by end product inhibition in *Escherichia coli*. This observation has since been made in a variety of different microorganisms[2–10]. UMBARGER AND BROWN[11] found that two different threonine dehydratases were synthesized in *E. coli*. Under aerobic conditions in a minimal salts medium the enzyme synthesized was controlled by L-isoleucine feedback inhibition and called the biosynthetic threonine dehydratase, but in a rich medium containing amino acids under anaerobic conditions the enzyme synthesized was insensitive to L-isoleucine feedback, stimulated by AMP and was termed the biodegradative threonine dehydratase. In all other organisms investigated so far, only one of these enzymes is found. Evidence has accumulated that the biosynthetic *E.coli* enzyme possesses separate binding sites for the substrate, the inhibitor and certain activators[12]. This enzyme is believed to be an active polymer consisting of subunits[12].

Most of the reports in the literature on the biosynthetic threonine dehydratase are concerned with the enzyme in *E. coli*[1,2,11–24], *Salmonella typhimurium*[4,25–27], *Saccharomyces cerevisiae*[5,28,29] and photosynthetic bacteria[6,8,10,30]. Only one report was published for the Bacillus enzyme[3]. Since it is evident now, that Bacillus is different from *E. coli* in several aspects and since the role of allosteric interactions during sporulation is not clear, we felt that an investigation of the enzyme in this genus would be important.

This paper reports the purification of threonine dehydratase from *Bacillus licheniformis*. Data are presented on the stability of the enzyme, its kinetic properties, and the effect of $HgCl_2$ and urea on the enzyme activity. The following paper reports the regulation of the enzyme during development[31].

EXPERIMENTAL PROCEDURE

Materials

Allo-free L-threonine, L-α-ketobutyric acid and pyridoxal phosphate were purchased from Sigma. Other amino acids were obtained from either Sigma or Calbiochem. The 2,4-dinitrophenylhydrazine was a product of Eastman and Sephadex gel was purchased from Pharmacia. All other chemicals were of the highest purity available and were obtained from either Fisher, Baker or Mallinckrodt.

Methods

Media and growth conditions. *B. licheniformis* A-5 was grown on the minimal salts medium previously described[32]. Cells were grown at 37° in either 1-l lots with shaking or in 15-l lots under forced aeration. With 20 mM glucose the cells reached the end of growth after 4 to 5 h with a generation time of about 75 min (ref. 31).

Preparation of extract. Cells were grown to near the end of the logarithmic growth phase, sedimented at 12 000 × *g* in a Servall centrifuge at 3° and washed once with 0.15 M NaCl. The washed cells of a 1-l culture were suspended in 10 ml of 1.0 M potassium phosphate buffer at pH 8.0, containing 10^{-3} M L-isoleucine and 10^{-4} M pyridoxal phosphate (hereafter called stabilizing buffer), or in 10 ml of water where indicated. The cells were disrupted by sonic oscillation for 3.5 min in a 9.0-kcycle Ratheon sonic oscillator and then centrifuged at 40 000 rev./min in a Spinco model L centrifuge for 60 min at 3°. The clear supernatant solution was filtered through a column of Sephadex G-100, equilibrated and eluted with 0.25 M potassium phosphate

buffer at pH 8.0. The active fraction was used as the enzyme in crude extract. For enzyme purification approx. 75 g of cells (wet weight) were obtained from a 15-l cell culture after the addition of crushed ice and continuous flow centrifugation in a Lourdes centrifuge at 4°. The sedimented cells were suspended in 150 ml of stabilizing buffer and extracted twice through a cooled Aminco-French pressure cell held at 10 000 lb/inch^2. The broken cell suspension was subjected to sonic vibration as above in 30-ml lots for 3 min to disrupt the nucleic acids. The extract was clarified by centrifugation in a manner similar to that used above.

Enzyme assay. The enzyme activity was estimated from the amount of α-ketobutyrate formed by a modification of the procedure of FRIEDEMANN AND HAUGEN[33]. The final volume of the standard reaction mixture was 0.3 ml and contained potassium phosphate at pH 9.2, 60 μmoles; L-threonine, 6 μmoles; and the appropriate amount of enzyme diluted with 0.2 M potassium phosphate buffer at pH 8.0. The enzyme solution was filtered through a 1.4 cm × 5 cm Sephadex G-100 column, equilibrated and eluted with 0.25 M potassium phosphate at pH 8.0, just before assay to remove all small molecular weight substances and the isoleucine contained in the stabilizing buffer. Control tubes contained all components except L-threonine. The reaction time was 10 min at 37° and the reaction was stopped by adding 0.1 ml of 40% trichloroacetic acid. After the addition of 0.4 ml of 0.1% 2,4-dinitrophenylhydrazine in 2 M HCl the mixture was further incubated for 10 min at 37°. Then 0.8 ml of absolute ethanol and 2.5 ml of 2.5 M NaOH were added followed by vigorous shaking. The absorbance of the solution was read immediately in a Zeiss PMQ II spectrophotometer at 515 mμ, and the amount of keto acid formed was calculated using a standard curve obtained with authentic L-α-ketobutyrate. One unit of activity is that amount of enzyme that produced 1 μmole of α-ketobutyrate in 1 min in the standard reaction mixture. Specific activity is defined as units of enzyme activity per mg of protein. The protein was estimated by the method of LOWRY *et al.*[34].

RESULTS AND DISCUSSION

Enzyme purification

1. *Extract.* The crude extract from 75 g of cells was prepared as described in *Methods*.

2. *Heat treatment at 55°.* The clear supernatant solution was distributed into 40-ml lots and exposed to 55° in a water bath. After 10 min with occasional swirling the solutions were cooled rapidly in an ice bath and the precipitated material was removed by centrifugation in a Servall RC-2B centrifuge at 45 000 × *g* for 15 min at 3°.

3. *Heat treatment at 65°.* The supernatant fluid from the 55° heat treatment was subjected to 65° for 10 min and the supernatant solution obtained as above.

4. *Fractionation with ammonium sulfate.* Ammonium sulfate (25.0 g) was added to the 65° supernatant fraction (100 ml) at 4° to give a saturation of about 40%. The mixture was stirred continuously during the addition and after a total time of 30 min the suspension was centrifuged at 30 000 × *g* for 15 min. To the supernatant fluid 5.5 g of ammonium sulfate was added to produce about 48% saturation. After stirring for 30 min, the precipitated protein was obtained by centrifugation.

5. *Heat treatment at 75°.* The precipitated protein was dissolved in a small volume of stabilizing buffer and exposed to 75° for 10 min. After rapid cooling the supernatant

fluid was obtained as in the previous heat steps and filtered through a Sephadex G-100 column or stored at 0°. It was important to use a concentrated protein solution (>5 mg/ml) for this treatment at 75° to retain the enzyme activity.

6. *Sephadex G-100 filtration.* Samples (0.5 ml) of the clear supernatant solution obtained after centrifugation were passed through a column (2.4 cm × 6 cm) of Sephadex G-100. The column was equilibrated and eluted with 0.25 M potassium phosphate at pH 8.0. The fraction containing the enzyme was kept at 0° and used immediately for analysis since the enzyme was unstable without isoleucine and pyridoxal phosphate. The specific activity of the purified enzyme was about 22 μmoles/min per mg of protein, representing a purification of about 50-fold over the starting material. A summary of the purification is given in Table I.

TABLE I

PURIFICATION OF THREONINE DEHYDRATASE

Fraction	*Volume (ml)*	*Units*		*Protein mg/ml*	*Specific activity (units/mg)*
		per ml	*Total*		
1. Spinco supernatant fluid	143	11.2	1600	26.5	0.42
2. First heat treatment, 55°	113	11.7	1322	18.1	0.65
3. Second heat treatment, 65°	98	10.8	1058	9.6	1.13
4. Ammonium sulfate, 40–48%	17	53.5	907	7.9	6.74
5. Third heat treatment, 75°	15	52.0	780	3.7	14.05
6. Sephadex G-100 filtration	30	23.0	690	1.05	21.90

The unstable nature of the enzyme demanded that the purification be carried out immediately after cell harvest. Storage of the enzyme was possible only after a cell-free extract was obtained in the presence of isoleucine and pyridoxal phosphate. After Sephadex filtration the half-life of the enzyme was about 5 h at 0° in 0.2 M potassium phosphate at pH 8.0. The use of column chromatography was not possible, due to the requirement for a high ionic strength environment by the enzyme.

The 50-fold purified enzyme appeared in a single fraction when sedimented in a sucrose gradient and coincided with the only protein fraction, indicating a fair degree of purity[31].

Properties of enzyme

1. *Stability of enzyme.* Threonine dehydratase was unstable when stored in sedimented whole cells as well as in crude extracts and in the purified form under a variety of experimental conditions. Cells from 2 ml of cell culture kept at −20° lost about 50% of their enzyme activity every 24 h The half-life of the enzyme activity in cells stored in larger lots increased somewhat.

In crude extracts prepared with water, the enzyme was inactivated rapidly with a half-life of about 2 h. The activity could be preserved with increasing concentrations of potassium phosphate at pH 8.0 (Fig. 1). A 1.0 M solution of this buffer increased the half-life of the enzyme about 7-fold. The stabilizing effect of high ionic strength solutions had also been found for the enzyme in crude extracts of other systems[5,16–18]. The enzyme activity in crude extracts was dependent on the hydrogen ion concen-

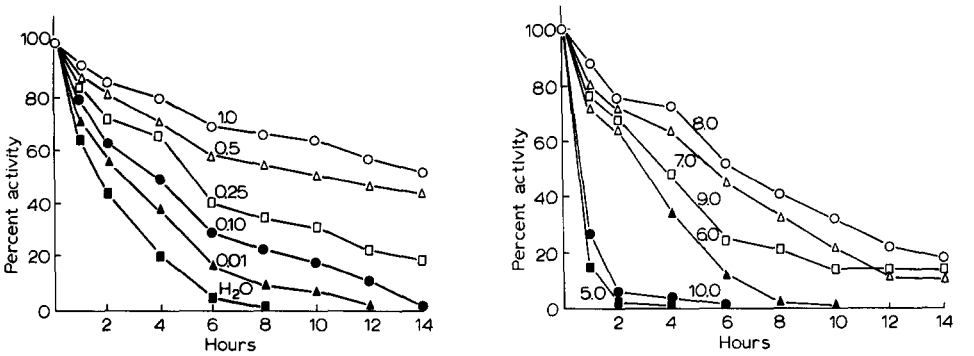


Fig. 1. Effect of increasing ionic strength on the enzyme stability. Aqueous crude extract (24 mg protein per ml) was added to an equal amount of potassium phosphate to yield the concentrations indicated at pH 8.0. Samples were removed, diluted 1:20 and assayed as described under *Methods*. The 100% activity equalled 0.42 μmole α-ketobutyrate per 10 min.

Fig. 2. Effect of pH on the enzyme stability. Aqueous crude extract (24 mg protein per ml) was adjusted to the indicated pH values with an equal amount of 0.2 M potassium phosphate or 0.2 M Tris. For assay see Fig. 1.

tration and was most stable at pH 8.0. Both high and low pH values resulted in rapid loss of enzyme activity (Fig. 2).

The enzyme in both the crude extract and in the purified form rapidly lost activity at temperatures of 25° and 37°. Temperatures of 0° and below prolonged the activity of the enzyme. These results are at variance with the data obtained for the enzyme from *S. typhimurium*[4], *Rhodospirillum rubrum*[6], and *Bacillus subtilis*[35] since these enzymes were fairly stable at room temperature or 37° but quite labile at 0°. The *B. subtilis* enzyme lost the cold sensitivity after partial purification and became more stable at 0° (ref. 35).

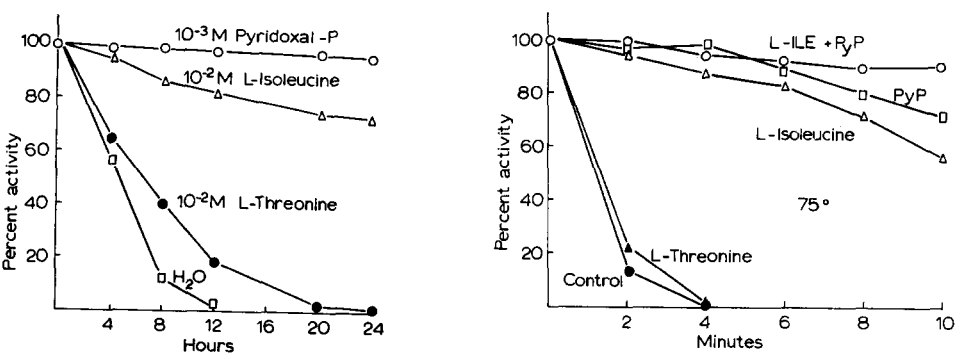


Fig. 3. Effects of substrate and inhibitor on the enzyme activity. The substances listed were added at double strength to the same volume of purified enzyme (2.0 mg protein per ml). Samples were removed and filtered through Sephadex G-100 (see *Methods*), diluted to a total of 1:40 and assayed as described under *Methods*. The 100% activity equalled 0.66 μmole α-ketobutyrate per 10 min. Enzyme in crude extract diluted 1:40 gave similar results.

Fig. 4. Protection of enzyme against heat denaturation. Purified enzyme (2.0 mg protein per ml) equilibrated with $2 \cdot 10^{-2}$ M amino acid or $2 \cdot 10^{-3}$ M pyridoxal phosphate (PyP) was exposed to 75°. Samples were removed and cooled rapidly. The samples were filtered, diluted and assayed as in Fig. 3. The 100% activity equalled 0.58 μmole α-ketobutyrate per 10 min. Enzyme in crude extract diluted 1:40 gave analogous results.

The enzyme was protected (half-life > 4 days) against inactivation at 0° by the addition of pyridoxal phosphate and L-isoleucine to the cell-free extract (Fig. 3). The substrate L-threonine or any other amino acid tested gave no significant protection. The enzyme from *E. coli*[2] and *S. typhimurium*[4] are also protected by L-isoleucine, but the yeast enzyme is not[29]. The yeast enzyme was protected by pyridoxal phosphate[29]. The threonine dehydratase of *B. licheniformis* was protected against heat inactivation by the same substances that preserved its activity at lower temperatures, namely, L-isoleucine and pyridoxal phosphate (Fig. 4). L-Threonine or any other amino acid as well as nucleotides failed to protect the enzyme.

TABLE II

INACTIVATION AND PROTECTION OF THE ENZYME FOLLOWING DILUTION

Enzyme in crude extract with a specific activity of 0.34 was diluted and kept at 0° with the additions indicated. After 4 h all samples were diluted to a total of 1:100, filtered through Sephadex G-100 (see *Methods*) and measured in the standard assay (see *Methods*).

Dilution	*Addition*	*Concn. (M)*	*Activity after 4 h (% of initial activity)*
—	—	—	59
1:10	—	—	49
1:25	—	—	39
1:100	—	—	19
1:100	L-Threonine	10^{-2}	78
1:100	L-Isoleucine	10^{-2}	52
1:100	L-Valine	10^{-2}	51
1:100	L-Leucine	10^{-2}	52
1:100	Pyridoxal phosphate	10^{-3}	30

The loss of enzyme activity following dilution has been investigated for the yeast enzyme[5]. The *B. licheniformis* enzyme diluted with 0.2 M potassium phosphate at pH 8.0 at 0° lost activity depending on the extent of dilution (Table II). Pyridoxal phosphate protected the concentrated enzyme from inactivation but had no influence on the loss of activity by the diluted enzyme. Only L-threonine had some protective effect and to a lesser extent all amino acids if added at high concentrations (Table II). This latter protection was probably unspecific.

It became quite apparent that the time of sonication of the cell samples was crucial. Small samples of 2 ml reached their highest enzyme activity after 20 sec of sonication and lost all their activity after 60 sec of sonication. For cells from 1-l cultures suspended in 10 ml the optimal time of sonication was 3.5 min. The suspending solution used was important since additional sonication for 5 min resulted in 50% loss of activity in an aqueous solution and only in 10% loss of activity in the stabilizing buffer. In all experiments the sonication time that resulted in optimal activity was used.

2. *Substrate specificity.* L-Threonine and L-serine can serve as substrates for the enzyme, but the activity for L-threonine was 5–8 times greater than for L-serine substrates. The enzyme in *E. coli*[36], Neurospora[37], yeast[23], and *Clostridium tetanomorphum*[38] also deaminated L-threonine more rapidly than L-serine. D-Isomers of threonine and serine did not serve as substrates in *B. licheniformis* in accordance with all other reports.

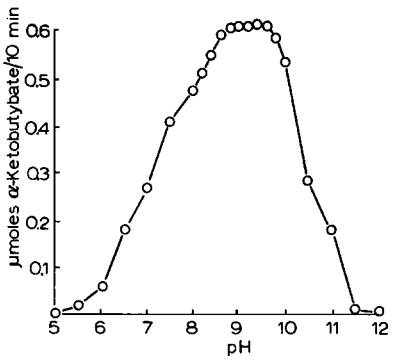


Fig. 5. The pH profile for the enzyme. Aqueous crude extract (23 mg protein per ml) diluted 1:40 with water was immediately added to the standard assay system (see *Methods*) that was adjusted to the indicated pH values with 0.2 M potassium phosphate or 0.2 M Tris. Purified enzyme diluted 1:50 gave similar results.

3. *Optimal assay conditions.* The enzyme reaction required an alkaline pH and the pH optimum was between pH 9.0 and 9.5 in potassium phosphate or Tris–phosphate buffer (Fig. 5). The rate of product formation was the same in either buffer. At the pH of 9.2 used for the standard assay the sensitivity of the enzyme toward L-isoleucine was greatly reduced (see Fig. 9). Although threonine dehydratase is a pyridoxal phosphate enzyme, the addition of the co-enzyme did not cause any significant increase in activity, presumably due to a firm binding of pyridoxal phosphate to the enzyme (Table III). Even the partially purified enzyme showed only a slight stimulation after pyridoxal phosphate addition. In other systems the pyridoxal phosphate requirement can be shown after extensive dialysis[38] or after partial purification[10,29]. Experiments with hydroxylamine-treated extracts indicated that the enzyme required one molecule of pyridoxal phosphate[11]. The addition of β-mercaptoethanol, glutathione or EDTA did not stimulate the enzyme from *B. licheniformis* (Table III).

4. *Kinetics with threonine as substrate.* The response of the enzyme to increasing

TABLE III

EFFECT OF PYRIDOXAL PHOSPHATE AND REDUCING AGENTS ON ENZYME

Enzyme in crude extracts with a specific activity of 0.44 and in the partially purified form with a specific activity of 16.8 were diluted 1:50 and the activity measured in the standard assay (see *Methods*) containing the substances listed.

Addition	*Concn. (M)*	*% Activity*	
		Crude extract	*Purified enzyme*
—	—	100	100
Pyridoxal phosphate	10^{-5}	98	102
Pyridoxal phosphate	10^{-4}	104	108
Pyridoxal phosphate	10^{-3}	97	112
β-Mercaptoethanol	10^{-5}	101	110
EDTA	10^{-4}	95	101
Glutathione	10^{-4}	96	98

substrate concentrations was the same for enzyme in crude extract and in the partially purified form (Fig. 6). The apparent K_m value for threonine was $3 \cdot 10^{-3}$ M. The shape of the curve did not follow strict Michaelis kinetics and it has been proposed that the substrate itself acts as a modifier for the enzyme[39]. If the initial velocities are plotted by the method of LINEWEAVER AND BURK[40] the line obtained is not linear even when plotted as $1/v$ *vs.* $1/[S]^2$ (plots not shown). Similar results were obtained for the *E. coli* enzyme[16–18] and it was concluded that the action of the enzyme was not strictly bimolecular. The enzyme from *S. typhimurium*[4], yeast[29], *E. coli*[12], and *Rhodopseudomonas spheroides*[10] gave normal Michaelis kinetics when L-valine was added, or when ADP was added to the *C. tetanomorphum* biodegradative enzyme[38]. The enzyme of *B. licheniformis* was not affected by L-valine or by nucleotides but slightly by L-aspartic acid. The enzyme of *S. typhimurium* investigated by other workers[27] was also found not to be stimulated by L-valine.

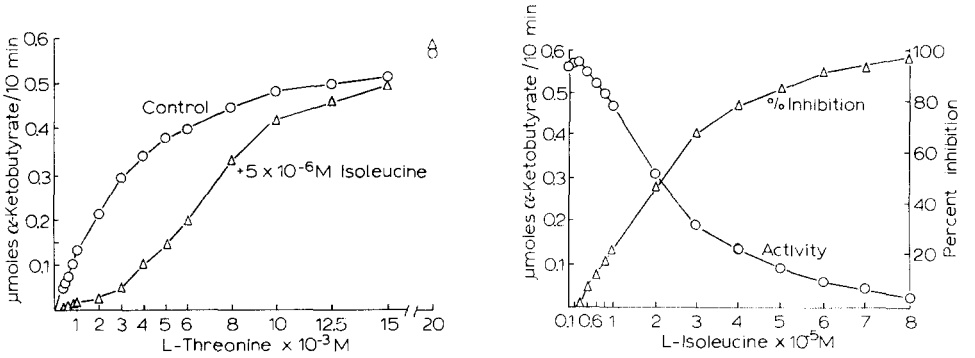


Fig. 6. Isoleucine effect with increasing substrate concentration. Crude extract (26 mg protein per ml) was diluted 1:50 with aqueous $5 \cdot 10^{-6}$ M L-isoleucine. Samples were added to the standard assay (see *Methods*), containing the indicated substrate concentration. Purified enzyme diluted 1:50 gave comparable results.

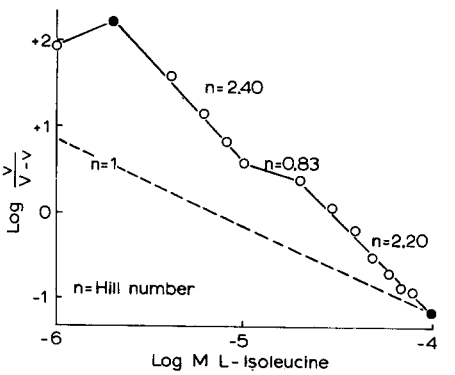
Fig. 7. Effect of increasing L-isoleucine concentration. Purified enzyme (2.0 mg protein per ml) was diluted 1:50 and samples added to the standard assay (see *Methods*) containing the L-isoleucine concentrations indicated. Enzyme in crude extract diluted 1:40 gave similar results. The % inhibition curve was calculated from the activities shown.

The Hill plot has been used to examine the effects that can occur between catalytic sites of the enzyme in *E. coli*[12,39]. The slope of the line obtained when $\log v/v_{max}-v$ is plotted against $\log[S]$ has been interpreted as an indication of the order of the reaction[41]. A Hill plot (not shown) of data obtained for the deamination at different concentrations of threonine had a slope of 1.12, indicating that the number of interacting sites is greater than 1. We do not know whether the slight variation of the substrate saturation curve from Michaelis kinetics and the consequent deviation of the slope of the Hill plot from unity is significant. Calculations[26] made for the enzyme from *S. typhimurium* showed that the slope of the Hill plot was equal to 1.0 if the inactivation of the enzyme during the assay time was considered, and that the assumption[4,12] of two interacting sites for the binding of L-threonine was not justified. This explanation cannot be used in the case of *B. licheniformis* since the inactivation of the enzyme during the 10-min assay in the presence of the substrate was negligible.

5. *Inhibition of enzyme by* L-*isoleucine.* The biosynthetic threonine dehydratase from many bacterial species is subject to end product inhibition by L-isoleucine[1-6, 8-10,27,30]. The inhibition by L-isoleucine of the *B. licheniformis* enzyme (Fig. 6) was quite specific, since all other amino acids tested did not inhibit the reaction at all or only at a 1000-fold higher concentration in the cases of L-leucine and L-valine. This latter inhibition was possibly due to isoleucine contamination. The effect of L-isoleucine was dependent upon substrate concentration and changed the apparent K_m, but not the v_{max}. This is an example of an allosteric K enzyme in which both the substrate and the effector are allosteric ligands[39]. The effect of increasing L-isoleucine concentration with a constant amount of substrate on the enzyme activity is shown in Fig. 7. At a substrate to inhibitor ratio of 10 000:1 the enzyme activity was inhibited to 50% (Figs. 6 and 7). The substrate to inhibitor ratio resulting in 50% inhibition held for a substrate concentration ranging from $2 \cdot 10^{-3}$ to $5 \cdot 10^{-2}$ M.

A double reciprocal plot of the data in Fig. 7 showed that the line obtained was not linear (plot not shown). A Hill plot of the same data gave a value for the slope of the best straight line equal to 2.0, although a line with 3 different slopes could be drawn (Fig. 8). The limitations of the Hill plots have been discussed[41] and all that is suggested from the Hill plot (Fig. 8) is that at least 2 molecules of isoleucine bind per enzyme molecule.

The extent of inhibition by L-isoleucine was dependent on the H^+ concentration (Fig. 9). At the lower pH values the inhibition was greatly enhanced and disappeared completely at a pH value of 10.5. CHANGEUX[16-18] has determined the affinity of L-isoleucine to the enzyme by a method that depends on the degree of protection of the enzyme against heat denaturation and found that the degree of inhibition with changing pH could be correlated with the affinity of isoleucine for the enzyme. The pH-dependent isoleucine effect indicated that the binding of isoleucine involves active groups of the enzyme that are independent of the catalytic site, since the enzyme is still active at pH 10.5, but insensitive to isoleucine inhibition. In addition to the stimulation



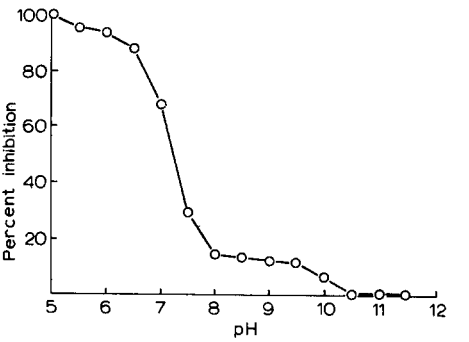


Fig. 8. Hill plot for L-isoleucine. The data from Fig. 7 are plotted as log $v/v_{max} - v$ *versus* log of the isoleucine concentration. v_{max} equalled 0.6 μmole α-ketobutyrate.

Fig. 9. Effect of pH on the isoleucine inhibition. Crude extract (26 mg protein per ml) was diluted 1:50 with aqueous 10^{-5} M L-isoleucine. Samples were added to the standard assay (see *Methods*) with pH values as indicated, adjusted with 0.2 M potassium phosphate or 0.2 M Tris. The enzyme activity varied at different pH values (see Fig. 5). At pH 9.2, 0.64 μmole α-ketobutyrate per 10 min were produced. Purified enzyme diluted 1:50 gave comparable results.

of the enzyme by L-valine, as discussed before, this amino acid can also reverse the isoleucine inhibition in cell-free extracts of several microorganisms[4,10,12,29]. This effect was negligible for the *B. licheniformis* enzyme. L-α-Aminobutyric acid partially overcame the isoleucine inhibition in *B. licheniformis* extracts at low substrate concentrations similar to the valine effect in other systems. This observation had also been made for the *B. subtilis* enzyme[3]. However, L-α-aminobutyrate did not stimulate the enzyme in the absence of isoleucine.

6. *Desensitization.* Selective inactivation of the inhibitor site has been achieved for the enzyme from *E. coli* by heat treatment at 55° (ref. 2) and is called desensitization. Attempts to desensitize the *B. licheniformis* enzyme by this method were unsuccessful since both the inhibitory and catalytic functions of the enzyme decreased to the same extent. The same observation was made for the yeast enzyme[29]. Mercurials desensitize the enzyme and it has been postulated[2,4] that blocking of –SH groups of the enzyme that are part of the isoleucine binding site cause this desensitization. The effect of $HgCl_2$ on the *B. licheniformis* enzyme is shown in Fig. 10. At low concentrations of $HgCl_2$ the enzyme is fully active and still quite sensitive to isoleucine inhibition. However, at a $HgCl_2$ concentration that inhibits the enzyme about 50% (10^{-6} M), the enzyme is completely insensitive to isoleucine inhibition. This concentration of $HgCl_2$ also prevented the protection by isoleucine, since 10^{-6} M $HgCl_2$ when added to the enzyme either in the presence or absence of L-isoleucine, showed the same degree of heat inactivation (Fig. 11). This was indicative of the same binding site for isoleucine and $HgCl_2$ as pointed out by other authors[4].

The inactivation by Hg^{2+} of the enzyme of *R. spheroides*[10] is partially reversed by the addition of β-mercaptoethanol and the enzyme showed identical kinetic behavior to that of the native enzyme. This reactivation was not observed for the *B. licheniformis* enzyme even when β-mercaptoethanol was added simultaneously with $HgCl_2$. The enzyme probably dissociates or alters its conformation irreversibly.

7. *Effect of urea.* CHANGEUX[12] reported the inactivation of the *E. coli* threonine

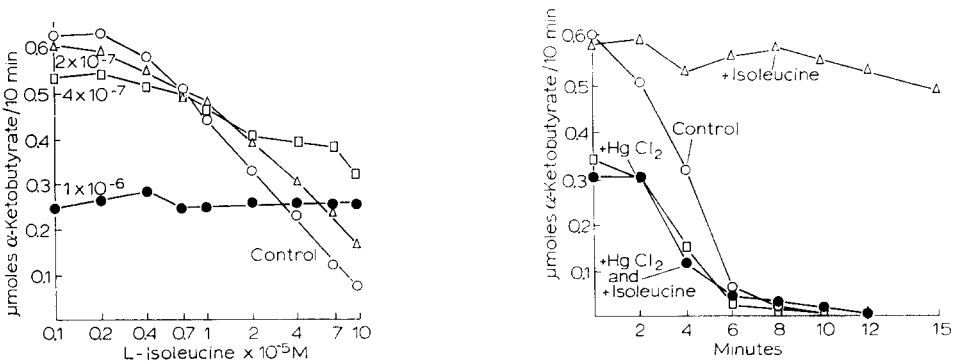
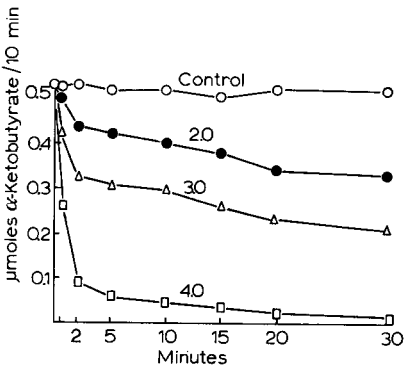


Fig. 10. Desensitization of the enzyme with $HgCl_2$. Crude extract (23 mg protein per ml) was equilibrated with the $HgCl_2$ concentrations listed. After 5 min at 0° samples were diluted 1:40 and measured in the standard assay (see *Methods*) containing the isoleucine concentrations indicated.

Fig. 11. Effect of $HgCl_2$ on the stabilizing effect of L-isoleucine. Crude extract (23 mg protein per ml) was equilibrated with 10^{-4} M L-isoleucine, 10^{-6} M $HgCl_2$ or both substances, and exposed to 65°. Samples were removed, cooled rapidly, diluted 1:40 and assayed as described under *Methods*. Purified enzyme diluted 1:50 gave analogous results.

dehydratase by urea and concluded from kinetic studies that an equilibrium was established in the presence of urea between an active polymer and inactive monomers. The enzyme of *B. licheniformis* is also subject to this inactivation (Fig. 12). The rate of the urea inactivation is high at first and then becomes much smaller after a few minutes. The effects of L-threonine, L-valine, L-isoleucine and L-α-aminobutyrate were tested on the urea-dissociated enzyme, since CHANGEUX[12] had observed that activators and substrates tended to dissociate the enzyme, while inhibitors associated it. The effect of these compounds on the *B. licheniformis* enzyme did not differ from the control significantly, although the deviations were in the same direction as observed by CHANGEUX. DATTA[10] also failed to see a protective effect of L-isoleucine on the urea inactivated enzyme of *R. spheroides*.

The urea inactivation was temperature dependent (Fig. 13). Low temperatures increased the fraction of enzyme that was denatured in the presence of urea. The equilibrium reached at different temperatures was reversible since the enzyme when



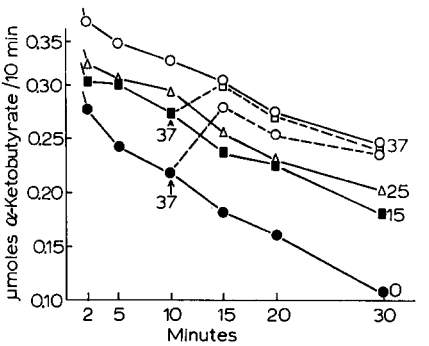


Fig. 12. Inactivation of enzyme by urea. Crude extract (26 mg protein per ml) was equilibrated to give the urea concentration indicated at 0°. Samples were removed, diluted 1:20 and assayed as described under *Methods*.

Fig. 13. Temperature dependence of urea inactivation. Crude extract (26 mg protein per ml) was equilibrated with an equal amount of 6 M urea and exposed to the indicated temperatures. Duplicate samples were used for transfer to higher temperatures. Samples were removed, diluted 1:20 and assayed as described under *Methods*.

transferred to a higher temperature after urea equilibrium was established, was reactivated to the activity that was found for the enzyme exposed to that higher temperature only (Fig. 13). Similar results had been obtained for the *E. coli* enzyme[12].

8. *Comparison.* From the data reported in the literature on the properties of the biosynthetic threonine dehydratase it is clear that, although there are some distinct differences, the enzyme in general has similar properties in different microorganisms. The differences are usually quantitative and in part probably due to variations in growing the organism, extraction procedures, enzyme analysis, and in the interpretation of the data obtained, since some variations are found for the enzyme from the same organism investigated by different workers. From the results reported in this paper it appears that the threonine dehydratase of *B. licheniformis* falls into the same pattern, since most of the enzyme properties are similar to the characteristics reported in the literature. The enzyme properties that differ significantly from the values re-

ported are the following: (a) The pH optimum is rather high (9.2). (b) Pyridoxal phosphate is not required even after partial purification. (c) L-Valine does not activate the enzyme or antagonize the isoleucine inhibition. (d) The enzyme is inhibited by isoleucine at a significantly lower concentration.

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