Threonine Deaminase of *Clostridium tetanomorphum*

**I. PURIFICATION AND PROPERTIES***

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**SUMMARY**

Threonine deaminase (L-threonine hydrolyase (deaminating), EC 4.2.1.16) has been purified approximately 700-fold from extracts of *Clostridium tetanomorphum*. Both threonine and serine can serve as substrates, but threonine is deaminated 5 to 10 times more rapidly than serine. Pyridoxal phosphate, a reducing agent, and alkaline pH are required for the deamination of either amino acid.

A plot of the rate of reaction at increasing substrate concentration gives a sigmoidal curve. Addition of adenosine diphosphate stimulates the reaction markedly at low substrate concentrations and yields a curve approaching a rectangular hyperbola. Kinetic analysis using the empirical Hill equation indicates the existence of cooperative effects between substrate sites in the absence of ADP and little interaction, if any, in the presence of ADP. ADP may be replaced by guanosine and inosine diphosphates and less effectively by uridine and cytosine diphosphates; dATP and thymidine diphosphates have little stimulatory activity. Experiments with 14C-ADP show that the enzyme binds ADP.

The enzyme is inhibited by pyridoxal phosphate binding agents, by sulfhydryl reagents, urea, detergents, and substrate analogues. ADP protects the enzyme against low concentrations of sulfhydryl inhibitors and hydrogen bond-breaking agents but not against other inhibitors. ADP (and the other nucleotides mentioned above), phosphate buffer at pH 7.0, divalent cations, and, to a lesser extent, monovalent cations, protect the enzyme against inactivation by heat and against loss of activity on dilution.

Dialysis of enzyme preparation at an alkaline pH decreases the ability of the enzyme to bind 14C-ADP and reduces the catalytic response to ADP. Conversely, addition of agents that combine with pyridoxal phosphate inhibits catalysis but does not inhibit the binding of 14C-ADP. These differential effects suggest that the substrate and allosteric effector are bound to separate sites on the enzyme.

A number of enzymes have been described, the activities of which are influenced by compounds having structures unrelated to those of the substrates, the end products, or the coenzymes participating in the reactions. In most instances, the role of such modifiers of enzyme activity ("allosteric effectors" (1)) has been linked to the regulation of biosynthetic and catabolic pathways and represents an important control mechanism in the cell. Threonine deaminase (or threonine dehydrase) of *Clostridium tetanomorphum*, an enzyme catalyzing the dehydration and deamination of threonine to α-ketobutyrate, ammonia, and water, is a degradative enzyme stimulated by adenosine diphosphate (2). Evidence has been presented (3) that the effect of ADP on this enzyme may also represent a regulatory mechanism which, in this instance, is linked to the energy metabolism of the cell.

This paper reports purification of the enzyme and further studies on the kinetics with threonine and serine as substrates. ADP has been found to protect the enzyme against some inhibitors whereas low concentrations of other compounds (e.g. urea) enhance the effect of ADP and promote enzyme activity. Nucleotides, divalent and monovalent cations, and buffers are compared with respect to their ability to stabilize the enzyme against inactivation by heat and dilution. Data are presented on the selective inactivation of the catalytic and allosteric sites. The following paper discusses the effect of substrates, nucleotides, mono- and divalent cations, and buffers on the dissociation-association reactions of the enzyme.

**EXPERIMENTAL PROCEDURE**

*MATERIALS*—*Clostridium tetanomorphum* (ATCC-3606) was grown in a complex glutamate medium (4) and harvested after 14 to 16 hours of incubation. In some experiments, cells were grown in a medium containing 0.75% glutamate, 0.2% yeast extract, 0.25% K₂HPO₄, and 0.01% MgSO₄ adjusted to pH 7.6 (simple glutamate medium).

Nucleotides were purchased from Sigma and Calbiochem; 14C-labeled ADP was obtained from Schwarz.

*METHODS*—One unit of activity is that amount of enzyme which produces 1 μmole of α-ketobutyrate in 20 min at 37° in a reaction mixture containing 100 μmoles of Tris buffer, pH 9.5, 50 μmoles of L-threonine, 20 μmoles of β-mercaptoethanol, 10 μg of pyridoxal phosphate, and further additions as noted in a final volume of 0.5 ml. α-Keto acids were determined by a modification of the Friedemann and Haugen (5) method. The reaction rate was constant for 8 to 12 hours with most preparations. Protein was estimated according to the method of Lowry et al. (6). Specific activity is defined as units of enzyme activity per mg of
and threonine deaminase but the incubation time for the latter determinations was increased to 1 to 12 hours depending on the enzyme in the assay. The starch slab was cut into blocks (0.25 x 0.5 x 2.0 cm), and the blocks were frozen, thawed, and squeezed dry. These preparations were assayed for protein and threonine deaminase but the incubation time for the latter determinations was increased to 1 to 12 hours depending on the activity of the preparations.

Starch slabs and gels were stained for protein with Amido Schwarz as described by Davis (9). The intensity of staining was determined by placing the gel in a closely fitting cylindrical quartz cuvette which was moved vertically in 1 mm increments, by means of a rack, in the light path of a Beckman model DU spectrophotometer. Optical density at 600 mÅ was recorded.

4. Sephadex G-50 Filtration—The precipitates collected by centrifugation were dissolved in water and aliquots of the 43.5 to 47% fraction were passed through a column (1.3 x 30 cm) of Sephadex G-50 (coarse mesh). The column was equilibrated and eluted with 0.05 M NaCl. The protein-containing fractions were pooled and either adsorbed to a column of DEAE-cellulose or stored at -20°. The ammonium sulfate precipitation and gel filtration yielded an approximately 14-fold increase in specific activity (Table I) and no loss in activity was observed in this fraction after 10 days of storage at -20°. In some experiments, the 47 to 50% fraction was also desalted by gel filtration and either combined with the desalted 43.4 to 47% fraction or chromatographed separately on DEAE-cellulose.

5. Chromatography on DEAE-cellulose—The fractions obtained from gel filtration were adsorbed to a column (1.3 x 25 cm) of DEAE-cellulose (coarse mesh, Sigma) and eluted with a linear gradient of NaCl (0.05 M to 0.30 M). Frequently, two peaks of activity were found, thus confirming the results of Hayashi, Gefter, and Weissbach (2). However, in the present investigation, the amount of enzyme eluted at the lower salt concentration (0.1 M NaCl) was variable and this "minor DEAE-cellulose peak" never contained more than one-fifth of the activity found in the "major DEAE-cellulose peak" eluted by 0.16 M NaCl. A comparison of the properties of the enzymes found in the two peaks showed that the first was less stable to storage at -20° than the second. In addition, several peaks of activity were discovered when the minor DEAE-cellulose peak material was sedimented in a sucrose gradient whereas the material from the major DEAE-cellulose peak was present as a single entity (discussed in the following paper). The properties of the enzymes from the two peaks were identical with respect to ADP activation. The effects of inhibitors are discussed in a later section.

6. Sephadex G-200 Filtration—Fractions from the major DEAE-cellulose peak having the highest activity were pooled, dialyzed, and concentrated with Aquacide (Calbiochem). They were either stored at -20° or passed through a column (1.3 x 30 cm) of Sephadex G-200 equilibrated with 0.1 M Tris-acetate buffer at pH 7.0. The same buffer was used to elute the enzyme. This step resulted in a 20-fold increase in specific activity with fair recovery of the material put on the column. Fractions having the highest activity were pooled, dialyzed against 200 volumes of distilled water for 2 hours, and lyophilized; these fractions had a half-life of approximately 4 days when stored at -20°. Chromatography of these prepara8ions on a second column of DEAE-cellulose yielded an additional 2-fold increase in specific activity but was not adopted as a routine step because the resulting fractions lost their activity rapidly (half-life of approximately 2 days). The final preparations (Step 6 of Table I) represent a 500- to 700-fold purification. In contrast, the two threonine deaminases of Escherichia coli (described below) are less stable than the C. tetanomorphum enzyme and have not been as amenable to purification (12-14).

7. Electrophoresis—Partially purified fractions having a
Specific activity of 3000 yielded several bands of enzyme activity in polyacrylamide gels. One or two sharp bands near the top of the gel \( E_f = \text{distance traveled by enzyme/distance traveled by dye marker} = 0.07 \) to 0.10) had low activity and three distinct bands (approximately 0.36, 0.47, and 0.55) had a much higher activity. As illustrated in Fig. 1, all of the protein bands corresponded in migration to areas of enzyme activity.

Electrophoresis of such preparations at pH 7.0 in starch, on the other hand, yielded a single zone of activity which coincided in position with the single band of protein (determined by elution and assay or by staining). Subsequent experiments showed that the pH used in acrylamide electrophoresis (pH 8.9) promotes dissociation of the enzyme.

**Properties of Enzyme**

1. **Substrate Specificity**—L-Threonine and L-serine, but not D-serine, D-threonine, \( m \)-allothreonine, or \( m \)-homoserine can serve as substrates for the enzyme. Since the reaction with either amino acid required the same conditions and the specific activity toward serine during enzyme fractionation paralleled that for threonine, it is assumed that the enzyme is nonspecific and can react with either compound, although threonine, as shown below, is the preferred substrate. Threonine deaminases from \( E. \ coli \) (15) and Neurospora (16) also deaminate both threonine and serine and react more rapidly with threonine than with serine.

2. **Kinetics with Threonine as Substrate**—Fractions of all degrees of purification showed an increased activity in the presence of ADP, with the greatest stimulation occurring at low concentrations of substrate. At high concentrations of substrate, the extent of stimulation by ADP depended on the preparation. Freshly prepared fractions of high specific activity (solid lines in Fig. 2) appeared to be slightly inhibited at high threonine concentrations in the presence of ADP, whereas fractions aged by storage at \(-20^\circ \) were not (dashed lines in Fig. 2). The lower activity of such stored fractions and the differences observed with respect to the stimulation by ADP may be attributed, in part, to dissociation of the enzyme on aging.

**Fig. 1.** Comparison of the migration of threonine deaminase activity and protein upon electrophoresis of a purified fraction. Threonine deaminase activity; this is given by micromoles of \( \alpha \)-ketobutyrate produced in the standard assay by a 1-mm gel slice after 6 hours of incubation. ---, optical density at 600 nm of a parallel tube stained for protein and scanned as described. \( E_f \) denotes distance traveled by protein or enzyme/distance traveled by dye.

However, differential inactivation of the catalytic and allosteric sites is also possible and may account for the different kinetic properties of aged fractions.

\( K_m \) values for threonine in the presence of \( 2.5 \times 10^{-4} \) M ADP were in the range of 1 to \( 4 \times 10^{-5} \) M; in the absence of ADP, \( K_m \) values were more variable, from \( 1 \times 10^{-2} \) to \( 1 \times 10^{-4} \) M. Extensively dialyzed fractions or preparations obtained after gel filtration showed a maximum 20-fold decrease in the \( K_m \) value for threonine in the presence of ADP. These values agree with the results reported previously by Hayaishi, Gefter, and Weissbach (2).

In contrast with earlier information (2), purified fractions yielded sigmoidal curves in response to increasing concentrations of substrate in the absence of ADP. Such sigmoidal curves and the corresponding nonlinear reciprocal plots (Fig. 2) have been assumed to indicate that the substrate itself acts as a modifier or allosteric effector; a kinetic analysis of such systems has been presented by Frieden (17).
3. Kinetics with Serine as Substrate — Serine was deaminated at a much lower rate than threonine and the reaction appeared to be more highly dependent on the addition of ADP than threonine deamination. \( K_m \) values in the absence of the nucleotide were 5 to 15 \( \times 10^{-4} \) M and 0.5 to 2.0 \( \times 10^{-2} \) M in the presence of ADP.

The deamination of serine (Fig. 3) by freshly prepared and aged fractions is, in general, similar to the deamination of threonine but differs in some respects. These differences may be summarized as follows. (a) The onset of activity in the absence of ADP requires a much higher concentration of serine than threonine; (b) ADP has a greater effect at intermediate and high concentration of serine than at the same concentrations of threonine; (c) as stated above, enzyme saturation requires a significantly higher concentration of serine than threonine; (d) the specific activity for serine is 5 to 10 times lower than for threonine; and (e) aged preparations (dashed lines in Fig. 3) are comparatively less active with serine than with threonine.

4. Homotropic Interactions — Sigmoidal curves in response to substrate or allosteric effector have been reported for many allosteric enzymes. Several explanations (18–21) have been proposed to account for kinetic data of this type. One interpretation is that cooperative effects can occur either between catalytic sites or between allosteric sites of the enzyme (18, 20).

The extent of such "homotropic interactions" (18) has been examined by means of the empirical Hill equation. The derivation of this equation, its application, and the limitations of this approach have been discussed in detail by Atkinson, Hathaway, and Smith (21). The slope of the line, \( n \), obtained when log \( V/V_{\text{max}} - v \) is plotted against log \( S \) is a function of the number of interacting binding sites per molecule and the strength of interaction between these sites. Thus, \( n \) has been interpreted as an indication of the order of the reaction (21) and as a measure of the total cooperative interactions (interaction coefficient (18, 20)).

As seen in Fig. 4A, the slope of the line obtained for a freshly prepared, highly purified fraction of threonine deaminase (solid lines) increases from \( n = 0.6 \) at low threonine concentration to \( n = 3.0 \) at high threonine concentrations. Crude extracts and partially purified fractions also gave changing slopes with maximum values for \( n \) ranging from 2 to 3. In contrast, analysis of data obtained with aged preparations of threonine deaminase (dashed lines) frequently yielded straight lines with \( n = 2 \). With all preparations, a definite decrease in slope was observed in the presence of ADP with values for \( n \) approximating 1, indicating complete independence of sites under these conditions. A Hill plot of data obtained for the deamination of serine (Fig. 4B) showed a marked change in the values for \( n \) at increasing serine concentration with both freshly prepared active fractions and aged preparations. It is apparent that the enzyme shows cooperative interaction which is not abolished by changes in enzyme activity. When ADP was added, \( n \) decreased to values near 1, duplicating the results obtained with threonine.
The same method of analysis for other enzymes has shown that cooperative interactions may exist with or without changes in $n$. Thus, an enzyme with strongly cooperative groups, the isocitric dehydrogenase of yeast (19), gave a value of $n = 4$ at all substrate concentrations. This value did not change when the positive effector, AMP, was added. Aspartic transcarbamylase (22), on the other hand, showed a very marked change in slope with a high degree of cooperative interaction ($n = 4$). When the allosteric inhibitor, CTP, was added, the value of $n$ was found to change slightly. The results with threonine deaminase of C. tetanomorphum resemble somewhat those obtained with aspartic transcarbamylase (i.e. $n$ may be high) but weak homotropic effects (possibly involving four substrate-binding sites) are indicated since $n$ decreases to 1 in the presence of ADP. The significance of the decrease in interactions on aging is not known at present but could reflect partial inactivation of threonine binding sites. In general, however, the effect of ADP fits the model proposed by Monod, Wyman, and Changeux (18) for "K systems."

These results may be compared with data available for other threonine deaminases. The isoleucine-sensitive threonine deaminase of E. coli (20) has been shown to have a lower value of $n$ ($n = 1.3$) and two substrate-binding sites have been postulated. Addition of isoleucine causes a decrease in the value of $n$ to 1.0. The isoleucine-inhibited threonine deaminase of Salmonella typhimurium exhibits Michaelis-Menten kinetics when assayed at high ionic strength (23, 24), but a sigmoidal curve is obtained at low ionic strength (23). The AMP-stimulated threonine deaminase of E. coli (13), on the other hand, displays apparently typical Michaelis-Menten kinetics with no evidence of cooperativity.

6. Nucleotide Specificity—Highly purified preparations showed the same activity with GDP and IDP as with ADP whereas UDP and CDP were less effective and dADP and TDP had a very slight effect. The Michaelis constant of activation, $K_a$, determined at low substrate concentration, was approximately $1 \times 10^{-3} \text{ M}$ for ADP, GDP, and IDP whereas $K_a$ for UDP and CDP was approximately $4 \times 10^{-4} \text{ M}$. $K_a$ values for dADP and TDP were higher ($4 \times 10^{-3} \text{ M}$). As seen in Fig. 5, the nucleotides differed greatly with respect to the extent of the reaction at saturating concentrations. A plot of these data according to the Hill equation gave slopes of 0.8 to 1.0 for ADP, GDP, and IDP and values of 0.5 to 0.7 for UDP and CDP. The value of $n$ for ADP was unchanged at three concentrations of substrate selected for maximum ADP effect but decreased at high concentrations of threonine. It will be recalled that ADP has less effect at high substrate concentrations.

Low concentrations of mono- and triphosphate nucleotide derivatives had no effect on the activity of highly purified preparations but a measurable stimulation was found at concentrations above $2.5 \times 10^{-3} \text{ M}$. ATP, GTP, and ITP stimulated the activity of crude and partially purified fractions, presumably because of contamination of these preparations with nucleotide triphosphatases.

6. Optimal Assay Conditions—The reaction required a reducing agent, pyridoxal phosphate, and an alkaline pH. An absolute requirement for a sulfhydryl compound was seen after partial purification; $\beta$-mercaptoethanol was satisfactory for this purpose. A broad pH optimum (8.5 to 10.5) was found in the absence of ADP; in the presence of ADP, the pH optimum was extended slightly (8.0 to 10.5). A more rapid reaction was found in Tris buffer than in phosphate or glycine buffers (at all pH values tested) despite the greater protection against inactivation observed with phosphate (discussed in a subsequent section). No evidence could be obtained for a stimulation by yeast extract at low pH values as reported by Hayaishi, Gelfter, and Weissbach (2).

The activity of all fractions was increased 5 to 10% by pyridoxal phosphate; a greater stimulation could be shown after dialysis of a partially purified fraction against 0.005 M cysteine.

7. Inhibitors—In view of the above requirements for enzyme action, inhibition by reagents capable of binding either pyridoxal phosphate or sulfhydryl groups was expected. Table II shows the activity of all fractions was increased 5 to 10% by pyridoxal phosphate; a greater stimulation could be shown after dialysis of a partially purified fraction against 0.005 M cysteine.

![Fig. 4](http://www.jbc.org/)

**Fig. 4.** Dependence of the reaction rate on substrate concentration and enzyme preparation. A, threonine as the substrate; --- and --- as in Fig. 2; data taken from Fig. 2. B, serine as the substrate; --- and --- as in Fig. 3; data taken from Fig. 3.

![Fig. 5](http://www.jbc.org/)

**Fig. 5.** Effect of nucleotide concentration on the deamination of threonine. Assayed as described in "Experimental Procedure" with 10 amoles per ml of L-threonine with an enzyme fraction having a specific activity of 450.
that hydroxylamine, cysteine, \( p \)-hydroxymercurobenzoate, and \( N \)-ethylmaleimide inhibited threonine deaminase. A concentration of approximately \( 1 \times 10^{-4} \text{ M} \) sodium borohydride also destroyed 50% of the activity (not shown in Table II).Arsenite, \( \text{Zn}^{2+} \), and \( \text{Cd}^{2+} \) (not shown in Table II) had no effect unless added at a final concentration of 0.05 to 0.20 \text{ M} \) and at this concentration many salts inhibited the reaction 20 to 30%.

It may be noted that the inhibition at high salt concentration was proportional at all substrate concentrations; i.e., the sigmoid nature of the substrate saturation curve observed in the absence of ADP was not altered by high ionic strength.

The presence of ADP had no effect on the activity observed with hydroxylamine, cysteine, and borohydride but a slight protection was afforded by ADP against the action of sulfhydryl reagents. The latter compounds and the pyridoxal binding agents were competitive inhibitors of the enzyme.

The enzyme was also inhibited competitively by \( \alpha \)-threonine, \( \text{dl} \)-allothreonine, \( \text{d} \)-serine, \( \text{l} \)-serine, \( \text{dl} \)-homoserine, \( \beta \)-hydroxybutyrate, and \( \alpha \)-aminobutyrate (not shown in Table II). However, these substrate analogues were poor inhibitors; a final concentration of \( 0.025 \) to \( 0.1 \text{ M} \) was required to produce a 50% inhibition. ADP did not protect the enzyme against inhibition by substrate analogues. In the presence of \( 0.1 \text{ M} \) \( \text{dl} \)-allothreonine, the sigmoidal character of the substrate saturation curve was either diminished or lost. A similar effect of analogues on the substrate-promoted interaction of sites has been reported for aspartic transcarbamylase (22) and for the isoleucine-sensitive threonine deaminase of \( \text{E. coli} \) (20).

The enzyme was also inhibited competitively by one of the products of the reaction, the ammonium ion. No inhibition was found when another product, \( \alpha \)-ketobutyrate, was added. Similar results have been obtained with the isoleucine-sensitive threonine deaminase of \( \text{S. typhimurium} \) (24).

As shown in Table II, the enzyme was inhibited by a number of hydrogen bond-breaking agents and was protected to varying degrees by ADP against the action of these compounds (Fig. 6). Sodium lauryl sulfate and guanidine (Table II and Fig. 6) were more efficient denaturing agents than urea and formamide.

TABLE II

| Effect of inhibitors |

Standard assay as described in "Experimental Procedure" with an enzyme fraction having a specific activity of 240 was used. Pyridoxal phosphate was omitted from the assay mixture for the first three compounds listed and \( \beta \)-mercaptoethanol was omitted in experiments with sulfhydryl inhibitors.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration yielding 50% inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Assayed without ADP</td>
</tr>
<tr>
<td>Hydroxylamine</td>
<td>( 5 \times 10^{-4} )</td>
</tr>
<tr>
<td>Cysteine</td>
<td>( 1 \times 10^{-2} )</td>
</tr>
<tr>
<td>( p )-Hydroxymercurobenzoate</td>
<td>( 2.5 \times 10^{-5} )</td>
</tr>
<tr>
<td>( N )-Ethylmaleimide</td>
<td>( 2 \times 10^{-4} )</td>
</tr>
<tr>
<td>Urea</td>
<td>( 5 \times 10^{-1} )</td>
</tr>
<tr>
<td>Formamide</td>
<td>1.0</td>
</tr>
<tr>
<td>Guanidine</td>
<td>( 5 \times 10^{-2} )</td>
</tr>
<tr>
<td>Sodium lauryl sulfate</td>
<td>( 3.4 \times 10^{-4} )</td>
</tr>
</tbody>
</table>

ADP protected the enzyme against urea, sodium lauryl sulfate, and formamide but very little protection was afforded against guanidine. A comparable protective effect by an allosteric effector (isoleucine) against inactivation by urea has been observed for threonine deaminase in \( \text{E. coli} \) (18). Ethanol and dioxane (not shown in Table II) are relatively poor inhibitors of the \( \text{C. tetanomorphum} \) enzyme with an even greater protective effect seen on adding ADP.

The deamination of threonine in the presence of ADP was slightly stimulated by a low concentration of urea, sodium lauryl sulfate, and formamide. In some experiments, activity was stimulated also at low concentrations of the inhibitors in the absence of ADP. Such effects have been interpreted to mean "loosening" or "relaxation" of aggregated forms of an enzyme. Stimulation was most reproducible with urea (maximally 2 to 5-fold decrease in \( K_m \) for threonine in the presence of ADP) and depended on the preparation. Aged, partially purified fractions were stimulated to a greater extent than freshly prepared fractions of high activity.

Protection against inactivation by urea or sodium lauryl sulfate was also observed when 0.025 \text{ M} \text{ MgCl}_2, 0.25 \text{ M} \text{ phosphate buffer at pH 7.0}, or 0.2 \text{ M} \text{ KCl} was added, although the degree of protection was less than that observed with ADP. This buffer could not replace phosphate buffer and no protection was observed on adding either substrate or substrate analogues, reducing agents, or pyridoxal phosphate.

A variable 10 to 30% inhibition was also observed upon the addition of certain amino acids (glycine, isoleucine, leucine, and lysine) but not after addition of 12 other amino acids tested singly or in combination. Isoleucine efficiently inhibits the "biosynthetic threonine deaminase" of \( \text{E. coli} \) (25-27), \( S. \).

In \( \text{E. coli} \) different threonine deaminases are produced in response to different conditions of growth (25, 26). Cultures grown aerobically in a synthetic medium form an isoleucine-sensitive enzyme (biosynthetic threonine deaminase) whereas cultures grown anaerobically in a complex medium form an AMP-stimulated enzyme (degradative threonine deaminase).
typhimurium (28), yeast (29), Bacillus subtilis (30), Rhodopseudomonas capsulata (31), and Acetobacter suboxydans (32); this is considered to be an important mechanism in the regulation of isoleucine biosynthesis. In E. coli, a 50% inhibition of the biosynthetic enzyme is observed with $1.2 \times 10^{-3}$ M isoleucine (27), whereas, as stated above, a much higher concentration is required to inhibit partially the C. tetanomorphum enzyme.

Since two threonine deaminases are produced in E. coli, the possibility existed that two enzymes are also produced by C. tetanomorphum and that the effect of isoleucine and other amino acids is due to the presence of a small amount of a second enzyme. To examine this possibility, cultures were grown in a medium containing lower concentrations of complex nutrients (simple glutamate medium) since a synthetic medium for the cultivation of C. tetanomorphum is not known. A slightly larger amount of threonine deaminase was produced following growth in this medium, but the response of both crude and purified enzyme fractions to ADP and to the amino acids in question was identical with that observed with crude and purified fractions derived from cells grown in the usual complex medium. All of the other properties of the enzyme were also identical with those of threonine deaminase from cells grown in the complex medium. Addition of either isoleucine, leucine, or lysine at a concentration of 0.1% to either simple or complex glumatate medium did not affect either the growth of the culture or the specific activity of crude cell-free extracts. It is concluded that a "biosynthetic threonine deaminase" was not produced under the conditions used and that the partial inhibition observed on adding certain amino acids was probably not related to an allosteric control mechanism.

8. Inactivation by Heat, Dilution, or Storage—The enzyme lost activity on dilution and heating but was protected against such losses by ADP (33). More detailed experiments (Tables III and IV) disclosed that a number of compounds may serve as protective agents. Complete protection against heat inactivation was afforded by a high concentration of phosphate buffer at pH 7.0 under the conditions used in Table III, and also by 0.5 M citrate or 0.5 M pyrophosphate at pH 7.0. Arsenate buffer was not as effective as phosphate and Tris buffer was deleterious. Exposure to heat in either phosphate, Tris, or arsenate at pH 9.5 (the pH used for the assay) resulted in rapid inactivation although some protection was observed at this pH when the buffer was supplemented with pyridoxal phosphate and $\beta$-mercaptoethanol. $\beta$-Mercaptoethanol alone or in the presence of any of the buffers investigated promoted inactivation.

The enzyme was protected by a number of cations but $1.5 \times 10^{-3}$ M EDTA and $10^{-3}$ M glycerol, and $10^{-3}$ M glucose (not shown in Table III) had only a slight effect. Lysine, leucine, or isoleucine also had no effect or a slight (2 to 5%) protective effect. Threonine protected the enzyme whereas serine and the substrate analogues did not.

As expected, the nucleotide diphosphates which were effective in stimulating threonine deamination (Fig. 5) could substitute for ADP in protecting the enzyme against inactivation by heat except that dADP was more effective as a protective agent than in stimulating enzymatic activity (Table IV). In general, compounds which protect the enzyme against inactivation by heat have been found to prevent enzyme dissociation, whereas those that increase inactivation by heat also promote dissociation of the enzyme. Data supporting this conclusion are presented in the following paper.

### Table III

<table>
<thead>
<tr>
<th>Addition</th>
<th>Concentration</th>
<th>Initial activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td></td>
<td>240</td>
</tr>
<tr>
<td>Phosphate buffer, pH 7.0</td>
<td>0.2</td>
<td>91.1</td>
</tr>
<tr>
<td>Phosphate buffer, pH 7.0</td>
<td>0.4</td>
<td>77.6</td>
</tr>
<tr>
<td>Arsenate buffer, pH 7.0</td>
<td>0.2</td>
<td>80.0</td>
</tr>
<tr>
<td>Phosphate buffer, pH 9.5</td>
<td>0.2</td>
<td>62.0</td>
</tr>
<tr>
<td>Tris buffer, pH 9.5</td>
<td>0.2</td>
<td>22.8</td>
</tr>
<tr>
<td>Phosphate buffer, pH 9.5, + supplement&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.2</td>
<td>11.1</td>
</tr>
<tr>
<td>$\beta$-Mercaptoethanol</td>
<td>0.1</td>
<td>36.7</td>
</tr>
<tr>
<td>MgCl&lt;sub&gt;2&lt;/sub&gt;</td>
<td>0.2</td>
<td>91.5</td>
</tr>
<tr>
<td>CaCl&lt;sub&gt;2&lt;/sub&gt;</td>
<td>0.2</td>
<td>74.8</td>
</tr>
<tr>
<td>KCl</td>
<td>0.2</td>
<td>40.5</td>
</tr>
<tr>
<td>L-Threonine</td>
<td>0.25</td>
<td>72.7</td>
</tr>
</tbody>
</table>

<sup>a</sup> Supplement, 10 µg per ml of pyridoxal phosphate and 0.1 M $\beta$-mercaptoethanol.

### Table IV

<table>
<thead>
<tr>
<th>Nucleotide</th>
<th>Initial activity at 2.5 X $10^{-4}$ M</th>
<th>Initial activity at 2.5 X $10^{-3}$ M</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADP&lt;sup&gt;a&lt;/sup&gt;</td>
<td>92.4</td>
<td>77.6</td>
</tr>
<tr>
<td>GDP</td>
<td>90.5</td>
<td>82.0</td>
</tr>
<tr>
<td>IDP</td>
<td>101.0</td>
<td>53.5</td>
</tr>
<tr>
<td>UDP</td>
<td>63.4</td>
<td>20.1</td>
</tr>
<tr>
<td>CDP</td>
<td>76.0</td>
<td>24.4</td>
</tr>
<tr>
<td>dADP</td>
<td>74.0</td>
<td>66.3</td>
</tr>
<tr>
<td>TDP</td>
<td>62.7</td>
<td>20.9</td>
</tr>
</tbody>
</table>

<sup>a</sup> With no addition the value was 9.1% of control.

Qualitatively similar results were obtained in experiments on the loss of activity following dilution and storage at 0°C. The loss in activity upon dilution was slow (half-life of 10 hours) when freshly prepared and highly purified fractions were tested, and more rapid (half-life of 4 hours) when aged preparations were assayed. No significant difference could be detected in the rates of inactivation at 20°C and 0°C. Comparison of these results with those of Freundlich and Umbarger (28) and Hirata et al. (13) indicate that the C. tetanomorphum enzyme is approximately 3 times more stable to dilution than the "degradative threonine deaminase" of S. typhimurium and E. coli.

The enzyme was stabilized to storage at $-20^\circ$C by solutions of high ionic strength (0.2 M phosphate buffer at pH 7.0 and 0.25 M MgCl<sub>2</sub> were especially effective) and by 10% sucrose and 30% glycerol. However, the addition of $2.5 \times 10^{-4}$ M ADP to preparations left at this temperature caused a marked decrease in activity. Possibly this inactivation at cold temperatures is...
related to the cold-enhanced effectiveness of allosteric compounds reported for other enzymes (34, 35). All of the above agents, including ADP, stabilized activity when preparations were stored at 5°.

Evidence for Separate Catalytic and Allosteric Sites

1. Loss of Response to ADP—Selective inactivation of an allosteric site has been achieved for several enzymes by means of heat, mercurials, or urea. For example, the sensitivity of aspartate transcarbamylase of E. coli to CTP can be completely abolished by exposure of the enzyme to p-hydroxymercuribenzoate (36, 37) whereas the biosynthetic threonine deaminase of E. coli has been desensitized to isoleucine by means of heat (20, 27). In both the examples cited, catalytic activity was enhanced by inactivation of the regulatory site.

Attempts to inactivate the allosteric site of the threonine deaminase of C. tetanomorphum by means of mercurials, urea, or heat were unsuccessful. p-Hydroxymercuribenzoate inhibits catalytic activity (Table II) whereas urea promotes dissociation of the enzyme at low concentrations (discussed in the following paper) and inhibits catalytic activity at higher concentrations (Table II). Inactivation by heat (90° for 20 to 30 min in the presence of 0.2 M phosphate buffer, pH 7.0) yielded preparations which showed little response to ADP but had lost 70 to 90% of the catalytic activity. Heat treatment, exposure to mercurials, and urea were also unsuccessful as inactivators of the allosteric site of the degradative threonine deaminase of E. coli (13).

Partial inactivation of the ADP site was apparently achieved by dialysis of the enzyme at an alkaline pH in the presence of pyridoxal phosphate and β-mercaptoethanol. The latter compounds may serve to protect the catalytic site from inactivation, although complete protection could not be achieved (a 30 to 75% loss in activity was usually observed). However, such preparations showed a decreased response to ADP (Fig. 7) indicative of partial desensitization. It is of interest that the sigmoidal curve typical of untreated fractions was somewhat modified. Longer periods of dialysis under the above conditions reduced the response to ADP still more but also resulted in a greater loss of enzymatic activity; shorter periods of dialysis (6 to 12 hours), on the other hand, yielded preparations having only a 10 to 20% decrease in catalytic activity and a corresponding loss in response to ADP. The over-all kinetic properties of the latter preparations resembled fractions aged by storage at -20°, indicating that partial inactivation of both catalytic and allosteric sites occurs readily under a variety of conditions. Complete desensitization to ADP was readily achieved with dissociated forms of the enzyme. These results and the effect of dialysis at an alkaline pH on the sedimentation properties of the enzyme are discussed in the following paper.

2. Binding of ADP—That ADP may be bound by threonine deaminase could be inferred from the $K_m$ values for threonine obtained with fractions incubated briefly with ADP and then

![Fig. 7. Effect of ADP on the kinetic properties of dialyzed and undialyzed fractions. Assayed as described in "Experimental Procedure." A fraction having a specific activity of 85 (undialyzed) was supplemented with 10 μg per ml of pyridoxal phosphate and dialyzed for 48 hours in the cold against 500 volumes of 0.2 M Tris buffer, pH 10.5, containing 0.1 M β-mercaptoethanol. The specific activity of the dialyzed fraction was 49.](http://www.jbc.org/)

### Table V

$K_m$ values for threonine for fraction pretreated with ADP

<table>
<thead>
<tr>
<th>Fraction</th>
<th>$K_m$ values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>4.2 x 10^-5</td>
</tr>
<tr>
<td>Treated</td>
<td>2.2 x 10^-3</td>
</tr>
</tbody>
</table>

### Table VI

Binding of $^{14}$C-ADP

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Additions</th>
<th>Binding of $^{14}$C-ADP</th>
<th>Specific activity of threonine deaminase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>None</td>
<td>8312</td>
<td>78.3</td>
</tr>
<tr>
<td>Dialyzed at pH 11.0</td>
<td>None</td>
<td>505</td>
<td>52.6</td>
</tr>
<tr>
<td>Heat-inactivated (untreated)</td>
<td>None</td>
<td>120</td>
<td>0</td>
</tr>
<tr>
<td>Untreated</td>
<td>Threonine, 0.2 μM</td>
<td>7550</td>
<td>84.1</td>
</tr>
<tr>
<td>Untreated</td>
<td>NH$_4$OH, 0.2 μM</td>
<td>7650</td>
<td>2.1</td>
</tr>
</tbody>
</table>

A fraction having a specific activity of 430 (untreated) was mixed with 2.5 x 10^-4 M ADP, left in ice for 5 min, passed through a column (1.3 x 15 cm) of Sephadex G-50, and eluted and assayed as described under "Experimental Procedure." The specific activity of the protein-containing fraction after gel elution was 455.
obtained in the absence of ADP. Supplementation of these filtered preparations with ADP did not change the \( K_m \) value in the presence of ADP. Furthermore, a typical Michaelis-Menten curve was observed in response to increasing concentrations of substrate (such as that illustrated in Fig. 2 for preparations assayed with ADP) rather than the sigmoidal curve obtained in the absence of ADP. Supplementation of these filtered preparations with ADP did not change the \( K_m \) value on the substrate saturation curve. After storage in the cold for 2 to 10 days, or dialysis against 0.005 M \( \beta \)-mercaptoethanol, the fraction gradually regained its original kinetic properties and displayed the usual sharp response to ADP at low threonine concentrations. These results and the experiments cited below on the binding of \(^{14}C\)-ADP suggest that the nucleotide is released from the enzyme slowly.

It has been shown earlier (31) that, if fractions were incubated with \(^{14}C\)-labeled ADP and then subjected to gel filtration, radioactivity was found in the protein-containing fractions. This radioactivity paralleled enzyme activity in the elution pattern. It should be noted that binding was detectable under nonequilibrium conditions, again suggesting a strong interaction between the enzyme and its allosteric effector. The amount of \(^{14}C\)-ADP bound to a given preparation depended on the amount of enzyme used and was related to the purity of the preparation. The properties of the binding reaction will be described elsewhere.

Experiments on the binding of \(^{14}C\)-ADP were also performed with fractions dialyzed under the conditions described above (Fig. 7) at an alkaline pH. As shown in Table VI, there was a considerable loss in the ability to bind ADP although much of the catalytic activity was retained. In general, the ability of the threonine deaminase fraction to bind ADP was correlated with the amount of enzyme and its allosteric effector. The amount of \(^{14}C\)-ADP bound to a given preparation depended on the amount of enzyme used and was related to the purity of the preparation. The properties of the binding reaction will be described elsewhere.

Table VI also shows that the binding of ADP was not affected or was inhibited only very slightly by the simultaneous addition of threonine. Addition of hydroxylamine, on the other hand, completely inhibited enzymatic activity but had relatively little effect on the binding of ADP. Similar results have been obtained with borohydride, another pyridoxal phosphate-binding agent. On the basis of these results, it may be suggested that ADP is not bound at the substrate site. The experiments with dialyzed fractions (Fig. 7 and Table VI) suggest that it is possible to inactivate this ADP-binding site preferentially. Hence, it appears that this enzyme, like many other allosteric enzymes (1, 27, 37), possesses separate allosteric and catalytic sites.

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