THE MECHANISM OF THREONINE DEHYDRASE OF ESCHERICHIA COLI

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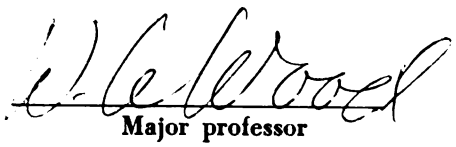

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ABSTRACT

THE MECHANISM OF THREONINE DEHYDRASE
OF ESCHERICHIA COLI

by Allen Thurman Phillips

L-threonine dehydrase from Escherichia coli grown anaerobically on an amino acid medium is activated by adenosine monophosphate (AMP) and reduced glutathione (GSH). This enzyme is presumed to function in the biodegradation of threonine.

The enzyme has been purified 50-fold from crude cell-free extracts and its properties investigated. Inhibition studies with KCN and hydroxylamine suggested that pyridoxal phosphate might also be required for activity. A partial reactivation could be observed upon addition of pyridoxal phosphate to the inhibited enzyme.

The purified enzyme exhibited an absorption maximum at 402 mμ and this absorption was considerably decreased by borohydride treatment. Hydrolysis of enzyme reduced with tritium-labeled borohydride allowed the isolation of N^6-pyridoxyllysine, indicating that pyridoxal phosphate was bound as a Schiff base to a lysyl residue in the native enzyme.

In order to elucidate the mechanism of activation by AMP and GSH, a detailed study of the reaction mechanism was undertaken. Experiments with D_2O, H_2O^{18} and α-tritiothreonine confirmed that the reaction proceeds first through dehydration to yield α-aminocrotonate, and this compound subsequently tautomerizes and hydrolyzes to yield α-ketobutyrate and ammonia. The observation of deuterium and O^{18}

back-incorporation into threonine during dehydration was taken as evidence that certain of the intermediate steps in the dehydration are reversible. Further evidence for a pathway of elimination involving unsaturated intermediates came from the isolation and identification of α-aminobutyrate from threonine dehydrase reaction mixtures which had been treated with borohydride. This α-aminobutyrate was formed by the reduction of α-iminobutyrate, an intermediate in the spontaneous conversion of α-aminocrotonate to α-ketobutyrate and ammonia.

Threonine dehydrase is also active on serine but this substrate results in a slow inactivation of the enzyme toward both substrates. When dehydrase was inactivated with L-serine-C^{14}, radioactivity was found associated with the protein after removal of the excess radioactivity by gel filtration. Total enzymatic hydrolysis of the labeled protein fraction, followed by paper chromatography, permitted the detection of C^{14}-lanthionine. This lanthionine would be formed by the addition of α-aminoacrylate, an intermediate in serine dehydration, to the SH group of a cysteinyl residue around the active site of the enzyme.

The role of AMP in threonine dehydrase was clarified by the following findings. The Michaelis constant for threonine decreased and the maximum velocity increased 6-fold in the presence of AMP at pH 8. Sucrose gradient centrifugations in the presence and absence of AMP revealed threonine dehydrase to have S_{20} values of 7.6 S and 5.0 S, respectively. If a spherical protein is assumed, these S_{20} values correspond to molecular weights of 153,000 and 83,000 respectively. Intermediate S_{20} values were noted as the AMP concentration was decreased from 3×10^{-3} M to zero. A gradient containing cytidine monophosphate, a less effective activator of this enzyme, showed threonine dehydrase to have an S_{20} of 6.9 S.

Assays conducted on the 7.6 S form of threonine dehydrase revealed no requirement for GSH, whereas the 5.0 S species required both AMP and GSH for activity. These data are consistent with the idea that one or more SH groups on threonine dehydrase are essential for the increases in sedimentation coefficient and activity observed in the presence of AMP.

Thus AMP and GSH activate threonine dehydrase by promoting the formation of a highly active species with an S_{20} of 7.6 S from a lesser active 5.0 S species. Because no evidence was obtained for AMP participation in the reaction mechanism and because no structural similarity exists between AMP and threonine, this compound must act as an allosteric effector in the regulation of threonine catabolism.

THE MECHANISM OF THREONINE DEHYDRASE OF *ESCHERICHIA COLI*

By

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VITA

Allen Thurman Phillips was born on October 30, 1938, in Vicksburg, Mississippi. He graduated from Menard Memorial High School in Alexandria, Louisiana, in May, 1956. He received the degree of Bachelor of Science in biochemistry from Louisiana State University in January, 1960, and a Master of Science degree from the same department in August, 1961. He accepted a graduate teaching assistantship in the Department of Biochemistry at Michigan State University, and in September, 1962, received a National Institutes of Health predoctoral fellowship for the remainder of his graduate training. The requirements for the Ph. D. degree will be completed in the fall of 1964.

Mr. Phillips is a member of the Division of Biological Chemistry of the American Chemical Society, the American Association for the Advancement of Science, the American Society of Microbiology, and Sigma Xi. He is married and has one child.

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INTRODUCTION

Hydroxyamino acid dehydrases have a widespread occurrence among microbial and mammalian species. Relatively little work has been conducted, however, on the details of catalysis by these enzymes. The reasons for this neglect of such a biologically important set of reactions are threefold: firstly, the mechanistic aspects of the reaction seemed to be straightforward from an organochemical point of view; secondly, many investigators chose to study the physiological and genetic factors which influence the formation and activity of these enzymes; and thirdly, the techniques for conducting such a detailed study of the reaction mechanism and enzyme chemistry were not available until recent years.

The investigations to be discussed were begun with the original intent of elucidating the mechanism of AMP activation of the L-threonine dehydrase from anaerobically grown Escherichia coli. In order to accomplish this, a procedure was developed for the partial purification of this enzyme and studies were initiated on the mechanistic aspects of the reaction; these were aimed at either implicating or eliminating a role for AMP at this level. Later, an investigation of enzyme conformational changes in the presence of AMP was undertaken. The results obtained from these two experimental approaches have been further utilized in a preliminary way to identify and study the nature of some catalytically important regions of this enzyme.

LITERATURE REVIEW

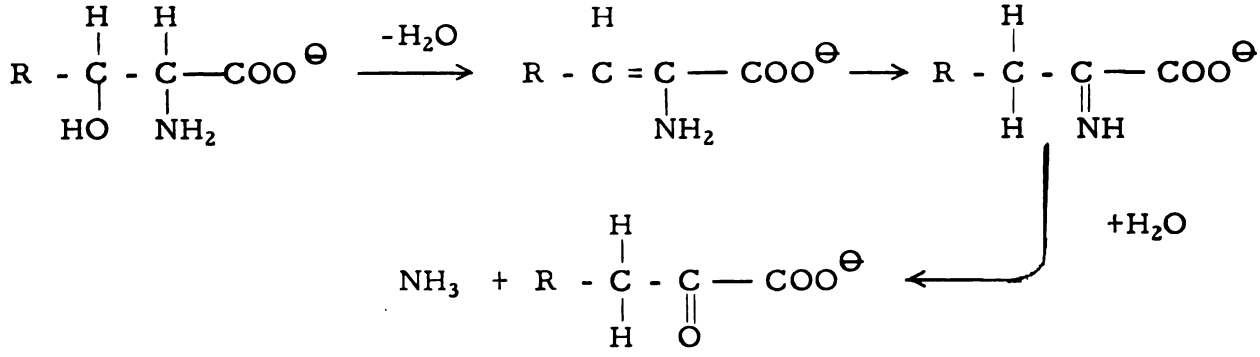
Serine and Threonine in Intermediary Metabolism

The synthesis and metabolism of the hydroxyamino acids, L-serine and L-threonine, have been subjects of considerable interest to biochemists over the last twenty years. Because recent and comprehensive reviews (1-4) on these broad topics are available, this discussion will be mainly concerned with the catabolism of these amino acids, and more specifically, with their catabolism via dehydrative deamination. This particular route of amino acid degradation assumes considerable importance in most biological systems, because serine and threonine are utilized poorly by many amino acid oxidases and amino transferases (transaminases). Catabolism of the hydroxyamino acids is thus dependent largely upon the action of specific dehydrases and aldolases, and to some extent, dehydrogenases.

Discovery, Distribution and Characteristics of Serine and Threonine Dehydrases

Following Gale and Stephenson's initial discovery of an anaerobic deamination of serine by Escherichia coli (5), Chargaff and Sprinson (6, 7) found that several other species of microorganisms (Proteus OX-19, Pseudomenas pyocyanae, Clostridium welchii), as well as rat, mouse, and rabbit liver extracts, were capable of producing an anaerobic deamination of serine and/or threonine. They characterized the products as ammonia and pyruvic acid and α-ketobutyric acid, respectively. Substitution of the hydroxylic hydrogen by various alkyl groups (ethyl or methyl) or phosphate or phosphatidic acid prevented

deamination. On the basis of such evidence, the authors proposed a mechanism for these reactions which involves the loss of water from serine or threonine and results in the formation of an α, β-unsaturated amino acid; this subsequently rearranges to the corresponding imino acid which then hydrolyzes to yield ammonia and an α-keto acid.



(R = H for serine, and CH_3 for threonine)

In this way, an enzymatic reaction which is in reality a dehydration results only in a deamination. A more detailed mechanism whereby this dehydration, tautomerism, and hydrolysis could occur was not postulated until sometime later.

Extensive study of this reaction was soon undertaken and in the next few years numerous reports appeared of anaerobic deamination of serine and/or threonine in a wide variety of organisms. Although the overall picture was far from clear, it soon became evident that there existed several types of dehydrase which differed markedly in substrate specificity and activator requirements.

Gale and Stephenson (5) had noted that the loss of serine dehydrase activity in whole cell preparations of E. coli could be prevented by the addition of reduced glutathione (GSH), by 5'-adenylic acid (AMP), or by boiled cell extracts. A brief report by Binkley (8) stated that serine dehydrase in cell-free extracts of E. coli and mouse liver was

inactivated by dialysis, but could be restored by certain divalent cations, notably zinc ions. Lichstein and Umbreit (9) reported that the L-serine and the DL-threonine dehydrases of resting suspensions of E. coli and Bacterium cadaveris were stimulated by yeast extract and biotin. Christman and Lichstein (10) found that AMP or biotin could stimulate serine and threonine dehydrases in E. coli or B. cadaveris cells which had been aged in phosphate buffer at pH 4 to inactivate these dehydrases. Certain of these observations were considerably clarified by the work of Wood and Gunsalus (11), who studied a partially purified dehydrase from anaerobically grown E. coli. This investigation indicated a marked stimulation of enzymatic activity by AMP and GSH. Furthermore, these authors showed that a single enzyme was responsible for the dehydration of both serine and threonine, and that serine, although a good substrate, caused a time-dependent total inactivation of the enzyme toward both substrates.

Research on the hydroxyamino acid dehydrases was soon extended to studies of the formation of these enzymes, and for E. coli, this eventually resulted in a clarification of the function of such dehydrases in amino acid metabolism. Pardee and Prestidge (12) found that a specific L-serine dehydrase was induced by serine, glycine, L-leucine and certain related compounds. Furthermore, they reported that L-threonine dehydrase behaved like a constitutive enzyme, but that additional activity could be induced by threonine as well. The inducibility of threonine dehydrase was also reported by Boyd and Lichstein (13). The apparent inconsistency between inducible and constitutive dehydrases was resolved by Umbarger and Brown (14, 15) with the finding of two L-threonine dehydrases in E. coli. One, a constitutive enzyme, was formed only in the absence of isoleucine, was inhibited by isoleucine, and was considered to function in the biosynthesis of isoleucine (hence its name "biosynthetic" dehydrase).

The enzyme was absent in isoleucineless mutants which could utilize α-ketobutyrate. They obtained indications of a requirement for pyridoxal phosphate for activity. The second dehydrase was induced only under anaerobic conditions during growth on an amino acid medium. This enzyme, designated the "biodegradative" dehydrase or the Wood and Gunsalus enzyme, required AMP and GSH, and there were indications that pyridoxal phosphate functioned as a coenzyme. It was not inhibited by isoleucine and hence was considered to function in the biodegradation of threonine. Both of these enzymes dehydrated serine and became inactivated during the process. In addition, they obtained sketchy evidence for a separate L-serine dehydrase.

Properties of the Purified Enzymes

The first attempts to purify threonine dehydrase were those of Wood and Gunsalus (11) using anaerobically grown E. coli as source of enzyme. They were able to show a requirement only for AMP and GSH; biotin, metals and pyridoxal phosphate did not activate. Shortly thereafter, Reissig (16) found that the L-threonine dehydrase from Neurospora required pyridoxal phosphate for activity. Later, Umbarger and Brown (15) utilized hydroxylamine inhibition to resolve their crude preparations of the "biosynthetic" and "biodegradative" dehydrases and thus were able to show that pyridoxal phosphate would partially reactivate these enzymes.

Sayre and Greenberg (17) and then Nishimura and Greenberg (18) partially purified the threonine dehydrase from sheep liver (final specific activity of 13 μmoles of α-ketobutyric acid formed per mg. of protein per minute). The yield through the seven step procedure was 10%. This enzyme was active on L-threonine, L-allo threonine and L-serine. Serine inactivated the enzyme, but this inactivation was overcome by the addition of pyridoxal phosphate. The latter authors (18)

speculated that an oxazolidine ring conjugate might be formed by reaction of the aldehydic group of pyridoxal phosphate with the hydroxyl and amino group of serine. No evidence, however, was obtained for this hypothesis. This threonine dehydrase was also stimulated by potassium or ammonium ions but not by any divalent cations. Mercuric ions and p-chloromercuribenzoate (PCMB) were inhibitory. The pH optimum was 8.2 to 8.6 and the Michaelis constant (K_m) for threonine was 8.0×10^{-3} M. Carbonyl reagents such as hydroxylamine and semicarbazide were inhibitory due to their reaction with the aldehydic group of pyridoxal phosphate. These reagents were used in resolving the holoenzyme free of coenzyme and in showing that pyridoxal phosphate was indeed the coenzyme of threonine dehydrase. Activation of the enzyme by AMP and GSH, first reported by Sayre and Greenberg, was not observed in the further purified preparations of Nishimura and Greenberg.

Walker (19) has reported the purification and properties of the L-threonine dehydrase of the rumen microorganism LC, now known as Peptostreptococcus elsdenii. In a 100-fold purified preparation, the K_m for threonine was 3×10^{-3} M whereas that for serine was 7×10^{-3} M; the optimum pH was 9.5. During purification the ratio of activities on threonine and serine remained constant, suggesting the existence of one enzyme. Freeze-drying in the presence of $(NH_4)_2SO_4$ or GSH produced a partial resolution of the enzyme-coenzyme complex. Activity could be restored only by the addition of pyridoxal phosphate. GSH was required for optimal activity.

Goldstein et al. (20) found that rat liver serine and threonine dehydrase activities were associated with the same protein, as judged by the similar inactivation rates obtained with ultraviolet irradiation, heat denaturation and proteolytic attack. Inducibility of the enzyme by threonine administration in the diet or in isolated perfused livers

could not be shown, but a high protein intake or cortisol administration did result in increased activity. The failure of these workers to show inducibility of threonine dehydrase is in conflict with the findings of Sayre, Jensen and Greenberg (21).

Considerable attention has recently been focused on the threonine dehydrase of Clostridium tetanomorphum. Hayaishi et al. (22-24) demonstrated a marked dependence on adenosine diphosphate (ADP), especially at low substrate concentrations. Yeast extract was also stimulatory in assays below pH 8.0. Their preparations were purified some 35-fold, but this specific activity was only equal to 0.1 μmole of α-ketobutyric acid produced per minute per mg. protein. K_m for threonine was found to be 6×10^{-3} M in the presence of ADP and 3×10^{-2} M in the absence of ADP. Experiments on the role of ADP will be discussed in a later section.

Most recently, Holzer and his co-workers (25) have studied a threonine dehydrase from the yeast, Saccharomyces cerevisiae. This enzyme was stimulated 6-fold by NH_4^+ ions. In addition to its stimulatory effect on activity, ammonia was found to repress the synthesis of the enzyme. An earlier publication of Holzer (26) had shown that this dehydrase was concerned with the biosynthesis of isoleucine.

The "biosynthetic" threonine dehydrase of E. coli, first studied by Umbarger (15), has recently been extensively investigated by Changeux (27). Purification of this enzyme from a 10-fold derepressed mutant of E. coli K12 has resulted in a 200-fold purification over the specific activity of extracts of the wild type. The partially purified enzyme is no longer competitively inhibited by isoleucine as was the crude enzyme. This "desensitization" was brought about by heating in the presence of high concentrations of isoleucine. A change in the kinetic properties was also noted upon purification. Whereas the native dehydrase did not follow simple Michaelis-Menten kinetics, the altered

dehydrase had normalized kinetics and a K_m of 5×10^{-3} M was calculated for threonine. Desensitization could also be accomplished by PCMB treatment; a protection against PCMB action was noted with L-threonine or L-isoleucine.

Further studies by Changeux (28) have indicated that at pH values above 10, the normal enzyme becomes insensitive to isoleucine and exhibits the usual type of kinetics observed for the dependence of velocity upon threonine concentration. In addition, L-allo threonine, which is not a substrate for the enzyme, inhibits threonine dehydration in a competitive manner even at pH values above 10. The interpretations given for the action of isoleucine and allo threonine will be presented in the later section on allosteric effectors.

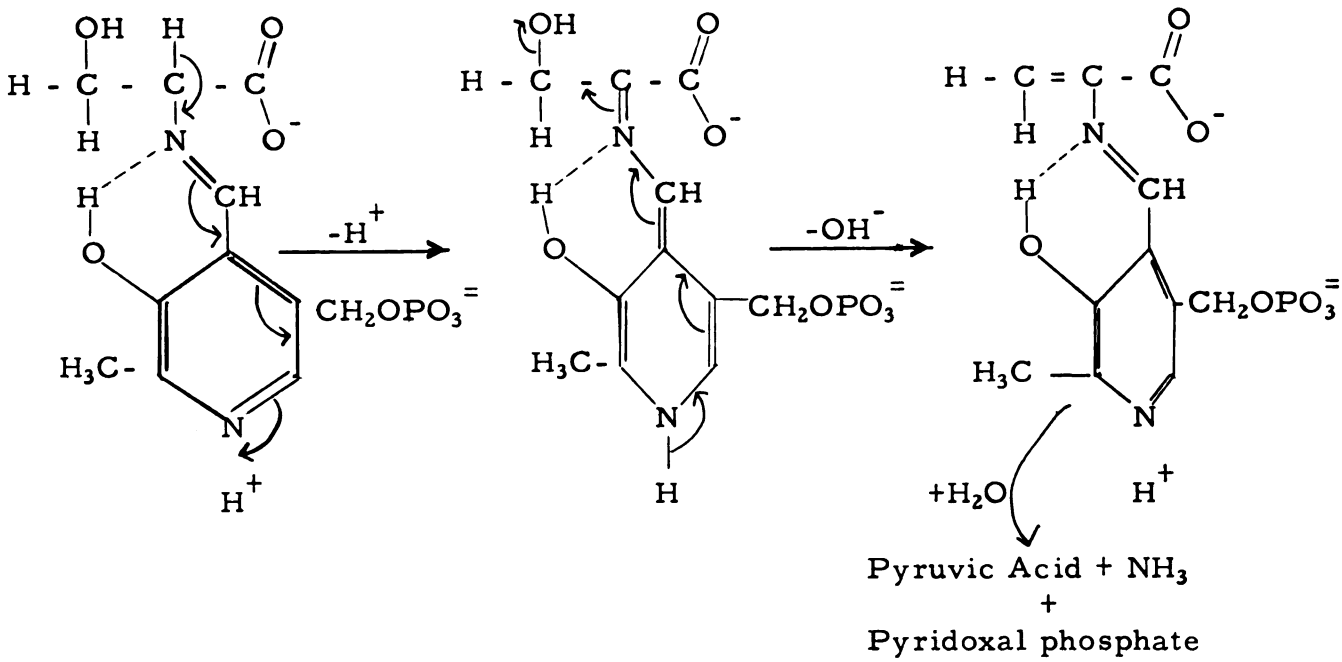
Changeux has also investigated the substrate specificity of the "biosynthetic" threonine dehydrase (29). Besides L-threonine and L-serine, the dehydrase slowly utilizes L-threonine amide and L-threonine methyl ester.

An AMP-stimulated threonine dehydrase was reported to be present in Streptomyces rimosus (30). This enzyme, however, was present in aerobic, glucose-grown mycelia and thus may play a different role than that assigned the nucleotide-activated enzymes found in anaerobic or facultatively anaerobic bacteria, i.e., anaerobic energy generation by substrate phosphorylation.

Mechanistic Aspects of the Hydroxyamino Acid Dehydrases

Little effort has been made to prove conclusively the scheme for serine and threonine dehydration proposed in 1954 by Metzler, Ikawa and Snell (31). Their original proposal was based on nonenzymatic experiments wherein serine, pyridoxal and certain divalent cations were incubated at elevated temperature. Evidence for Schiff base

formation between serine and pyridoxal was found. Therefore, the electron-withdrawing properties of the pyridine ring were invoked to form a long conjugated double bond system which could facilitate the loss of a particular atom or groups of atoms which were in close proximity to the α-carbon atom. The mechanism for serine dehydration which would be in accord with this theory is that shown below (4):



Braunstein and his collaborators (32), from consideration of enzymatic reactions, have independently arrived at a scheme which is very similar to that described above.

Some related aspects of pyridoxal catalysis should be mentioned at this point. The success of the Schiff base idea in explaining the wide variety of enzymatic pyridoxal-catalyzed reactions and the demonstration that nonenzymatically, pyridoxal does catalyze these reactions via a Schiff base has served to direct attention toward the additional contribution of the enzyme protein to the catalytic action.

Metzler (33) has found that Schiff bases between pyridoxal and many amino acids exhibit an absorption peak at 410-415 mμ.

By analogy, several highly purified pyridoxal enzymes which exhibit absorption peaks in the absence of substrate in the range of 410-430 mμ are considered to contain a Schiff base-like structure formed between pyridoxal and some amino group on the enzyme. Homoserine deaminase (34) and serine transhydroxymethylase (35) exhibit peaks at 427 mμ and 415 mμ, respectively, and these peaks are unchanged in the pH range of 5 to 10. On the other hand, phosphorylase (36) and glutamate-aspartate aminotransferase (37) exhibit maxima at 415 mμ and 430 mμ, respectively, but only at pH values below 5. These differences may reflect slight variations in the mode of binding of pyridoxal to the enzyme, but the interpretation still remains that the 415-430 mμ absorption corresponds to Schiff base formation.

The nature of the protein amino group participating in Schiff base formation has been investigated in the two best characterized pyridoxal enzymes, glycogen phosphorylase (38) and glutamate-aspartate aminotransferase (59). Upon reduction of both enzymes with borohydride, covalently bound pyridoxal phosphate was found associated with the proteins. Hydrolysis of the proteins yielded the fragment, N^6-pyridoxyllysine, indicating that the ϵ-amino group of a lysyl residue was the amino participant. Although two instances do not provide sufficient evidence for a generalization, it is considered likely that many other pyridoxal phosphate-containing enzymes will prove to have pyridoxal phosphate bound to a lysyl residue. The reason for this possibility will now be discussed.

It has been generally considered essential that the aldehydic group of pyridoxal phosphate be intact so that Schiff base formation with the amino group of the substrate will occur. This view was favored in spite of the above mentioned instances where the aldehydic group was not free, but rather was bound in an internal Schiff base. It was presumed that either the incoming amino group of the substrate

displaced the protein amino group or reacted with the small amount of free aldehyde form present, thereby shifting the equilibrium towards the free group and away from the Schiff base.

Recent work by Jencks and Cordes (40) has indicated, however, that non-enzymatically, the pyridoxal-valine Schiff base reacts more rapidly with semicarbazide than does free pyridoxal. This and other similar model experiments led them to conclude that the Schiff base is more reactive than free pyridoxal, and thus, in the case of a pyridoxal phosphate enzyme, it would be energetically more favorable for the coenzyme to be bound as a Schiff base prior to reaction.

While the requirement for pyridoxal phosphate in hydroxyamino acid dehydration is reasonably well established, no information is available from the literature as to the groups involved in binding of coenzyme to the apo-dehydrase. Only two studies have been conducted and these only indirectly suggest the nature of the groups involved in dehydration.

Davis and Metzler (41) have studied the pH dependence of the kinetic parameters of threonine dehydrase from sheep liver. They concluded from the variation in K_m with pH that the unprotonated amino group of threonine combines with the enzyme. The lack of any significant variation in the maximum velocity over the pH range 6 to 10 was interpreted as indicating that no groups with pK values in the range 6 to 10 participate in the catalysis of the rate-limiting step. Another possibility would be that some step subsequent to the dehydration, such as release of bound α-aminocrotonate, is the slowest step in the reaction sequence.

Further investigation of the pH dependence of K_m revealed that the free enzyme is reversibly converted to an inactive or non-binding form by simultaneous dissociation of three protons at about pH 9.1 and at 37° C. The authors speculated that this might reflect an alteration in the binding of pyridoxal phosphate.

Russell et al. (42) have conducted similar studies on the variation of maximum velocity and Michaelis constant with pH for the threonine dehydrase reaction. Their data confirm that the unprotonated amino group of threonine is the form bound to the enzyme, but in addition indicate that some basic group on the enzyme ionizes around pH 8.0. No indication was given as to the nature of this group. After consideration of current knowledge and theories of the mechanism of dehydration, the results of these kinetic experiments remain largely uninterpretable.

Nucleotide Activation in Other Systems and the Allosteric Effector Concept

A review by Monod, Changeux and Jacob (43) has thoroughly discussed the subject of allosteric proteins as metabolic regulators. The model proposed by these authors for such proteins features two or more stereospecifically different, non-overlapping receptor sites. The regions of most interest are the "active site," where substrate is bound, and the "allosteric site," where some regulatory metabolite is specifically and reversibly bound. The binding of the "allosteric effector" is thought to produce some sort of reversible change in the protein structure so altering the substrate interactions as to produce either a more or a less favorable situation for catalysis. Furthermore, since the allosteric effector plays no role in the reaction brought about by the protein, this effector need bear no structural relationship to the substrate.

This model serves well to explain a variety of activations and inhibitions observed in a number of enzymes where the effects are produced by compounds which bear little if any resemblance to the substrate. Included in this category are certain enzymes which have

nucleotide activators or inhibitors. Some of the more thoroughly studied examples of such enzymes will now be discussed along with the isoleucine-inhibited threonine dehydrase which has served as a model for allosteric effector studies.

Prior to the inception of the present study, three enzymes which exhibited a nucleotide activation had been extensively studied. These enzymes were glycogen phosphorylase from muscle, glutamate dehydrogenase from liver and aspartate transcarbamylase (ATCase) from E. coli. Because it would not be possible to discuss thoroughly the research conducted on the role of nucleotides in the activation or inhibition of these enzymes, only a brief summary of recent findings will be attempted.

Two excellent reviews on glycogen phosphorylase have been written by Krebs and Fischer (44, 45). In these reviews the phosphorylase molecule is pictured as a tetramer of molecular weight 500,000 (referred to as phosphorylase a) held together by at least two disulfide bridges and unknown interactions between two serine phosphate pairs. This form of the enzyme is only slightly activated by AMP. Cleavage of the serine phosphate bonds (achieved by a specific phosphatase) results in the formation of a dimer of molecular weight 250,000 (referred to as phosphorylase b). This latter form of phosphorylase is virtually inactive in the absence of AMP. Work by Kent et al. (36) indicated on the basis of sedimentation equilibrium experiments that phosphorylase b in the presence of AMP reverted to the tetrameric state with a doubling of the 250,000 molecular weight. This process was likened to the reformation of active phosphorylase a with the exception that removal of the loosely bound AMP resulted in a dissociation back to the dimeric or phosphorylase b form. In addition to a structural-induced change, AMP decreased the K_m for glucose-1-phosphate or glycogen.

In recent months, however, new evidence concerning the function of AMP has been presented. Monod et al. (43) cite unpublished sucrose gradient experiments by Vagelos in their laboratory which showed that formation of the tetramer in the presence of AMP did not occur. This writer has also learned from Dr. Carl Frieden (personal communication) that the original sedimentation experiments of Kent et al. (36) on the aggregation of phosphorylase b in the presence of AMP were in error and that no such phenomenon is observed in light-scattering experiments. Thus no specific structural change can yet be attributed to AMP for phosphorylase b.

Further kinetic effects produced by AMP have been reported by Helmreich and Cori (46) and also by Madsen (47). Both groups have observed that AMP and glucose-1-phosphate have reciprocal abilities to decrease each other's K_m values, as do AMP and the other two substrates, glycogen and inorganic phosphate. In addition, it has been found (46) that the degree of stimulation of phosphorylase a by AMP is dependent upon substrate concentration; thus it, too, is dependent upon AMP but only at low substrate concentrations.

Glutamate dehydrogenase has been extensively studied, expecially with regard to the relationship between enzymatic activity and molecular structure. A recent paper by Frieden (48) summarizes much of what is known in this area. Glutamate dehydrogenase undergoes a reversible, concentration-dependent association to form an enzymatically active tetramer of molecular weight one million from 250,000 molecular weight sub-units which are also active. A number of nucleotides including guanosine triphosphate (GTP), adenosine triphosphate (ATP) and adenosine diphosphate (ADP) are activators or inhibitors. Generally GTP and ATP are inhibitors while ADP is an activator. The mechanism whereby the inhibition or activation is accomplished has not been elucidated, but it is clear that the nucleotides have a specific binding

site on the enzyme separate from the substrate binding site and that they can influence enzyme activity as well as enzyme structure. Inhibition of the enzyme leads to a configurationally-altered subunit which cannot undergo the concentration-dependent association reaction.

Tomkins et al. (49) have contended that the state of aggregation of glutamate dehydrogenase does play an important role in metabolic regulation. They cite work which indicates that the 250,000 molecular weight species is active in catalyzing an alanine dehydrogenation reaction, whereas the one million weight species is inactive towards alanine. Agents such as GTP, ATP, reduced nicotinamide adenine dinucleotide (NADH) and diethylstilbesterol which favors disaggregation stimulate alanine dehydrogenase activity, and ADP which protects against disaggregation prevents alanine dehydrogenase activation. Even dilution-induced disaggregation favors alanine dehydrogenase activity. Immunological experiments by Talal (49) have detected three antigenic forms of glutamate dehydrogenase, and these forms each have a characteristic substrate specificity; one has glutamate dehydrogenase activity, one has alanine dehydrogenase activity, and a third has both. This and other evidence seem strongly to favor the concept that the type of activity displayed is a direct function of the state of aggregation.

Aspartate transcarbamylase from E. coli is inhibited by cytidine triphosphate (CTP) in a manner analogous to the isoleucine inhibition exhibited by the "biosynthetic" threonine dehydrase of E. coli. CTP is bound to ATCase at a site which is kinetically distinguishable from the binding site for aspartate and carbamyl phosphate. The most recent investigations of Gerhart and Pardee (50) indicate that the binding of CTP at its "regulatory" or allosteric site strengthens subunit interactions in the enzyme and distorts the active site, resulting in a decreased substrate affinity. ATP is capable of antagonizing the CTP

inhibition (51); this effect is presumed to be achieved by ATP binding at the regulatory site without a concomitant increase in subunit interaction.

Only a brief investigation of induced conformation changes has been made, but simultaneous sedimentation of two enzyme solutions, one containing carbamyl phosphate plus an aspartate analogue, and the second containing only enzyme, indicated that a 5% decrease in sedimentation occurred in the presence of substrates. This finding was considered indicative of a weakening of subunit interactions in the presence of substrate.

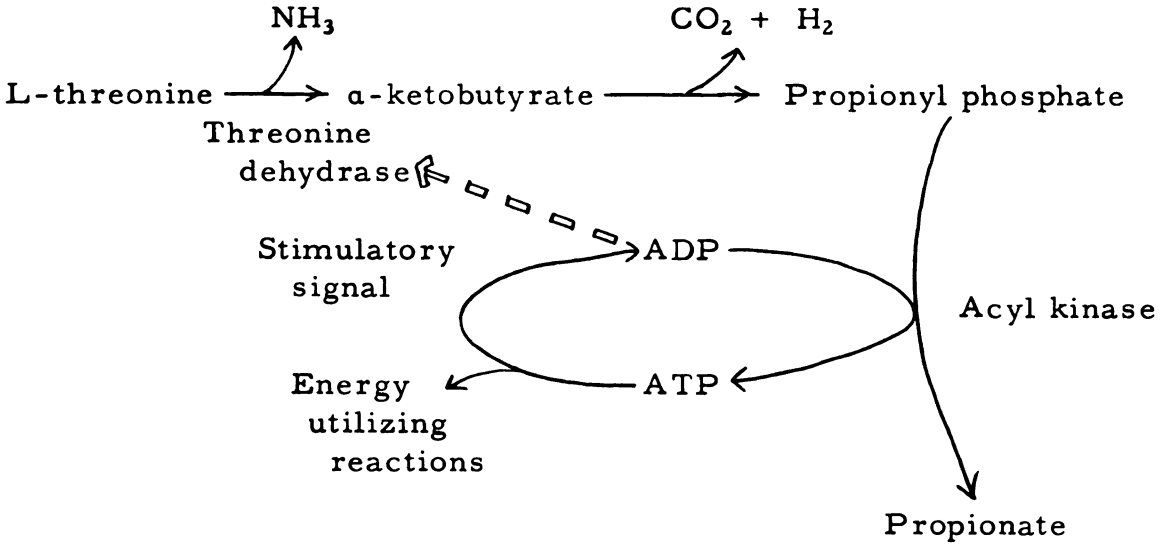
Although these examples are the most thoroughly studied for such nucleotide allosteric effectors, several other enzymes have recently been investigated for their nucleotide effectors.

NAD-linked isocitrate dehydrogenase from yeast has been studied by Atkinson and his co-workers (52). This enzyme is stimulated by AMP, but only at low isocitrate concentrations. The K_m for isocitrate was markedly affected by the presence of AMP. A very similar situation exists in the case of liver NAD-isocitrate dehydrogenase studied by Chen et al. (53). This enzyme was activated by ADP rather than AMP, but the activation was qualitatively similar in other respects.

A third NAD-requiring isocitrate dehydrogenase has been investigated by Sanwal et al. (54). This enzyme was partially purified from Neurospora crassa and was found to be markedly activated by AMP. At pH 6.5, however, the AMP stimulation decreased as the substrate concentration increased. Furthermore, at this pH, the enzyme exhibited normal kinetic behavior; whereas at higher pH values, abnormal kinetics were observed. These findings were considered indicative that two sites, a substrate site and an activator site, were present, but that the activator site was either modified or destroyed at pH 6.5.

A study most pertinent to the present work is that reported by Hayaishi et al. (22) on an ADP-activated threonine dehydrase from Clostridium tetanomorphum. The 35-fold purified enzyme showed a marked dependence on ADP at low substrate levels, but the effect decreased as substrate level increased. Only guanosine diphosphate and inosine diphosphate were able to substitute for ADP; AMP was only 5% as active as ADP. Experiments with C^{14}-labeled ADP failed to show any utilization or breakdown of ADP during activation. Whitely and Hayaishi (24) also found that ADP protected threonine dehydrase against inactivation by heat and dilution. In addition, they found that ADP was loosely bound to the enzyme, but was associated with the enzyme fraction after passage through a Sephadex G-50 column.

A control mechanism has been proposed by Tokushige et al. (23) for energy production in C. tetanomorphum in which threonine metabolism is regulated by the ADP concentration. This postulate is based on experiments which indicate that C^{14}-threonine is converted to C^{14}-propionate via α-ketobutyrate and propionyl phosphate. Their scheme is as follows:



Thus they consider that the degradation of threonine in C. tetanomorphum is inversely linked to ATP synthesis; that is, that as ATP is utilized, the ADP level increases to activate threonine dehydrase so as to provide the means for more ATP synthesis.

No discussion of allosteric effectors and their protein receptors would be complete without mention of the work of Changeux (27-29) on the allosteric interactions in the biosynthetic threonine dehydrase. As discussed earlier, considerable evidence has been accumulated to show that isoleucine can bind to at least one site on this enzyme which is kinetically and physically distinct from the substrate binding site. The findings that isoleucine inhibition can be destroyed by various treatments without corresponding destruction of enzymic activity, and that allo threonine, a substrate analogue, can inhibit enzyme activity under conditions where isoleucine sensitivity is present or absent are considered to support strongly the hypothesis of separate regulator and substrate sites (29).

Still further evidence for the presence of a "regulatory" site in addition to the active site has been described by Monod et al. (43) and Freundlich and Umbarger (55). Valine, presumably an isoleucine analog, activates rather than inhibits the reaction by increasing the affinity of the enzyme for threonine. When valine is incorporated into dehydrase reaction mixtures containing isoleucine, the inhibitory effect of isoleucine is prevented. A similar behavior is observed with norleucine or methionine in place of valine.

Because this enzyme has resisted purification, the more common techniques for observing a conformational alteration have been unsuitable. Sucrose gradient centrifugation experiments (29) have indicated that no detectable change in sedimentation velocity occurs in the presence of isoleucine or allo threonine. Urea inactivation, however, has produced a kinetically detectable disaggregation of the native enzyme and this

effect is reversed by isoleucine and stimulated by allo threonine--a situation somewhat similar to that found in ATCase, where subunit interactions are weakened in the presence of substrate.

METHODS AND MATERIALS

Bacteriological

Escherichia coli, Crookes strain, ATCC 8739, was maintained in stationary liquid culture at 37°C. and was transferred biweekly. The culture medium consisted of 1% tryptone, 0.5% yeast extract and 0.5% K_2HPO_4.

For the isolation of threonine dehydrase, E. coli was grown in 18 liters of the above medium dispensed in 20 liter glass carboys, or in the maximum volume of a 10 gallon stainless steel fermenter (New Brunswick Scientific Company). Growth from a 10% inoculum proceeded for 20-24 hours at 37°C. without agitation or aeration.

Clostridium tetanomorphum, ATCC 3606, was maintained anaerobically at 30°C. on a medium containing 500 g. of beef liver, 10 g. of peptone, 1 g. of K_2HPO_4, 5 g. of yeast extract and 1 l. of distilled water; the final pH of the medium was adjusted to 7.0.

Larger quantities of cells were obtained from a glutamate medium as described by Tokushige et al. (23). This procedure also includes the method for preparing cell-free extracts.

Chemical

Materials

C^{14}-L-threonine, sodium glutathione, 5'-AMP, 2'-AMP and 3'-AMP were purchased from Schwarz Bioresearch, Incorporated. Polyadenylic acid was obtained from the Miles Chemical Company. ADP, ATP, cytidine monophosphate (CMP), inosine monophosphate

(IMP), NAD, NADH, and NADPH were products of Pabst Laboratories. All amino acids, protamine sulfate, vitamin B_6 derivatives, and other nucleotides or nucleotide analogs were from the California Corporation for Biochemical Research. Bovine serum albumin and α-ketobutyric acid were products of the Sigma Chemical Company.

Enzymes used as excess reagents were obtained as follows: crystalline lactic dehydrogenase, D-amino acid oxidase and catalase were from the Worthington Biochemical Corporation; fungal protease Type VI (Pronase), L-amino acid oxidase, Type III, alkaline phosphatase, Type I, of calf mucosa, and yeast hexokinase were purchased from the Sigma Chemical Company; triose phosphate isomerase-α-glycerophosphate dehydrogenase (mixture of crystals) was from C. F. Boehringer and Sons; L. Light and Company supplied the glucose-6-phosphate dehydrogenase.

C^{14}-L-serine, $NaBH_4$-H^3 and a C^{14}-benzoic acid standard were purchased from New England Nuclear Corporation. Tritiated water, specific activity = 1 curie/g., was purchased from Volk Radiochemical Company; a tritiated water standard was obtained from the Packard Instrument Company. Deuterium oxide, 99.7%, was purchased from the Liquid Carbonic Division of the General Dynamics Corporation. Oxygen-18 enriched water (5.58 atom % O^{18}) was purchased from the Yeda Research and Development Company, Weizmann Institute, Rehovoth, Israel.

All ion-exchange resins were obtained from the California Corporation for Biochemical Research. Sephadex was purchased from Pharmacia, Limited.

N^6-pyridoxyllysine was first prepared by adapting the procedure for the synthesis of N^6-β-glyceryllysine (56):25 mg. of pyridoxamine dihydrochloride and 165 mg. of 5-δ-bromobutylhydantoin (57) were dissolved in 10 ml. of 70% ethanol, adjusted to pH 9.5 with saturated

NaOH, and refluxed for 40 hours. The pH was checked at 12-hour intervals and solid Na_2CO_3 added to restore the pH to approximately 9 when necessary. After 40 hours, the solvent was removed in vacuo, and the residue resuspended in 1 ml. of cold water. The precipitate which did not go into solution (unreacted bromobutylhydantoin) was removed by centrifugation and discarded. The supernatant was then passed through a Dowex-50 (H^+) column, and the column was washed with water until no material absorbing at 290 mμ was eluted. This eluate was concentrated in vacuo, made 2 M in NaOH and hydrolyzed at 80° C. for 8 hours under nitrogen in a Thunberg tube. The solution was then neutralized with 6 M HCl and passed through a column of AG 11A8 ion-retardant resin to remove NaCl. The ninhydrin-positive fraction from this column was concentrated in vacuo and characterized.

This material was homogeneous on cellulose acetate electrophoresis at two pH values, but paper chromatography of larger amounts (n-butanol-acetic acid-water, 60:15:25) revealed two minor components in addition to the major spot. This preparation of N^6-pyridoxyllysine was strongly fluorescent under ultraviolet light and had maximum absorption at 325 mμ at pH 7.0 (45).

An alternative and more convenient procedure for the preparation of N^6-pyridoxyl lysine was that recently described by Krebs and Fischer (45). This procedure involved the $NaBH_4$ reduction of a Schiff base formed between pyridoxal and N-α-carbobenzoxy-L-lysine (58). Removal of the carbobenzoxy group by treatment with 2 N HBr in glacial acetic acid at room temperature for 2 hours and subsequent evaporation in vacuo yielded N^6-pyridoxyllysine. This material had the same electrophoretic and spectral characteristics as that described above, but paper chromatography revealed a small lysine contamination.

Lanthionine sulfoxide and sulfone were prepared as described by Zahn and Osterloh (59). Over-oxidation to unknown products seemed to cause some difficulty, but this was largely overcome by careful temperature control.

For the preparation of N^6-(DL-2-amino-2-carboxyethyl)-L-lysine, commonly referred to as lysalanine, N-acetyldehydroalanine was prepared (60, 61) and 60 mg. were reacted with excess cupric complex of L-lysine at pH 10, 50° C., and under flowing N_2 gas. After 12 hours, the solution was acidified with concentrated HCl and H_2S bubbled through to remove Cu^{++}. After removal of CuS by centrifugation, the solution was evaporated to 0.1 volume and the precipitates of any unreacted N-acetyldehydroalanine and L-lysine were centrifuged off and discarded. The final supernatant contained N-acetyl lysalanine and some lysine. Concentrated HCl was added to a final concentration of 2 N and the solution was refluxed for 2 hours to remove the N-acetyl group. This solution was evaporated to dryness in vacuo and redissolved in water.

Lysine-copper complex was replaced in later syntheses by N-α-carbobenzoxy-L-lysine (58). Removal of the carbobenzoxy group was achieved by treating with 2 N HBr in glacial acetic acid at room temperature for 2 hours.

Alpha-tritio threonine was prepared by performing the racemization of DL-allo threonine as described by Akabori et al. (62), in the presence of T_2O. The preparation was carried out on a 2 mmole scale in the presence of tritiated water of specific activity of 0.6 mcurie per mmole of H_2O. The thrice-recrystallized mixture of threonine isomers was obtained in 23% yield and had a specific activity of approximately 0.06 mcurie per mmole. DL-threonine was separated from DL-allo threonine by preparative paper chromatography on Whatman 3 MM paper in the solvent system 2-butanone, n-butanol, water and cyclohexylamine (5:5:2.5:1), as recommended by Flavin and Slaughter (63).

Determinations and Procedures

Threonine in culture media was determined by the colorimetric procedure of Desnuelle and Naudet (64). For a more sensitive threonine determination, the procedure of Flavin and Slaughter (65) was used. This involved oxidation of threonine to acetaldehyde with periodic acid, followed by spectrophotometric determination of acetaldehyde as NADH oxidation in the presence of excess yeast alcohol dehydrogenase. All threonine isomers are measured equally. A specific method for L-threonine was developed utilizing purified threonine dehydrase which was shown to have high specificity (Table IV). This assay was similar to that used for determination of threonine dehydrase activity, but employed excess dehydrase. Thus the amount of NADH oxidized was a measure of the amount of L-threonine present (see below). Serine was estimated colorimetrically by the method of Lambert and Neish (66).

Alpha-keto acid determinations were performed either by the semicarbazide method of Macgee and Doudoroff (67) or by a spectrophotometric-enzymatic method similar to that used for α-ketobutyrate determination in threonine dehydrase assays. When desired, the separation of α-ketobutyrate from reaction mixtures was achieved by column chromatography on Dowex-1-chloride. Following a brief washing with water, the α-ketobutyrate was eluted with a linear gradient of 0 to 0.15 M HCl.

Threonine was isolated from reaction mixtures, purified and crystallized as described by Flavin and Kono (68).

Electrophoresis experiments were performed on cellulose acetate strips (2.2 x 12 cm.). The equipment included an electrophoresis chamber (Colab) and a power supply (Heath Co.). Electrophoresis, drying and staining were carried out as described by Scherr (69).

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Additional experiments were conducted with a Veronal buffer, pH 7.5, in place of the acetic acid-formic acid buffer, pH 2.0, of Scherr.

Spectrophotometric assays were conducted in silica cuvettes of 1.0 cm. light path. Absorbance changes were measured in a Gilford Multiple Sample Absorbance Recorder attached to a Beckman DU monochromator.

Linear sucrose gradients (5 - 20% w/w) were prepared with a gradient maker constructed from Lucite as described by Martin and Ames (70). The total volume of each gradient was 4.3 ml., and 0.10 ml. of enzyme sample was usually layered carefully on each gradient contained in 0.5 x 2 inch lusteroid centrifuge tubes. Centrifugation was carried out in a pre-cooled Spinco SW-39 rotor in the Spinco Model L ultracentrifuge. After a slow manual acceleration, the speed was set at 35,000 rpm and centrifugation continued for 15-17 hours. The sample temperature was maintained between 10 and 15° C. by adjusting the coolant temperature to 13° F. The rotor was allowed to coast to a stop and the gradients removed; 30-35 fractions of 10 drops each were collected by puncturing the centrifuge tubes with a 22-gauge hypodermic needle without Luer fitting mounted on a ring stand support (70). The fractions were stored in ice until all the assays were completed.

Isotopic

All quantitative radioisotope measurements, either tritium or carbon-14, were performed in a Packard Tri-Carb liquid scintillometer. Three scintillation systems were used, depending largely on the isotope to be measured and the volume to be sampled. The system of Kinard (71) was used primarily for counting C^{14} in aqueous samples of up to one ml.; the system of Werbin (72) was used for counting tritium and C^{14} when the volume of aqueous samples was 1 to 3 ml. The latter

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system was also employed when radioactivity on cellulose acetate strips was to be determined, because the strips were soluble in this solvent system and geometric considerations were eliminated; the same system was used for counting tritium on paper strips because of its high efficiency. However, reproducible positioning of the paper was essential in this case. For routine counting of C^{14} on paper strips, the system and procedure of Wang and Jones (73) was used because of its simplicity and low cost.

Efficiencies for C^{14} and H^3 were determined by adding known amounts of radioactive standards to already counted samples in bottles and then recounting.

When a preliminary location of C^{14} on paper chromatograms was desirable, a Nuclear Chicago thin-window gas flow counter[1] was used. Radioautography was performed on Kodak No-Screen X-ray film in standard film holders.

Combustion of samples and analyses for deuterium content by the falling drop method were performed by Mr. Josef Nemeth, 303 W. Washington Street, Urbana, Illinois.

Samples for 0^{18} analysis were combusted with $HgCl_2$ as described by Rittenberg and Ponticorvo (74). The CO_2 obtained was then analyzed on the Michigan State University mass spectrometer. The 0^{18} content in the H_2O^{18} used in these experiments was determined at the termination of each incubation by removing the water in vacuo and trapping it in a dry ice-acetone bath. The method of Boyer et al. (75) was employed for the conversion of 0^{18} in H_2O^{18} to $CO_2{}^{18}$, and the CO_2 was then analyzed as described above. Several standard samples of 0^{18}-enriched KH_2PO_4 were generously supplied by Dr. Clarence Suelter, as were instructions and assistance in performing these combustions.

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[1]Dr. N. E. Tolbert graciously permitted the use of this instrument.

Enzymatic

A number of commercially available enzymes were employed as standards in this study. Yeast and horse liver alcohol dehydrogenases were obtained as lyophilized powders from Worthington Biochemical Corporation and were assayed as described by Vallee and Hoch (76), but with a 15-fold reduction in volume. Malate dehydrogenase and fructose diphosphate aldolase from muscle were obtained from Worthington Biochemical Corporation as suspensions in $(NH_4)_2SO_4$. Malate dehydrogenase was assayed by the method of Mehler et al. (77); aldolase was assayed by the triose phosphate isomerase-α-glycerophosphate dehydrogenase method of Baranowski and Neiderland (78). A 15-fold reduction in volume was used in both malate dehydrogenase and aldolase assays.

2-Keto-3-deoxy-6-phosphogluconic (KDPG) aldolase was supplied as a crystalline suspension in $(NH_4)_2SO_4$ by Dr. H. P. Meloche and Mr. J. M. Ingram of this laboratory (79). Assays were conducted as described by Kovachevich and Wood (80).

Adenylate kinase was assayed by the rate of formation of reduced nicotinamide adenine dinucleotide phosphate (NADPH) in a coupled assay consisting of ADP, glucose, excess hexokinase from yeast and glucose-6-phosphate dehydrogenase (81). This assay was also scaled down 15-fold from the published procedure.

Assay and Preparation of Threonine Dehydrase

A coupled assay based on the oxidation of NADH was used for estimation of threonine dehydrase activity:

$$\text{Threonine} \xrightarrow{\text{Threonine Dehydrase}} \alpha\text{-ketobutyrate} + NH_3$$

$$\alpha\text{-ketobutyrate} + \text{NADH} \xrightarrow[\text{dehydrogenase}]{\text{excess lactic}} \alpha\text{-hydroxybutyrate} + \text{NAD}$$

The assay mixture for threonine dehydrase which was used for the major part of this work contained the following: 1.0 μmole of AMP, 1.0 μmole of GSH, 0.08 μmole of NADH, 0.025 mg. of lactic dehydrogenase (rabbit muscle, Worthington Biochemical Corporation), 15 μmoles of potassium phosphate buffer, pH 8.0, and a suitable dilution of the dehydrase. After a 5 minute pre-incubation of all components, 4.0 μmoles of L-threonine, pH 8.0, were added to initiate the reaction. All assays were run in a total reaction volume of 0.20 ml. Absorbance changes were measured at 340 mμ; the reaction temperature was usually 25° C.

A unit of threonine dehydrase is defined as that amount of enzyme which will produce an absorbance change of 1.0 per minute under the prescribed conditions. Based on a molar extinction coefficient of 6.22×10^3 for NADH, this unit equals 0.032 μmoles of threonine dehydrated per minute. Specific activity is defined as units per mg. of protein (protein determined by the method of Lowry et al. (82) with crystalline bovine serum albumin as standard).

The assay was shown to be highly reliable and linear with enzyme content for rates up to 0.2 OD change per minute; dilutions for assay were usually made in 0.1 M phosphate buffer, pH 8.0, containing bovine serum albumin, AMP, and GSH at 1 mg. per ml. concentration each.

For purification of threonine dehydrase, anaerobically grown E. coli were collected by continuous centrifugation, washed once with 0.1 M KCl, and resuspended in an equal weight of 0.1 M phosphate buffer, pH 8.0, containing 3×10^{-3} M each of AMP and GSH. The suspension was then subjected to sonic oscillation for 10 minutes at 10 Kc and 200 watts. The crude extract thus obtained was centrifuged at 30,000 x g and 3° C. for 30 minutes. Purification of the enzyme was conducted in the following manner. All steps were carried out at 0 to 3° in the presence of 3×10^{-3} M 2-mercaptoethanol.

Step 1. The protein content of the crude extract was diluted to 12 mg. per ml. with distilled water and $(NH_4)_2SO_4$ was added to 0.1 M concentration. One-fifth volume of 2% protamine sulfate was added with stirring, the precipitate collected by centrifugation and discarded.

Step 2. To the supernatent from Step 1, $(NH_4)_2SO_4$ was added to 1.2 M, and any precipitate which formed was collected and discarded. A further addition of $(NH_4)_2SO_4$ to 1.8 M precipitated the enzyme. This precipitate was redissolved in distilled water and the salt content was adjusted to 0.04 M (as determined from conductivity measurements) by further dilution with distilled water.

Step 3. $Ca_3(PO_4)_2$ gel was added to the diluted solution from Step 2 until nearly all of the enzyme was adsorbed by the gel; this usually required 1 g. $Ca_3(PO_4)_2$ per 100,000 units of enzyme. The gel containing the enzyme was washed once with 0.04 M $(NH_4)_2SO_4$ and then the enzyme was eluted from the gel by 2 washings with 0.10 M PO_4, pH 8.0. Approximately 50 ml. of eluant were required to elute 100,000 units.

Step 4. The eluate from $Ca_3(PO_4)_2$ was again treated with $(NH_4)_2SO_4$ and the fraction precipitating between 1.2 and 1.8 M was collected, dissolved in water, and the solution diluted with H_2O to a salt content of 0.02 M.

Step 5. Alumina C_γ gel was added (350 mg. per 100,000 units). When the major portion of the enzyme was absorbed, the gel was collected by centrifugation and the enzyme immediately eluted with 3 portions of 0.05 M phosphate, pH 7.0 (120 ml. per 100,000 units). When the majority of the enzyme was not adsorbed, the gel was collected and discarded. Only one batch of alumina C_γ gel tested failed to adsorb the enzyme, but in that instance, negative adsorption produced a purification comparable to that obtained with the positive adsorption.

Step 6. Protamine sulfate (2%) was added dropwise with stirring to the enzyme solution from Step 5 (1.25 ml. per 10 ml. of the enzyme solution). The sticky yellow precipitate which formed within 30 min. was collected by centrifugation and transferred to a beaker containing 25 ml. of 0.25 M phosphate buffer, pH 7.0. This suspension was slowly stirred at 0° C. for 12 hours.

Step 7. The slightly turbid solution from Step 6 was centrifuged and the clear supernatent was treated with $(NH_4)_2SO_4$. The fraction precipitating between 1.2 and 1.8 M was dissolved in 0.05 M phosphate buffer, pH 7.0, containing 3×10^{-3} M each of AMP and GSH. This solution was either used in the experiments to be described, or fractionated further.

Step 8. (Optional). The enzyme solution from the preceding step was diluted with water to a salt concentration of 0.05 M. This solution was transferred to the top of an ECTEOLA-cellulose column which had been previously equilibrated with 0.05 M phosphate buffer, pH 7.0. The column was washed with 20 ml. of the equilibration buffer, and then gradient elution was begun. All buffers contained 1×10^{-3} M AMP and 2-mercaptoethanol. A linear gradient (0 to 0.5 M phosphate, pH 7.0) was used as eluant. Threonine dehydrase eluted at a molarity of approximately 0.25 M phosphate.

Threonine dehydrase purified through Step 7 was quite stable when rapidly frozen in a dry ice-acetone bath and stored at -15° C. Activity remained essentially unchanged for at least 3 months, and over 50% of the original activity was present after 1 year. However, freezing of the enzyme in the presence of mercaptoethanol consistently destroyed the activity. Thus GSH was used in any step just prior to freezing.

RESULTS

Characteristics of Purified Threonine Dehydrase

Purification

A summary of the purification of threonine dehydrase is presented in Table I. A 54-fold purification was obtained with a 51% recovery of activity. No reliable values for the purification achieved by ECTEOLA-cellulose chromatography were obtained because the Lowry method for protein determination was unsuitable in the presence of high concentrations of AMP and 2-mercaptoethanol; chromatography in their absence resulted in considerable loss of activity. The data in this table were obtained from the fractionation of a crude extract of cells grown in 40 liters of medium, but essentially similar results were obtained on 2-fold larger or smaller scales.

Table I. Purification of threonine dehydrase from E. coli.

Step	Total Units	Specific Activity	Recovery (Percent)	Fold
Crude extract	67,000	35	100	--
Protamine treatment	85,300	--	127	--
$(NH_4)_2SO_4$ ppt. 1.2-1.8 M	60,000	62	90	1.8
$Ca_3(PO_4)_2$ gel eluate	45,000	188	68	5.5
$(NH_4)_2SO_4$ ppt. 1.2-1.8 M	48,500	346	72	10
Alumina C_γ gel eluate	39,600	1400	57	41
Protamine precipitate	40,000	--	60	--
$(NH_4)_2SO_4$ ppt. 1.2-1.8 M	34,000	1900	51	54
ECTEOLA chromatography	20,000	--	33	--

Effect of pH and Buffers

Threonine dehydrase exhibits a fairly sharp pH optimum near pH 8.0 in 0.06 M phosphate buffer. Of a variety of buffers tested, only cacodylate afforded more activity than phosphate. However, since cacodylate has a low buffering capacity at pH 8.0, phosphate was used for almost all of the tests. The enzyme stability optimum in phosphate buffer is near pH 7.0.

Stability

Stability of the enzyme in dilute solutions was markedly enhanced by AMP, GSH or 2-mercaptoethanol, and bovine serum albumin. Heat treatment, charcoal, organic solvents and pH values below 5 all had adverse effects on activity. The insoluble addition product of protamine and threonine dehydrase was very stable at pH 7.0. Dehydrase activity was reasonably stable upon dialysis when 2-mercaptoethanol was present in all solutions.

Inhibitors

KCN at 5×10^{-3} M concentration inhibited threonine dehydrase activity 90%. Hydroxylamine, pH 8, 5×10^{-4} M concentration, destroyed 95% of the dehydrase activity as measured in an enzymatic assay. These compounds were inhibitory only toward the dehydrase and, at the concentrations employed, did not interfere with the measurement of very small amounts of α-ketobutyrate by the lactate dehydrogenase assay system. This conclusion has been verified by comparison of the rates of ammonia release with and without inhibitor. Parachloromercuribenzoate (PCMB) inhibited threonine dehydrase 90% at 2.5×10^{-5} M concentration when tested in the absence of added GSH and compared to a similar assay containing no PCMB.

Activity was neither inhibited nor stimulated by the following: Fe^{++}, 1×10^{-3} M; F^{-}, 1×10^{-2} M; Ca^{++}, Mg^{++}, or Mn^{++}, 5×10^{-4} M; arsenite, 1×10^{-4} M; isoleucine, 1×10^{-2} M; and propionate, 1×10^{-2} M.

Activation by Adenosine monophosphate

A comparison of the ability of various nucleotides and nucleotide analogs to activate threonine dehydrase is shown in Table II.

Table II. Specificity of activation of threonine dehydrase. (Assays were performed as described in the text. The final concentration of activator was 5×10^{-3} M except where noted.)

Compound Tested	Percent of Activity with AMP
5'-AMP	100
CMP	48
Deoxy AMP	38
2'-AMP or 3'-AMP	27
ADP or ATP	20
GMP	17
UMP or IMP	4
3', 5'-cyclic AMP	0
Adenosine monosulfate	0
Adenosine monoacetate	0
Polyadenylic acid (2 mg/ml)	0

The values for ADP and ATP have been roughly corrected for the amount of AMP contamination as determined by chromatography (83). Of the nucleotides tested, only CMP and deoxy AMP exhibited appreciable activating ability. Lesser activation was displayed by 2'-AMP, 3'-AMP, ADP, ATP and GMP. It is worthy of mention that, unlike the situation with phosphorylase b (44), IMP has virtually no activating ability.
It is evident from the table that certain groups are required for activity,

notably an amino group on a purine or pyrimidine ring and a phosphoric acid residue esterified at position 5' on the nucleoside moiety.

Although a number of nucleotides could activate threonine dehydrase to varying extents, it was nevertheless considered desirable to examine commercial AMP for possible contamination by a highly active trace component. For this, 50 mg. of AMP were neutralized with KOH and streaked on Whatman 3 MM paper. Development of the chromatogram as described by Cormier (83) permitted the detection of two trace components in addition to AMP. Each of the three compounds was eluted and concentrated in vacuo prior to tests for activating ability. The results, presented in Table III, indicate that the AMP fraction was effective in activating threonine dehydrase and that the trace components were inactive at approximately 1/20 the AMP concentration (a concentration many fold greater than that present in AMP preparations). The exact identity of the trace components was not determined, but from other work (83), it is likely that one of the unknowns was 3',5'-diphosphoadenosine.

Table III. Chromatography of commercial AMP and activating ability of the various components. (The AMP examined was Schwarz Bioresearch Lot No. 6025. Chromatography was carried out as described in the text; concentrations are based on a molar extinction coefficient of 15.4×10^3 at 259 mμ. Each assay contained the same amount of dehydrase and rates were measured after 5 minutes preincubation.)

Component	Concentration	Activity (Δ A/min.)
$R_{AMP} = 0.30$	5×10^{-5} M	0.002
$R_{AMP} = 0.41$	6×10^{-5} M	0.002
$R_{AMP} = 1.0$	1×10^{-3} M	0.015
AMP (commercial)	1×10^{-3} M	0.016
None	---	0.002

Substrate Specificity

The ability of threonine dehydrase to dehydrate other hydroxy-amino acids and analogs is shown in Table IV. In agreement with the specificity of the biosynthetic threonine dehydrase of E. coli (29), the biodegradative dehydrase was found to be active only on L-serine and L-threonine. A trace of activity was observed on DL-allo threonine, but this could result from the presence of traces of L-threonine. In contrast, however, the threonine dehydrase from sheep liver is appreciably active on L-allo threonine (18). The K_m for threonine and serine were identical, 5×10^{-3} M, and very nearly identical maximum velocities were obtained. Since serine inactivates the dehydrase, it is important to consider only initial rates; otherwise it would be concluded (as has been done in the past) that serine is a poorer substrate (11, 15, 18).

Table IV. Substrate specificity of threonine dehydrase. (Excess crystalline malate dehydrogenase replaced lactate dehydrogenase in the usual assay when the β-hydroxyaspartate isomers were tested. All other compounds were evaluated in the normal assay system.)

Compound	Concentration	Percent of Threonine Activity
L-threonine	1×10^{-2} M	100
D-threonine	1×10^{-2} M	0
DL-allo threonine	2×10^{-2} M	3
L-serine	1×10^{-2} M	100
DL-homo serine[a]	2×10^{-2} M	0
DL-erythro-β-hydroxy-aspartate	2×10^{-2} M	0
DL-threo-β-hydroxy-aspartate	2×10^{-2} M	0
(DL + allo) - cystathionine	2×10^{-2} M	0
L-penicillamine	1×10^{-2} M	0
DL-homocysteine	2×10^{-2} M	0

[a]Inhibitory to normal threonine dehydration.

Activation by Pyridoxal Phosphate

A requirement for pyridoxal phosphate in threonine dehydration has been previously indicated (see Literature Review). The ability of pyridoxal phosphate to reactivate hydroxylamine-inactivated threonine dehydrase but not serine-inactivated enzyme is seen in Table V.

Table V. Resolution and pyridoxal phosphate reactivation of threonine dehydrase.

Inhibitor, Concentration	Activity after dialysis	Activity after Incubation		Stimulation by pyridoxal p
		Without pyridoxal p	With pyridoxal p[a]	
NH_2OH[b]:	Percent	Percent	Percent	Fold
1×10^{-4} M	77	81	72	< 1
1×10^{-3} M	35	15	52	3.5
5×10^{-3} M	5	4	27	6.7
1×10^{-2} M	0.1	0.1	6.3	63
None	100[d]	100[d]	95	< 1
Serine[c]:				
5×10^{-2} M	53	35	31	< 1
1×10^{-1} M	37	25	25	1
None	100[d]	100[d]	100	1

[a] The pyridoxal phosphate concentration was equal to 2×10^{-4} M.
[b] The incubation time was 2 hours.
[c] The incubation time was 4 hours.
[d] Arbitrarily set at 100%.

These experiments were performed in individual tubes, each containing 1 μmole of AMP, 1 μmole of NaGSH, 10 μmoles of potassium phosphate buffer, pH 8.0, approximately 50 units of dehydrase, and neutralized NH_2OH or L-serine at varying concentrations. The final volume was 0.2 ml. After 2 or 4 hours of incubation at 15° C., the dehydrase

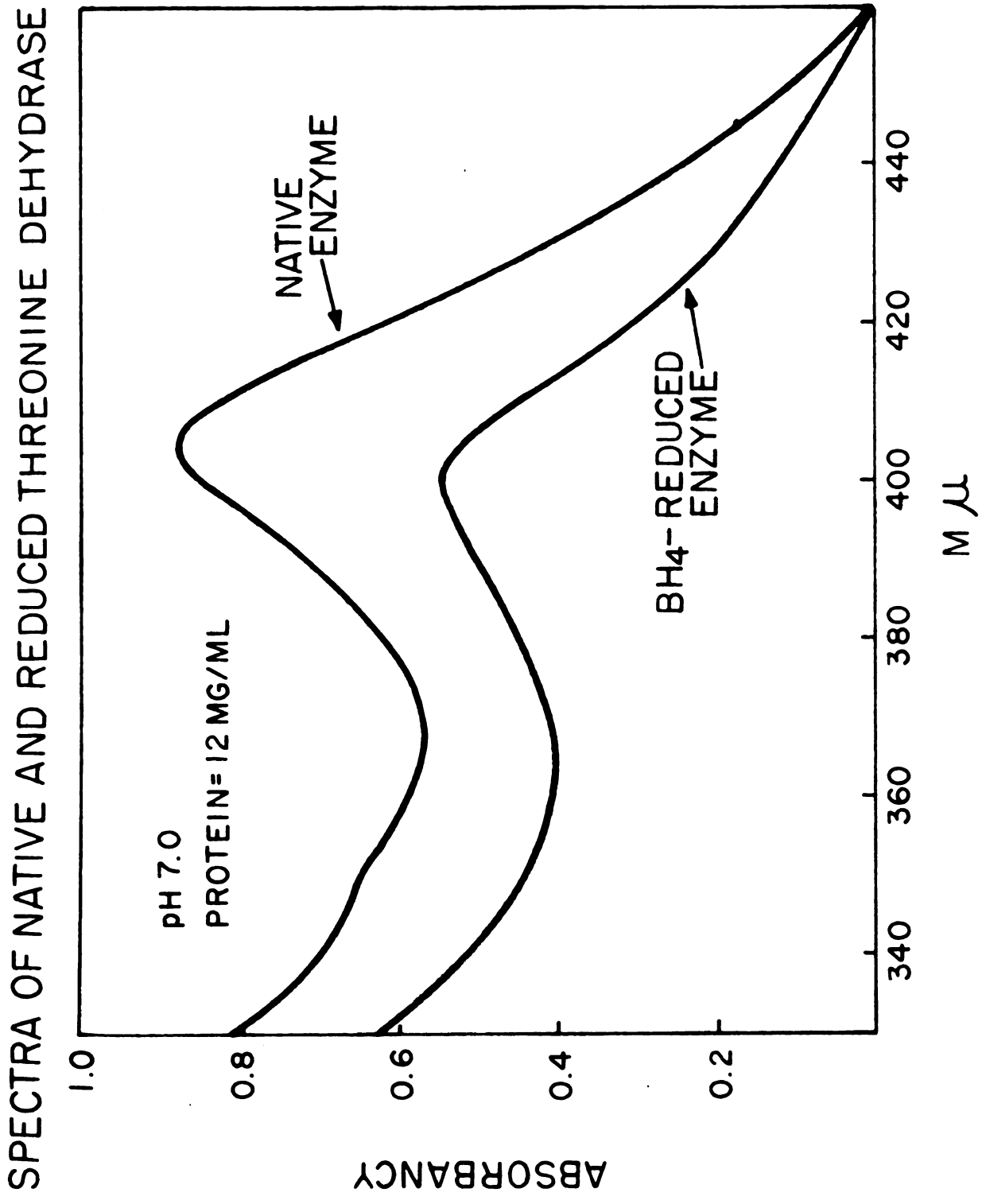
activity was determined on aliquots, and then the contents of each tube were dialyzed at 0° C. for 2 hours against 2 liters of 0.05 M phosphate buffer, pH 7.0, containing AMP and 2-mercaptoethanol, each 1×10^{-3} M. Aliquots of the dialyzed solutions were assayed, and then incubated with 2×10^{-4} M pyridoxal phosphate, and reassayed. The extent of reactivation was arbitrarily measured after 1 hour incubation at 15° C.

The results indicate that reactivation with pyridoxal phosphate was appreciable but by no means complete. Similar partial reactivation was observed by Umbarger (15). Attempts at complete resolution and reactivation at 37° C. as described by Nishimura and Greenberg (18) for the sheep liver threonine dehydrase were consistently unsuccessful, presumably because of enzyme instability at 37° C. The difference between the inactivating ability of NH_2OH in an enzymatic assay (95% inactivation at 5×10^{-4} M reported above) and in these mixtures (95% inactivation at 5×10^{-3} M NH_2OH) is ascribed to the differences in enzyme concentration under these two conditions.

Because the foregoing results were considered inconclusive proof of pyridoxal phosphate participation, additional experiments were performed. Fifty-fold purified threonine dehydrase was observed to have a pronounced yellow color at neutral pH. A limited spectrum analysis revealed an absorption maximum at 402 mμ (Figure 1, upper curve). This absorption maximum falls between that normally seen for free pyridoxal phosphate (388 mμ) and pyridoxal phosphate bound to protein as a hydrogen-bonded Schiff base (415-430 mμ). The free aldehyde form of pyridoxal phosphate bound to a protein has a maximum around 360 mμ (39).

Threonine dehydrase, (4.1 mg. of protein, specific activity of 1,900 units per mg.), was treated at pH 7 and 3° C. with a total of 10 μmoles of $NaBH_4 \cdot H^3$ (150 μc per μmole), followed by 8 μmoles of acetic acid. These were added in 4 equal portions at 3 minute intervals,

Figure 1. Spectra of native and borohydride-H^3 reduced threonine dehydrase. Spectral measurements were made on a Cary Model 15 recording spectrophotometer equipped with a beam masking system which permitted the use of 1.5 x 10 mm. microcuvettes with a volume of 0.35 ml. A solution containing all components except enzyme was used in the blank compartment. The enzyme used had a specific activity of 1,900 units per mg. of protein; spectra were made on 4.2 mg. of protein contained in a volume of 0.35 ml.


SPECTRA OF NATIVE AND REDUCED THREONINE DEHYDRASE
NATIVE ENZYME
BH4- REDUCED ENZYME
pH 7.0
PROTEIN= 12 MG/ML
ABSORBANCY
1.0
0.8
0.6
0.4
0.2
340
360
380
400
420
440
M μ

as described by Grazi et al. (84). After reduction, the intensity of absorption at 402 mμ decreased and activity was lost. The lower curve in Figure 1 is that exhibited after treatment with borotritide. Excess radioactivity was then removed from the reduced enzyme by four precipitations with ammonium sulfate and dialysis against 0.1 M phosphate buffer, pH 7.0; the protein concentration was readjusted to that prior to reduction and the spectrum recorded. The extent of reduction was approximately 72% as determined from the incorporation of 3.0×10^6 dpm of tritium[1] and assuming an enzyme purity[2] of 33% and three moles of bound pyridoxal phosphate per 80,000 g. of protein.

The nature of the component reduced by borotritide treatment was investigated through total enzymatic hydrolysis and chromatography of fragments. The reduced enzyme (4.1 mg. of protein) was mixed with 60 μg of a non-specific fungal protease and ethanol (10% final concentration). After 24 hours of incubation at 40° C., the solution was evaporated to a small volume and chromatographed in n-butanol - acetic acid - water (60:15:25) on Whatman No. 1 paper. The tritium was located in a narrow strip by counting directly cut sections. The radioactivity was found to be almost entirely located in one peak having an R_f value of 0.02. The corresponding area of the rest of the paper was eluted and further characterized by electrophoresis on cellulose acetate.

As shown in the upper part of Figure 2, at pH 7.5, the radioactivity migrated toward the cathode whereas synthetic N^6-pyridoxyllysine

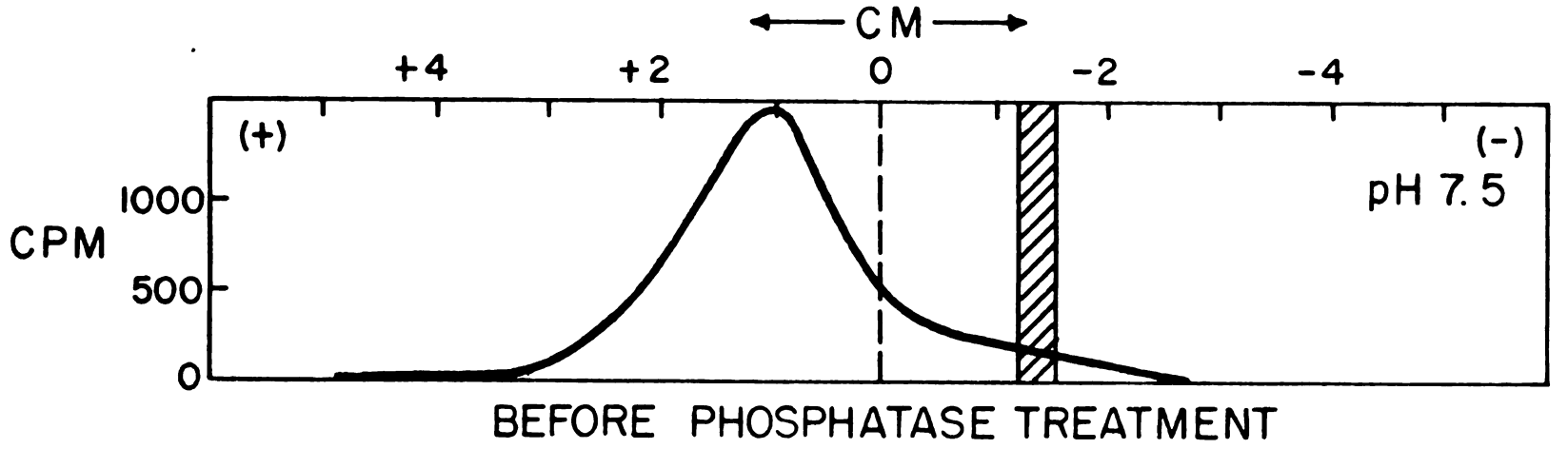
---

[1] $$\text{Percent reduction} = \frac{100 \times (3 \times 10^6 \text{ dpm T incorporated}) \times \frac{(80,000 \text{ μg protein})}{\text{μmole enzyme}}}{0.33 \text{ purity} \times 4,100 \text{ μg} \times \left(\frac{82 \times 10^6 \text{dpm T}}{\text{μatom T in } BT_4}\right) \times \left(\frac{3 \text{ moles pyridoxal}}{\text{mole enzyme}}\right)}$$

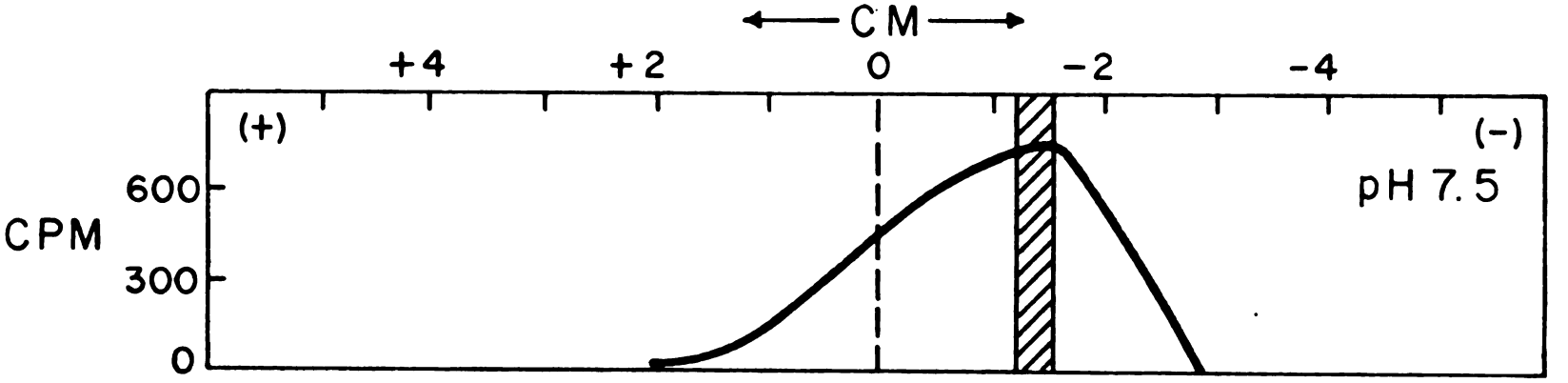
[2] Enzyme purity was based on analyses in the Model E analytical ultracentrifuge which revealed the presence of three components in nearly equal amounts in dehydrase preparations of specific activity 1,900. The basis for the assumption of three moles of pyridoxal phosphate per 80,000 g. of protein will be discussed later.

Figure 2. Identification of borotritide reduction product from threonine dehydrase. Cellulose acetate electrophoresis of synthetically-prepared N^6-pyridoxyllysine (shaded area, ninhydrin stained) and radioactive product obtained from proteolytic digestion of borotritide-reduced dehydrase (heavy solid line). These experiments were performed at pH 7.5 with 300 volts and 1.0 milliamps for 20 minutes. Three microliters of radioactive product were applied to each strip along with 1-2 μl. of synthetic pyridoxyl lysine (approximately 5 mg/ml).

IDENTIFICATION OF BOROTRITIDE REDUCTION PRODUCT FROM THREONINE DEHYDRASE



BEFORE PHOSPHATASE TREATMENT



AFTER PHOSPHATASE TREATMENT

located with ninhydrin moved toward the anode. Of the 2,500 cpm applied, 2,300 cpm were recovered in the peak. Then 25 μl. of the enzyme hydrolyzate (containing 25,000 cpm) was treated with 100 μg. (0.1 unit) of alkaline phosphatase from calf mucosa, 0.15 μmole of $MgCl_2$, and 10 μmoles of tris-chloride buffer, pH 9.0, in a final volume of 40 μl. After 3 hours at 20° C., the mixture was heated briefly and the protein removed by centrifugation. As shown in the lower diagram of Figure 2 after electrophoresis of the supernatant solution, the radioactivity maximum migrated identically with the color band of authentic N^6-pyridoxyllysine. In electrophoresis at pH 2.0, however, the synthetic compound and the radioactive product before phosphatase treatment migrated identically toward the cathode, and phosphatase treatment did not alter the direction of migration of the radioactive product. This result would be expected of phosphorylated or non-phosphorylated N^6-pyridoxyllysine since the phosphate group is not appreciably ionized at pH 2.

From the foregoing experiments, it was concluded that borohydride reduction of native threonine dehydrase leads to the reduction of the Schiff base between pyridoxal phosphate and the ε-amino group of lysine to form N^6-pyridoxyl phosphate lysine. The isolation of this fragment was considered important for the following reasons: (a) the presence of pyridoxal phosphate in native enzyme was thereby established; (b) a main linkage of pyridoxal phosphate to the native enzyme at neutral pH values is the Schiff base linkage, and this, in turn, indicates that a transaldimination mechanism for catalysis is probable; and (c) the amino group participating in the Schiff base structure is the ε-amino group of a lysyl residue.

Mechanistic Studies of Threonine Dehydration

One mechanism for α, β-elimination involves an intercarbon shift of a hydride ion. Such a mechanism has been observed by Abeles (85) in the dehydration of diols, and the process requires dimethylbenzimidazolylcobamide coenzyme. If this type of mechanism functions in threonine dehydration, hydrogen ions from water would not be incorporated into α-ketobutyrate and the α-hydrogen of threonine would migrate to the β-carbon of α-iminobutyrate and subsequently be found in α-ketobutyrate (Figure 3, left).

If desaturation is the primary event in threonine dehydration and α-aminocrotonate and α-iminobutyrate are intermediates as postulated by Chargaff and Sprinson (6), then all of the α-ketobutyrate formed should contain 1 atom of hydrogen from water as a result of the tautomerism of α-aminocrotonate to α-iminobutyrate (Figure 3, right) (4). Furthermore, the α-hydrogen atom of threonine should be lost to water during the formation of α-aminocrotonate.

Experiments were undertaken to distinguish between these two mechanisms by an examination of the origin of the β-hydrogens of α-ketobutyrate and the fate of the α-hydrogen of threonine.

Tritium Incorporation from T_2O

When threonine dehydration was conducted in tritiated water and the reaction interrupted after 50% utilization of threonine (15 minutes) or after 20 hours, and the α-ketobutyrate isolated, the results presented in Table VI were obtained. Virtually no incorporation was observed (0.013 and 0.07 gram atom of T per mole of α-ketobutyrate). The slightly higher incorporation noted in the long time incubation probably indicates a small amount of incorporation by non-enzymatic enolization

Figure 3. Alternative pathways for threonine dehydration. Hydrogens arising from solvent are shown in bold type; the α-hydrogen of threonine is shown in the shaded circles.

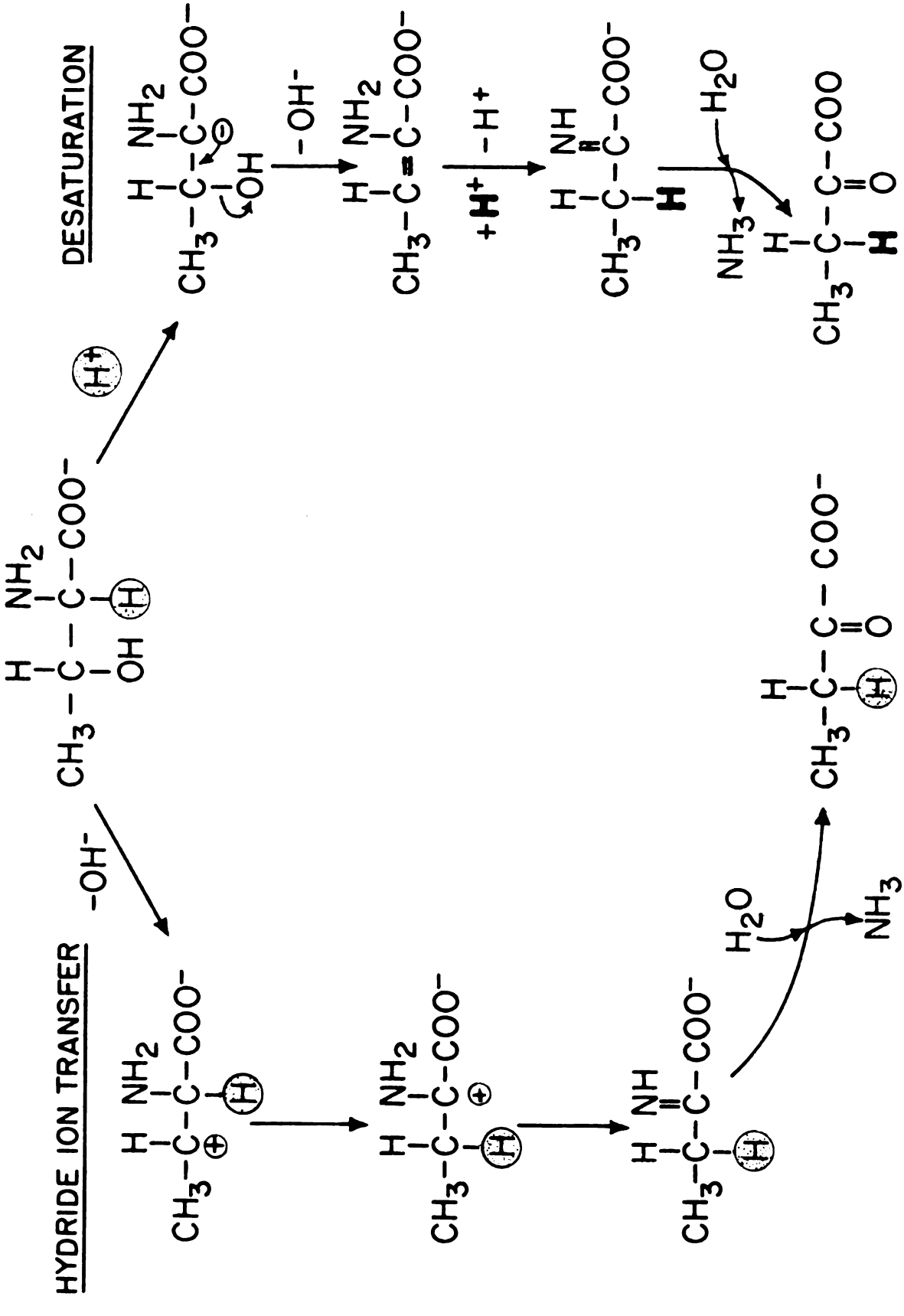

DESATURATION
HYDRIDE ION TRANSFER
-OH⁻
+H⁺
-OH⁻
-H⁺
H₂O
NH₃
H₂O
NH₃

Table VI. Threonine dehydrase reactions in tritiated water. (Each incubation mixture contained 40 μmoles of L-threonine, 5 μmoles of AMP, 5 μmoles of NaGSH, 40 μmoles of phosphate buffer, pH 8.0, and 40 units of dehydrase in a final volume of 0.40 ml.)

Incubation Time (Hrs)	Specific Radioactivity T_2O dpm/μatom	Specific Radioactivity Keto Acid dpm/μmole	Keto Acid Recovery	μgatoms T Incorporated per μmole
0.25	1.25×10^6	1.55×10^4	90% [a]	0.013
20.0	1.25×10^6	8.75×10^4	80	0.070

[a] Based on 20 μmoles of α-ketobutyrate present at 50% reaction.

of α-ketobutyrate. As judged by this lack of incorporation, the α-hydrogen of threonine should be transferred by the hydride transfer mechanism (Figure 3, left).

The Fate of Tritium in the Dehydration of α-Tritio threonine

DL-α-tritio threonine, synthesized as described in "Methods and Materials," was incubated with threonine dehydrase until the L-isomer was completely dehydrated as determined by α-ketobutyrate assay (semicarbazide method). When dehydration was complete, the incubation mixture was poured onto a Dowex-1-chloride column (1.1 x 5 cm.) and washed with 50 ml. of water. The wash was then concentrated to dryness using an acetone-dry ice trap to yield a volatile fraction (water) and a residue (D-threonine). The α-ketobutyrate was eluted from the column in the usual fashion. Radioactivity was determined in the water, threonine and the α-ketobutyrate (Table VII).

These results clearly show that the α-hydrogen of threonine was lost to the water, and virtually no transfer of this hydrogen to the

Table VII. Enzymatic dehydration of α-tritio threonine. (Each reaction contained 5 μmoles of AMP, 5 μmoles of NaGSH, 100 μmoles of phosphate buffer, pH 8.0, 250 units of dehydrase (specific activity = 1,300), 2.5 μmoles of L-threonine and 5 μmoles of DL-α-tritiothreonine in a final volume of 1.0 ml.)

Enzyme	Disintegrations per Minute				Isotope Recovery (Percent)
	Substrate	Keto Acid	HTO	Residue	
Native	362,000	650	172,000	168,000	96
Boiled	362,000	0	1,950	358,000	99

β-position of α-ketobutyrate occurred. A control with boiled dehydrase handled in the same manner showed that no spontaneous exchange took place between α-tritio threonine and water.

The conflict between the lack of incorporation of tritium from T_2O and the release of tritium from α-tritio threonine appeared to result from an isotope discrimination in which hydrogen incorporation was considerably favored over that of tritium because of the extremely low molar quantity of tritium present. This discrimination could be eliminated by running the dehydration in 99.7% D_2O. In this case, if sources of exchangeable H were minimized, the rate of dehydration could be lower but discrimination in favor of H would be impossible or greatly reduced.

Experiments with Deuterium oxide

After enzymatic dehydration of 1.5 mmoles of threonine in 99.7% D_2O for 20 hours, approximately 1 mmole of α-ketobutyrate had been formed. Alpha-ketobutyrate was isolated by chromatography on a 1.4 x 14 cm. column of Dowex-1-chloride. The pooled fractions containing α-ketobutyrate were treated with 0.33 volume of 1% 2,4-dinitrophenylhydrazine in 2 N HCl and the α-ketobutyric acid dinitrophenylhydrazone

recovered. One hundred thirty mg. of compound were obtained after four recrystallizations from a 3:1 methanol-water mixture. The melting point was 200.5-201° C. (uncorrected).

A control reaction containing 1.0 mmole of potassium α-keto-butyrate (86) in place of L-threonine was carried through the same procedure in order to partially correct for enzymatic and nonenzymatic enolization of the keto acid. After four recrystallizations, 99 mg. of the authentic α-ketobutyric acid dinitrophenylhydrazone were obtained. The melting point was 199.5-200° C. (uncorrected).

Table VIII tabulates the results of the deuterium analyses and indicates that after subtraction of a rather substantial control value, approximately 0.79 gram atom of deuterium was incorporated per mole of α-ketobutyrate. This incorporation is in basic agreement with a route involving desaturation, and tautomeric rearrangement of α-amino-crotonate as was also the release of tritium from α-tritio threonine shown in Table VII.

Table VIII. Threonine dehydrase reactions in deuterium oxide. (Aqueous solutions containing L-threonine, 1.5 mmoles, 40 μmoles of AMP, 60 μmoles of NaGSH and 750 μmoles of phosphate buffer were lyophilized together and redissolved in 7.0 ml of 99.7% D_2O. Approximately 1,000 units of dehydrase (specific activity =1,300) were precipitated with $(NH_4)_2SO_4$ and the well-drained precipitate dissolved in 0.2 ml. of 99.7% D_2O. Alpha-ketobutyrate, 1.0 mmole of the K^+ salt, replaced L-threonine in the control reaction.)

Compound Analyzed	Atom Percent Excess D	Hydrogen Atoms Per Mole	Atoms D Per Mole	Δ
α-Ketobutyrate DNP-hydrazone (enzymic)	14.12	10	1.41	0.79
α-Ketobutyrate DNP-hydrazone (control)	6.18	10	0.62	

The value of 0.79 gram atom per mole of α-ketobutyrate is considered to be a minimum figure with the true value approaching one gram atom of deuterium incorporated per mole. This conclusion is based on the assumption that the amount of incorporation in this type of control would be higher than the corresponding spontaneous incorporation in the enzymatically produced α-ketobutyrate because: (a) the concentration of α-ketobutyrate available for enolization was initially higher in the control than in the enzymatic reaction which started at zero α-ketobutyrate, and (b) the rate of deuterium incorporation by enolization into the added β-diprotio-α-ketobutyrate of the control would be faster than deuterium incorporation by enolization with the enzymatically produced β-protio-β-deuterio-α-ketobutyrate, which already contained 50% of the maximum deuterium content. In the latter case, a substantial proportion of the exchanges would only involve an exchange of D for D.

Back-incorporation of Deuterium from D_2O and and O^{18} from H_2O^{18} into Threonine

The data presented in Table IX for deuterium and O^{18} back-incorporation were obtained in separate experiments. Each experiment was interrupted when exactly 50% of the 2 mmoles of threonine had been utilized as measured by threonine disappearance by the alcohol dehydrogenase assay. Reisolation of the remaining threonine and crystallization (68) yielded from 40 to 90 mg. of the nearly pure dipolar ion of threonine. For the experiments with deuterium, 99.7% D_2O was used; for experiments with H_2O^{18} the O^{18} final concentrations were 4.77 atom percent excess for the native enzyme incubation and 4.53 atom percent excess for the boiled control. The results in Table IX indicate that about 25% of the threonine molecules had incorporated deuterium and an equal amount had incorporated O^{18}. These data indicate that some dehydrated species such as α-aminocrotonate was rehydrated to form threonine containing deuterium or O^{18}.

Table IX. Enzymatic back-incorporation into threonine of O^{18} and deuterium from H_2O^{18} and D_2O. (Reactions mixtures were identical to those in Table VIII, except all reactants and volumes were increased one-third. Control reactions contained boiled dehydrase.)

Compound Analyzed	Mass Ratio $\frac{44}{46}$	Atom Percent	Atom Percent Excess	Atom Percent Excess in Water	Atoms O or H Per Mole	Atoms Incorporated Per Mole	Δ
Threonine from Enzyme + H_2O^{18}	0.01266	0.629	0.422	4.77	3	0.266	0.248
Threonine from H_2O^{18} Control	0.00470	0.234	0.027	4.53	3	0.0179	
CO_2^{18} Natural Abundance	0.00414	0.207	---	---	---	---	
Threonine from Enzyme + D_2O	---	---	3.03	> 99.5	9	0.272	0.260
Threonine from D_2O Control	---	---	0.13	> 99.5	9	0.012	

Non-enzymatic rehydration of α-aminocrotonate was ruled out in the case of O^{18} incorporation by the observation that the isolated O^{18}-threonine was pure L-threonine as measured by the excess threonine dehydrase assay, and contained no D-threonine or DL-allo threonine which would be expected if non-enzymatic rehydration were occurring.

Isolation of an Intermediate in Threonine Dehydration

Further evidence that the dehydration proceeds via an unsaturated intermediate was obtained by treating dehydrase reaction mixtures containing C^{14}-L-threonine with borohydride. The reaction mixtures contained 1 μmole of AMP, 1 μmole of NaGSH, 30 μmoles of potassium phosphate buffer, pH 8.0, 10 units of dehydrase and 4 μmoles of C^{14}-L-threonine (specific activity = 0.1 μcurie per μmole) in a final volume of 0.25 ml. During the reaction at 3° C., 10 μmoles of KBH_4, followed by 8 μmoles of acetic acid, were added as described previously for borotritide reduction. The reaction mixture was then chromatographed on Whatman No. 1 paper in 77% ethanol - water or t-butanol - 2-butanone - water - cyclohexylamine (40:20:40:4) and amino acids located by ninhydrin spray or radioautographs developed after 72 hours exposure. A major spot corresponding to threonine was observed, but there was in addition a new ninhydrin-positive and radioactive compound which subsequently co-chromatographed exactly with authentic DL-α-aminobutyrate in the above two solvent systems. The only other labeled compound was identified as C^{14}-α-ketobutyrate or C^{14}-α-hydroxybutyrate.

No α-aminobutyrate could be detected after incubation of mixtures of α-ketobutyrate, ammonia, enzyme and KBH_4 and no dilution of radioactivity in C^{14}-α-aminobutyrate was observed when C^{14}-L-threonine was dehydrated enzymatically in the presence of a large pool of unlabeled

α-ketobutyrate. Both of these results are consistent with the postulate that α-aminobutyrate was not formed from α-ketobutyrate, but rather was formed from an intermediate produced enzymatically.

Since the moles of α-aminobutyrate formed far exceeded the moles of enzyme present, the reduction of a free intermediate was indicated. The intermediates postulated to exist between threonine and α-ketobutyrate are α-aminocrotonate and α-iminobutyrate. Although either of these compounds could be reduced by suitable agents to α-aminobutyrate, the nature of borohydride as a reducing agent favored α-iminobutyrate as the immediate precursor of α-aminobutyrate. To establish the nature of the precursor, reduction was carried out with H^3-labeled $NaBH_4$. The tritiated aminobutyrate was isolated and purified by chromatography as above. This material was then oxidized to α-ketobutyrate with a mixture of D- and L-amino acid oxidases. When the oxidation was complete, the mixture was poured on a column of Dowex-1-chloride and washed with 50 ml. of water. The wash fraction was lyophilized and the radioactivity estimated in both the volatile fraction (water) and the non-volatile residue (unreacted substrate). Keto acid was recovered by the usual gradient technique and analyzed for radioactivity. A control with boiled oxidases was treated in the same manner.

If α-aminocrotonate were reduced by borotritide, the tritium would be present in the α and β carbons of α-aminobutyrate and would yield β-tritio-α-ketobutyrate upon oxidation. If α-iminobutyrate were reduced to α-tritio-α-aminobutyrate, oxidation would yield unlabeled α-ketobutyrate. Table X shows that after oxidation by D- and L-amino acid oxidases, tritium was found only in water, indicating that α-tritio-α-aminobutyrate was oxidized. Although only a small part of the substrate was oxidized, these results indicate that the precursor of α-aminobutyrate was α-iminobutyrate rather than α-aminocrotonate. The control reaction showed that no tritium exchange with water had occurred in the absence of active oxidases.

Table X. Oxidation of tritiated α-aminobutyrate. (The tritium-labeled α-aminobutyrate, isolated from chromatograms of reaction mixture treated with $NaBH_4 \cdot H^3$ as described in the text, was incubated with 1.5 units of L-amino acid oxidase, 0.4 unit of D-amino acid oxidase, 30 units of catalase, 25 μmoles of tris-chloride buffer, pH 7.8, 25 μmoles of KCl, and 2.5 μmoles of unlabeled DL-α-aminobutyrate for 4 hours at 38° C. The above enzyme units from the manufacturer's labels are equal to 1 μmole of substrate decomposed per minute.)

	Disintegrations per Minute				Isotope Recovery
Enzymes	Substrate	Keto Acid	HTO	Residue	(Percent)
Native	254,000	0	68,000	148,000	85
Boiled	254,000	0	3,100	162,000	65

In a second experiment of the same type, a dehydrase reaction mixture containing C^{14}-L-threonine as substrate was treated with $NaBH_4 \cdot H^3$ as described previously. The H^3, C^{14}-α-aminobutyrate was isolated and purified by paper chromatography in an ethanol - water (77%) solvent system and a n-butanol - acetic acid - water solvent (60:15:25). Oxidase treatment was performed as before but a more active preparation of D-amino acid oxidase was available for this experiment. At the completion of the reaction, the flask contents were lyophilized directly and the reaction solvent (water) recovered. The dry residue was then redissolved in H_2O, placed on a Dowex-1-chloride column, and washed with 25 ml. of H_2O to yield a fraction containing unreacted materials. The α-ketobutyrate was eluted from the column by the usual gradient technique, and both C^{14} and H^3 radioactivities were estimated in all fractions. The results of this oxidation are shown in Table XI. These results confirmed those obtained from the first experiment, and indicated that almost all of the tritium is released from α-aminobutyrate into water

Table XI. Oxidation of H^3, C^{14}-α-aminobutyrate. (The α-aminobutyrate was isolated from reaction mixtures as described in the text. This material (0.1 ml.) was incubated with 2 units of L-amino acid oxidase, 2 units of D-amino acid oxidase, 30 units of catalase, 100 μmoles of tris-chloride buffer, pH 7.8, and 5.0 μmoles of unlabeled DL-α-aminobutyrate for 3 hours at 37° C. The final volume was 1.0 ml. Unlabeled potassium α-ketobutyrate (5.0 μmoles) was added to the control mixture after incubation was complete.)

Enzymes	Disintegrations per Minute								Isotope Recovery	
	Substrate		Keto Acid		HTO		Residue			
	H^3	C^{14}	H^3	C^{14}	H^3	C^{14}	H^3	C^{14}	H^3	C^{14}
									(Percent)	
Native	145,000	16,300	5,100	3,500	118,000	2,180	16,900	10,500	96.5	99
Boiled	145,000	16,300	0	140	9,500	420	117,800	12,100	88	82

by oxidase treatment, and thus is in agreement with an α-tritio-α-aminobutyrate structure. The use of both H^3 and C^{14} labels and the ratio of activities served to indicate the purity of the α-aminobutyrate used in this experiment. In addition, the location of the various compounds during the chromatographic phase of the experiment was considerably simplified by the use of the C^{14} label.

Identification of a Cysteinyl Residue in Proximity to the Active Site of Threonine Dehydrase

The nature of the inactivation of threonine dehydrase during dehydration of serine has been a mystery in view of the fact that dehydration of threonine exhibits no such inactivation. A recent postulate (18) that an oxazolidine ring conjugate was formed between serine and pyridoxal phosphate does not explain the inability of threonine to behave similarly. The only support for such a postulate was the observation that added pyridoxal phosphate overcame the inactivation. This finding, however, could not be confirmed in the present study (see Table V).

A more plausible explanation could be predicted from the results of treating dehydrase reaction mixtures with borohydride. The hydrolysis of α-iminobutyrate is sufficiently slow to allow a substantial reduction of the α-imino acid to α-aminobutyrate with borohydride as shown above. Since addition of borohydride during the dehydration of serine did not yield detectable alanine, it is apparent that α-iminopropionate is much more rapidly hydrolyzed. Thus it could be predicted also that α-aminoacrylate would be much more reactive than α-aminocrotonate.

The propensity for nucleophilic addition to the double bond of α-aminoacrylate derivatives is well-known (87, 88). Consequently, it was considered likely that serine inactivation could result from the addition of α-aminoacrylate to some nucleophilic group on the enzyme.

The most likely candidates as nucleophiles are SH groups of cysteinyl residues and ϵ-NH_2 groups of lysyl residues. In the former case, the expected product of aminoacrylate addition would be lanthionine; the latter condensation would yield lysalanine.

To investigate this possibility, the dehydrase was inactivated by dehydration of C^{14}-L-serine and after removal of the free C^{14}, hydrolysis and chromatographic identification of any radioactive components was undertaken. For inactivation, the reaction mixture contained 0.5 ml. of C^{14}-L-serine (50 μcuries, 0.4 μmole), 50 μmoles of C^{12}-L-serine, 300 μmoles of phosphate buffer, pH 8.0, 1 mg. of bovine serum albumin and 60 units of AMP-free threonine dehydrase (specific activity = 1,000 units per mg.). Further additions were made as follows: after 10 minutes reaction, 1 μmole of AMP was added; at 30 minutes, 60 additional units of threonine dehydrase were added; at 40 minutes, 10 μmoles of C^{12}-serine were added; at 45 minutes, 1 μmole of AMP was added; at 50 minutes, 30 units of threonine dehydrase were added. After 1 hour of reaction, the entire mixture (final volume = 1.0 ml.) was transferred to a 1.1 x 26 cm. Sephadex G-25 column. The column was developed with water and 10-drop fractions were collected.

The effluent equal to the void volume from the column was pooled and evaporated to 1 ml. The protein was precipitated with 1 ml. of 10% trichloroacetic acid (TCA) containing 1 mg. per ml. C^{12}-serine, centrifuged and the precipitate washed 3 times with 2 ml. of 5% TCA containing 1 mg. per ml. C^{12}-serine and then washed twice with 2 ml. of ether. The washed and dried pellet was resuspended in water and treated with 50 μg. of fungal protease as described previously. After 24 hours, the mixture was evaporated to dryness and taken up in 10% isopropanol. For further characterization beyond chromatography, certain chromatograms were eluted and subjected to a 6 hour hydrolysis at 121° C. in 6 N HCl in a sealed, evacuated tube. This material was then evaporated to dryness *in vacuo* and the chromatographic identification repeated.

Paper chromatography was conducted in the following solvent systems: n-butanol - acetic acid - water, 60:15:25; n-butanol-pyridine - water, 1:1:1; phenol - water, 100:20, with and without a 0.3% NH_3 atmosphere; and 77% ethanol - water. Radioactivity was located by direct counting of cut paper sections (1 x 2 cm.).

Table XII presents the R_f values obtained for unknown radioactive compounds and for authentic standard samples.

Table XII. R_f values of C^{14}-labeled products from enzymatic hydrolyzates of C^{14}-serine-inactivated threonine dehydrase.

Compound	R_f in Solvent [a]				
	1	2	3	4	5
Cysteic acid	0.070	0.24	0.10	0.045	0.070
Cystathionine	0.11	0.15	0.20	0.185	0.12
Lanthionine	0.10	0.090	0.060	0.080	0.18
Serine	0.22	0.30	0.48	0.27	0.32
Lanthionine sulfoxide	0.075	0.14	0.14	0.17	0.15
Lanthionine sulfone	0.040	0.10	0.12	0.15	0.40
Lysalanine	0.17	0.19	0.32	0.57	0.33
C^{14}-enzyme hydrolyzate	0.095	0.095	0.055 + 0.12	0.090 + 0.17	- [b]
HCl hydrolyzate of C^{14}-material from Solvent 3			0.050 + 0.10 + 0.80		0.17 + 0.74

[a] Solvent No. 1 = n-butanol : acetic acid : H_2O; 60:15:25
No. 2 = n-butanol ; pyridine ; H_2O; 1:1:1
No. 3 = phenol : H_2O ; 100:20
No. 4 = same as No. 3 but with NH_3 atmosphere, 0.3%
No. 5 = Ethanol : H_2O ; 77:23

[b] Not suitable for crude hydrolyzate because of considerable streaking.

The C^{14}-containing material migrated to essentially the same position as lanthionine in the butanol - acetic acid - water and butanol - pyridine - water solvents (systems 1 and 2 of Table XII). No radioactivity was found to correspond with authentic lysalanine or serine, indicating that the labeled material had undergone chemical modification and that the ϵ-NH_2 group of a lysyl residue did not add across the double bond of α-aminoacrylate. Chromatography in phenol - water - ammonia (system 4), however, gave rise to two radioactive components; one could be identified as lanthionine and the other had an R_f value similar to that of lanthionine sulfoxide. When the phenol solvent was used without the NH_3 atmosphere (system 3), again two radioactive peaks were present. In this instance, one component was identified as lanthionine while the second component migrated to a location which did not permit a decision among lanthionine sulfoxide, lanthionine sulfone and cysteic acid. Elution of this radioactive material, followed by a brief HCl hydrolysis, yielded components identified as cysteic acid and lanthionine when rechromatographed in phenol - water (system 3) or lanthionine when chromatographed in ethanol - water (system 5). In addition, a third compound was observed on the chromatograms. This compound had a high R_f in phenol - water and ethanol - water solvents, as would be predicted for S-ethylcysteine. However, positive identification could not be made due to the lack of a standard.

On the basis of the foregoing chromatographic evidence, the radioactive compound was considered to be lanthionine formed by the addition of α-aminoacrylate to the SH group of a cysteinyl residue in peptide linkage. The appearance of lanthionine sulfoxide and cysteic acid on phenol-water chromatograms is not surprising because this solvent often causes oxidation of thiols and thioethers (89). The formation of S-ethylcysteine or similar compound must be due to a decomposition of lanthionine during acid hydrolysis, in spite of the precautions taken to prevent such destruction.

The incorporation of radioactivity from C^{14}-serine into the protein fraction could further be utilized for an estimation of the number of active sites of the enzyme, provided that each site had one cysteinyl SH group in proximity. On the assumption that each site inactivated binds serine, the number of moles of serine-C^{14} bound per mole of dehydrase could be determined from the total counts bound and the specific activity of serine-C^{14}. The number of moles of serine-C^{14} bound would then equal the number of moles of active sites. For this, serine inactivation of threonine dehydrase was carried out in the usual manner with the exception that the loss of dehydrase activity was followed. When 72 units of the 430 units of dehydrase had been lost, the reaction mixture was passed through a Sephadex G-25 column and fractions collected as usual. The protein-containing fractions were pooled, evaporated to 0.35 ml. and dialyzed against distilled water. The protein-bound radioactivity was then measured and calculated to be 18,700 dpm total.[1] If the assumptions of 100% recovery of radioactive protein from the column, a molecular weight of 80,000 and a purity of 33% are made, a total of 2.9 moles of α-aminoacrylate (serine-C^{14}) was bound per mole of threonine dehydrase. Because of the large number of assumptions, the value of 3 moles of α-aminoacrylate per mole of dehydrase is probably only a rough estimate for the number of active sites.

The Mechanism of AMP Activation

The preceding investigations of the mechanism of dehydration, the mode of pyridoxal phosphate binding, and the serine inactivation offer no insight as to the role of AMP activation of threonine dehydrase.

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[1]The radioactivity bound was approximately 0.0017% of the input radioactivity, 1.1×10^8 dpm.

It is quite likely, then, that AMP does not directly participate in the catalytic action in a manner analogous to pyridoxal phosphate or any other normal coenzyme, but rather functions less directly in a controlling capacity.

A kinetic basis for the activation by AMP is presented in Figure 4. Double reciprocal plots of velocities observed with a range of substrate concentrations at pH 7.0, 8.0, and 8.6, and in the presence or absence of AMP, indicate that when AMP is present, there are marked decreases in K_m as well as increases in V_{max}. At pH 8.0, K_m and V_{max} values in the presence of AMP were 5×10^{-3} M and 0.27 Δ A per minute, respectively; in the absence of AMP, K_m and V_{max} values were 3×10^{-2} M and 0.034 ΔA per minute, respectively. Qualitatively similar K_m and V_{max} changes were noted at other pH values. It is interesting to observe that in the absence of AMP, K_m values were identical at pH 7.0, 8.0, and 8.6, suggesting that K_m is independent of pH over this range. In the presence of AMP, however, the K_m values were markedly pH dependent.

More conclusive evidence for a role of AMP was found through sucrose gradient centrifugation experiments conducted in the presence and absence of AMP. This technique was ideally suited for the study of fairly drastic conformational changes because only microgram quantities of enzyme were required to locate activity peaks and complete purity was not essential.

Sedimentation patterns of threonine dehydrase[1] in sucrose

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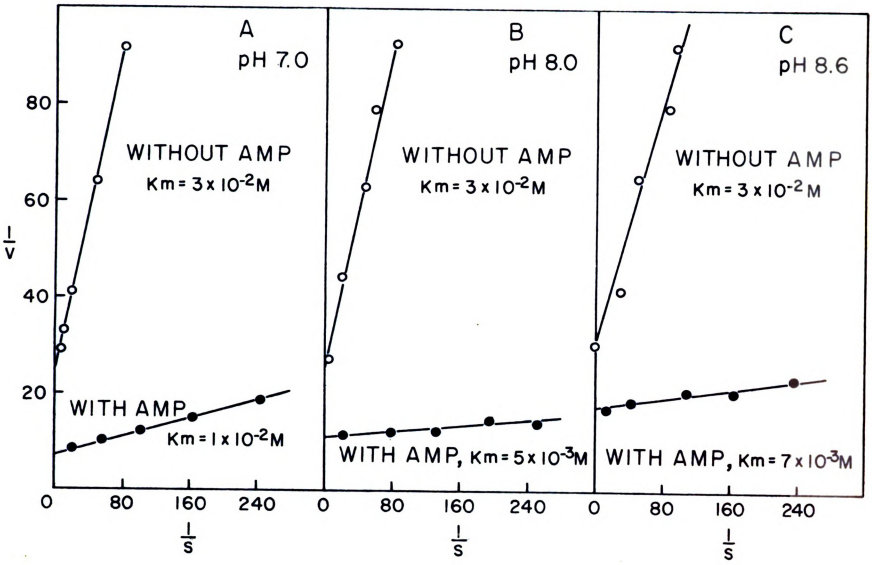
[1]Marker enzymes as well as threonine dehydrase were incorporated into the 0.10 ml. sample that was placed on each gradient. Because of the different specific activities and stabilities of the enzymes, the actual amount of each enzyme placed on the gradient was determined empirically. The following amounts of enzymes were used per gradient: 2 units of yeast alcohol dehydrogenase, 1 unit of liver alcohol dehydrogenase, 1 unit of KDPG aldolase, 1 unit of muscle aldolase and 2 units of malate dehydrogenase. Generally only 2 or 3 marker enzymes were

Figure 4. K_m and V_{max} determinations at pH 7, 8 and 8.6. Threonine concentrations are expressed in moles/liter; velocities are expressed as Δ absorbance per minute as determined in the NADH-lactic dehydrogenase coupled assay. Data have not been corrected to account for differences in velocity at the different pH values. Phosphate buffer was used for all experiments.


A
pH 7.0
WITHOUT AMP
Km = 3 x 10-2M
WITH AMP
Km = 1 x 10-2M
B
pH 8.0
WITHOUT AMP
Km = 3 x 10-2M
WITH AMP, Km = 5 x 10-3M
C
pH 8.6
WITHOUT AMP
Km = 3 x 10-2M
WITH AMP, Km = 7 x 10-3M
1/v
1/s
0
20
40
60
80
160
240

gradients containing 3×10^{-3} M AMP or 3×10^{-3} M IMP yielded results shown in Figure 5. When no nucleotide was added to the gradient and care taken to remove traces of AMP from the enzyme preparation, the dehydrase migrated as in an IMP gradient. The average sedimentation coefficient for threonine dehydrase in the presence of 3×10^{-3} M AMP was 7.6 S; in the presence of 3×10^{-3} M IMP or no nucleotide, the average S_{20} was 5.0 S (Table XIII). It was thus concluded that the presence of AMP induced a conformational change of sufficient magnitude to produce a drastic increase in sedimentation velocity. If the assumptions of identical partial specific volumes and spherical shapes are made for marker enzymes and threonine dehydrase, a rough estimate of molecular weight can be obtained from sedimentation coefficients[2] alone (70). When this calculation was performed, a molecular weight of 153,000 was found for the 7.6 S species, and 83,000 for the 5.0 S species. This corresponds to an apparent dimerization in the presence of AMP.

In a similar experiment in which cytidine monophosphate (CMP), a less efficient activator, was substituted for AMP at 3×10^{-3} M concentration, threonine dehydrase had an S_{20} of 6.9 S; this is in accord with the idea that ability to activate the enzyme is related to the ability to produce a dimerization.

Figure 6 shows the sedimentation velocity (expressed as the peak position after 16 hours of centrifugation) versus AMP concentration

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used in each gradient. Malate dehydrogenase was discontinued in later experiments because the particular preparation available had a sedimentation coefficient (S_{20}) of 4.8 S rather than 3.6 S as reported (90); this increased S_{20} was confirmed in independent experiments using the Spinco Model E analytical ultracentrifuge. The enzyme units referred to above are expressed in μmoles of substrate decomposed per minute.

[2] $$\frac{S_{20} \text{ of unknown}}{S_{20} \text{ of known}} = \left(\frac{\text{Molecular weight of unknown}}{\text{Molecular weight of known}} \right)^{2/3}$$

Figure 5. Sucrose gradient centrifugation of threonine dehydrase. Marker peak positions were: yeast alcohol dehydrogenase, fraction 13; liver alcohol dehydrogenase, fraction 18; KDPG aldolase, fraction 20. See text for conditions of centrifugation.

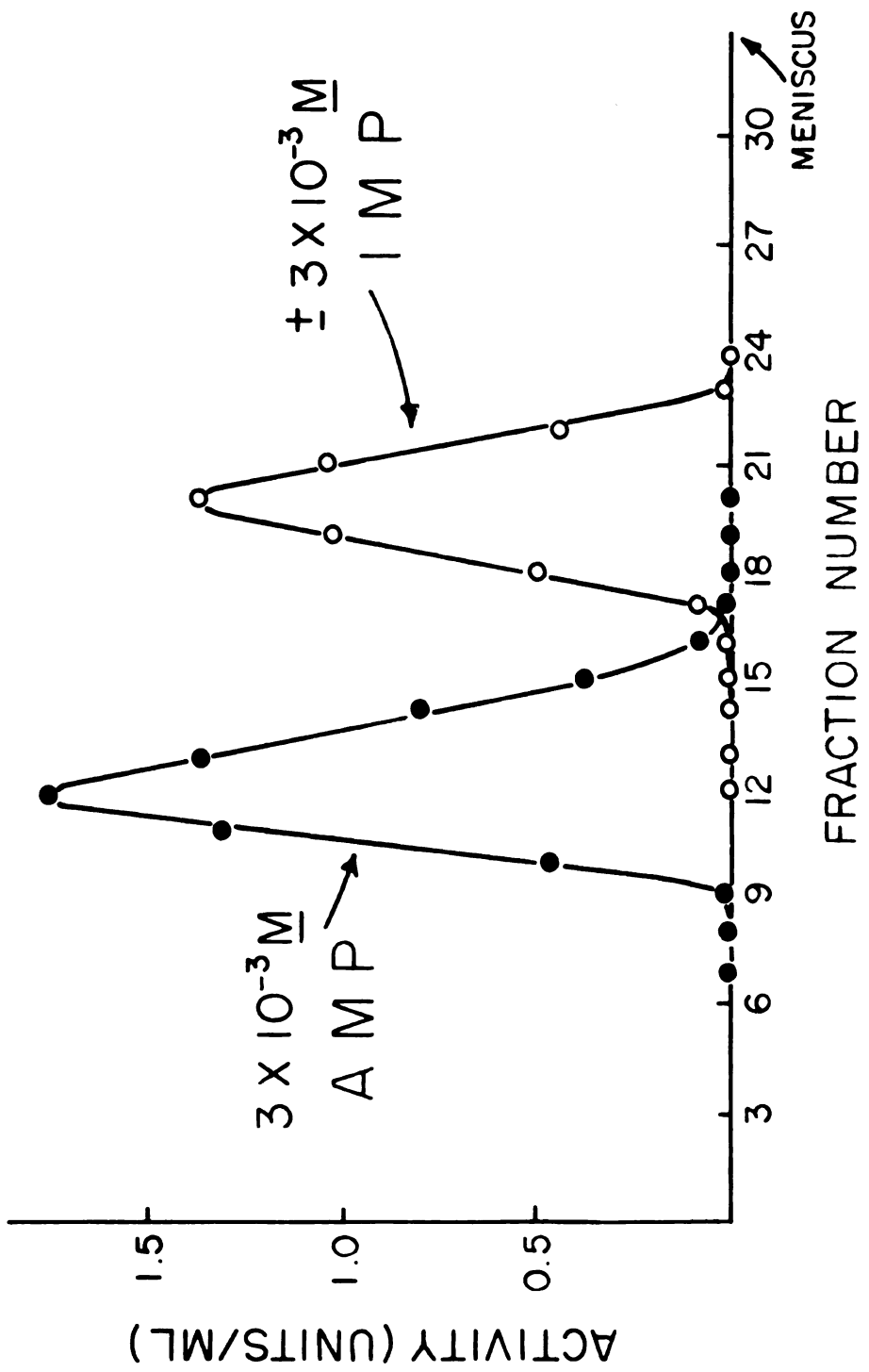

ACTIVITY (UNITS/ML)
1.5
1.0
0.5
3 X 10⁻³ M
A M P
± 3 X 10⁻³ M
I M P
3
6
9
12
15
18
21
24
27
30
MENISCUS
FRACTION NUMBER

Table XIII. Sedimentation coefficients and molecular weights of threonine dehydrase in the presence and absence of AMP.

	Standard Marker Enzymes				Threonine Dehydrase [a]			
					With AMP		Without AMP	
Enzyme	Fraction	S_{20}	Mole. Wt.	Ref.	S_{20}	Mole. Wt.	S_{20}	Mole. Wt.
Yeast ADH	13	7.5	150,000	70	7.9	161,000	5.2	88,000
Liver ADH	18	5.2	84,000	91	7.3	140,000	4.8	75,000
Muscle Aldolase	10	8.2	180,000	92	7.5	158,000	5.0	85,000
KDPG Aldolase	20	4.5	90,000	d	7.6	---[b]	5.0	---[b]
Average					7.6	153,000	5.0	83,000

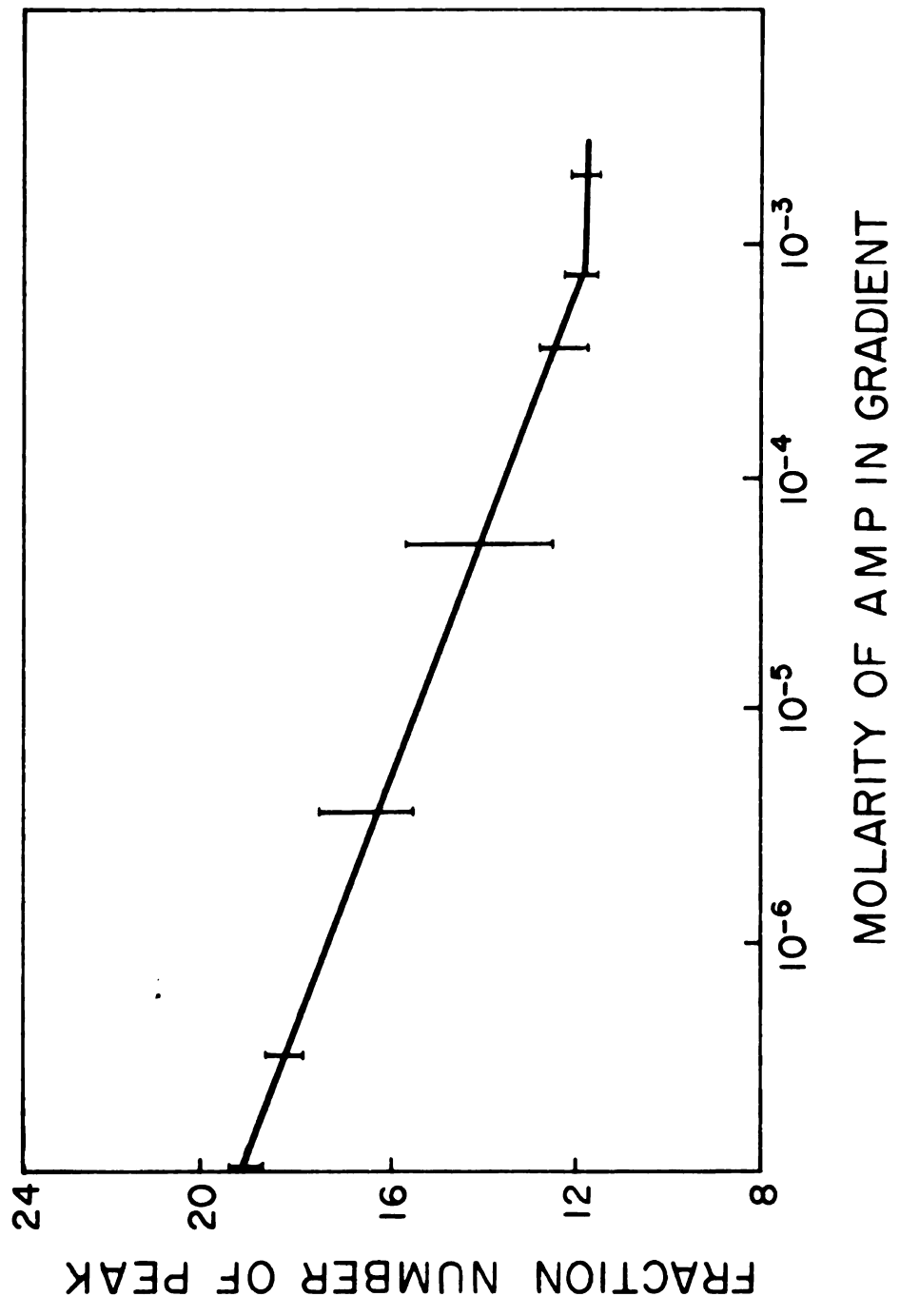
[a] Based on peak positions of fraction 12 and 19 in the presence and absence of AMP, respectively, and a total of 33 fractions in the gradient.

[b] Not calculated because of the non-spherical shape of KDPG aldolase.

[c] Fraction Number 33 corresponds to the meniscus.

[d] Meloche, H. P. and Wood, W. A. J. Biol. Chem., in press.

Figure 6. Sedimentation rate of threonine dehydrase as a function of AMP concentration. Conditions were the same as in Figure 5 except the AMP concentration was varied as indicated. The vertical lines indicate the peak width.


FRACTION NUMBER OF PEAK
24
20
16
12
8
10^{-6}
10^{-5}
10^{-4}
10^{-3}
MOLARITY OF AMP IN GRADIENT

in the sucrose gradient (enzyme concentration was nearly constant for all experiments). There was a series of intermediate S_{20} values at intermediate AMP concentrations and a pronounced broadening of the peak (as indicated by the vertical lines on Figure 6) was apparent. The intermediate S_{20} values and the absence of multiple peaks corresponding to varying ratios of 7.6 S and 5.0 S species suggest that the two species are rapidly interconverted at limiting concentrations of AMP. Gilbert (93) has discussed the theoretical sedimentation patterns for a monomer-dimer system in rapid equilibrium and concluded that no resolution of the two components is possible and that only a single, asymmetric peak would be obtained when an equal concentration of monomer and dimer are present.

On this basis, it is proposed that the following equilibrium exists:

$$2\,[5.0\text{ S "monomer"}] + n\text{AMP} \rightleftharpoons [7.6\text{ S "Dimer"} - n\text{AMP}]$$

With the knowledge that dimerization occurred in the presence of AMP and that reversible dissociation was favored by lowered concentrations of AMP, experiments were designed which would provide further insight into the mechanism for this structural alteration.

The sucrose gradient technique was utilized to obtain small quantities of both the "monomeric" or "dimeric" species in their pure forms. These fractions were first assayed in four ways: (a) with both AMP and GSH in the cuvette, (b) with AMP only, (c) with GSH only, and (d) with both omitted. When a valid rate had been obtained, any omitted component was then added and the new rate observed. The results of assays conducted with these species are tabulated in the upper part of Table XIV.

The more prominent facts gathered from assays of the "dimeric" or 7.6 S fraction and the "monomeric" or 5.0 S fraction are as follows:

Table XIV. Activities exhibited by the 7.6 S and 5.0 S species of threonine dehydrase.

	Fraction	First Addition[1]	Rate (ΔA /min.)	Relative Velocity (Percent)	Second Addition[2]	Rate (ΔA/min.)	Relative Velocity (Percent)
I	S_{20} = 7.6	None	0.030	7	+Both	0.14	34
		+GSH	0.041	10	+AMP	0.25	61
		+AMP	0.36	88	+GSH	0.38	93
		+Both	0.41	100	+None	0.41	100
	S_{20} = 5.0	None	0.003	7	+Both	0.012	27
		+GSH	0.006	13	+AMP	0.034	75
		+AMP	0.007	16	+GSH	0.017	38
		+Both	0.045	100	+None	0.045	100
II	S_{20} = 7.6	+AMP, GSH	0.122	100	+None	0.122	100
		+AMP, GSH, GSSG	0.056	46	+None	0.056	46
		+AMP, GSSG	0.010	8	+GSH	0.025	20
		+GSSG	0.006	5	+AMP	0.008	6
		+GSSG	0.006	5	+AMP, GSH	0.018	15

[1]Preincubated 5 minutes before threonine added.

[2]After 20 minutes with threonine.

1) The 7.6 S fraction exhibited nearly the same activity with or without GSH, but had a greatly reduced activity in the absence of added AMP, suggesting that a rapid dissociation of the dimer may occur in the enzymatic assay in the absence of AMP. A sizable reactivation occurred when AMP was added to a reaction mixture which had been preincubated with only GSH.

2) The 5.0 S fraction exhibited dependence on both AMP and GSH. Assay of this fraction in the presence of only GSH yielded a low activity; partial activation was seen upon the addition of AMP. In the reverse incubation, considerably less activation was seen upon GSH addition, suggesting that activation is accomplished best by GSH treatment first, followed by addition of AMP. This fact is considered to indicate that the 5.0 S fraction as obtained from the sucrose gradients contains at least one disulfide bond which must be reduced before AMP can induce dimer formation.

3) Assay of the 7.6 S fraction without both AMP and GSH resulted in very low rates; addition of both of these components failed to activate to the same extent as when only GSH was present, and suggests that the dimer form in the absence of both AMP and GSH is first converted to a monomer and then to an additional inactive form. This latter form is quite likely identical with the 5.0 S fraction isolated in sucrose gradients containing no AMP. This form of the enzyme requires both AMP and GSH for activation, and can be activated only some 30% when AMP and GSH are added <u>after</u> threonine. Assay of the 7.6 S fraction with oxidized glutathione (GSSG) at 3 m<u>M</u> concentration in the absence of AMP and GSH revealed little activity (Table XIV, lower part). The addition of AMP to this mixture did not produce appreciable activation unless GSH was also added. Simultaneous preincubation of AMP and GSSG, followed by GSH addition upon assay, resulted in a slightly greater activation. These findings are considered evidence that the

monomer-dimer equilibrium can be upset in favor of the monomeric species by the presence of an oxidizing agent (GSSG), but that AMP can partially antagonize this action, presumably by maintaining the dimeric structure.

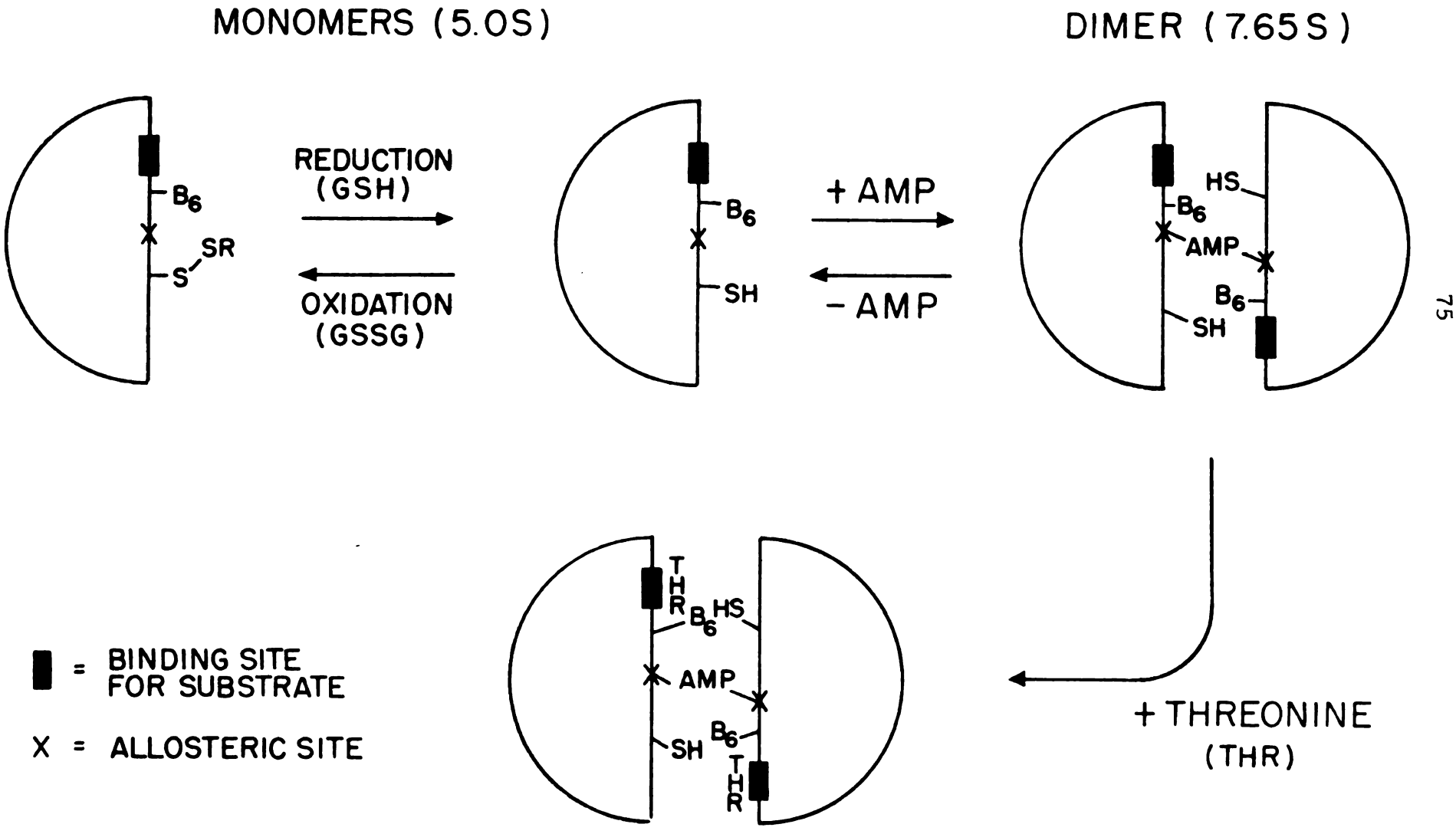
These facts formed the basis for the tentative model proposed in Figure 7. Although additional proof of the accuracy of this model has not been obtained, the model is open to experimental confirmation. One point yet to be experimentally verified is the prediction that a 5.0 S fraction isolated from a gradient containing GSH but no AMP would not require further GSH treatment in order to be activated by AMP. Furthermore, p-chloromercuribenzoate treatment of this fraction in the absence of GSH should prevent AMP activation due to the combination of PCMB with the SH groups which are essential for the formation and maintenance of the dimer structure. A further prediction of this model is a second-order dependence upon protein concentration for dimer formation in the presence of excess AMP.

AMP Activation as a Metabolic Control Mechanism

Having found a basis for the AMP activation, consideration was given its significance as a possible control mechanism in the anaerobic metabolism of *E. coli*. Tokushige *et al.* (23) concluded from experiments with the ADP-activated threonine dehydrase of the anaerobe, *C. tetanomorphum*, that ATP synthesis depressed threonine dehydrase activity by the removal of ADP and inversely, that ADP build-up activated threonine dehydrase (see a discussion of their proposal in the Literature Review). This mechanism could be feasible for anaerobically-grown *E. coli* where ATP synthesis via substrate phosphorylation rather than oxidative phosphorylation would predominate.

Figure 7. Proposed model for activation of threonine dehydrase.

PROPOSED MODEL FOR ACTIVATION OF THREONINE DEHYDRASE



A major point of variance, however, was that ADP rather than AMP activates the dehydrase of C. tetanomorphum. Thus it was postulated that E. coli possesses an adenylate kinase which links the AMP, ADP and ATP levels through the reaction:

$$2\ ADP \underset{\text{Kinase}}{\overset{\text{Adenylate}}{\rightleftharpoons}} ATP + AMP$$

In this case, AMP rather than ADP could accumulate and thus be available to activate threonine dehydrase. Through the action of the dehydrase, α-ketobutyrate would be formed and this keto acid could produce ATP through a dismutation of α-ketobutyrate to propionyl phosphate and α-hydroxybutyrate. A corollary of this postulate would be the fact that C. tetanomorphum is lacking in adenylate kinase.

The results presented in Table XV show that adenylate kinase is found in reasonable amounts in both organisms. Thus this postulate for the difference in nucleotide specificity is inadequate. No further work on the in vivo control of threonine dehydration has been conducted.

Table XV. Adenylate kinase content of extracts of E. coli and C. tetanomorphum. (The preparation of extracts and assays were performed as described in the text. Ten μl. of a 1:10 dilution of the crude extracts was used for the assays.)

Extract	Units[a]/ml	Protein (mg/ml)	Specific Activity
E. coli	164	14.8	11.1
C. tetanomorphum	188	19.4	9.7

[a]A unit is defined as that amount of enzyme which produces an absorbance change of 1.0 O.D. per minute under the described assay conditions.

DISCUSSION

The results obtained from isotope experiments on the mechanism of threonine dehydration confirm that an α, β-elimination mechanism involving unsaturated intermediates is operative. The data which support this conclusion are as follows:

(a) Dehydration in the presence of D_2O resulted in the formation of α-ketobutyrate with nearly one atom of D per molecule of ketobutyrate.

(b) When α-tritio threonine was used as substrate, the tritium was recovered in water while the α-ketobutyrate was unlabeled.

(c) Treatment of dehydrase reaction mixtures with borohydride produced C^{14}-α-aminobutyrate from C^{14}-threonine. Reduction with H^3-labeled borohydride resulted in the formation of α-tritio-α-aminobutyrate, indicating that α-iminobutyrate was the immediate precursor of α-aminobutyrate.

(d) The results of O^{18} and D back-incorporation into threonine during dehydration suggest a reversibility of the dehydration, at least prior to the release of α-aminocrotonate from the enzyme. These data also indicate that hydrolysis of the imino acid might be the only irreversible step in the entire sequence.

In addition, the presence of pyridoxal phosphate bound as a Schiff base to the ε-amino group of a protein lysyl residue strongly suggests that this compound plays an important role in dehydration, presumably through a transaldimination reaction between the pyridoxal-lysine Schiff base and the amino group of threonine.

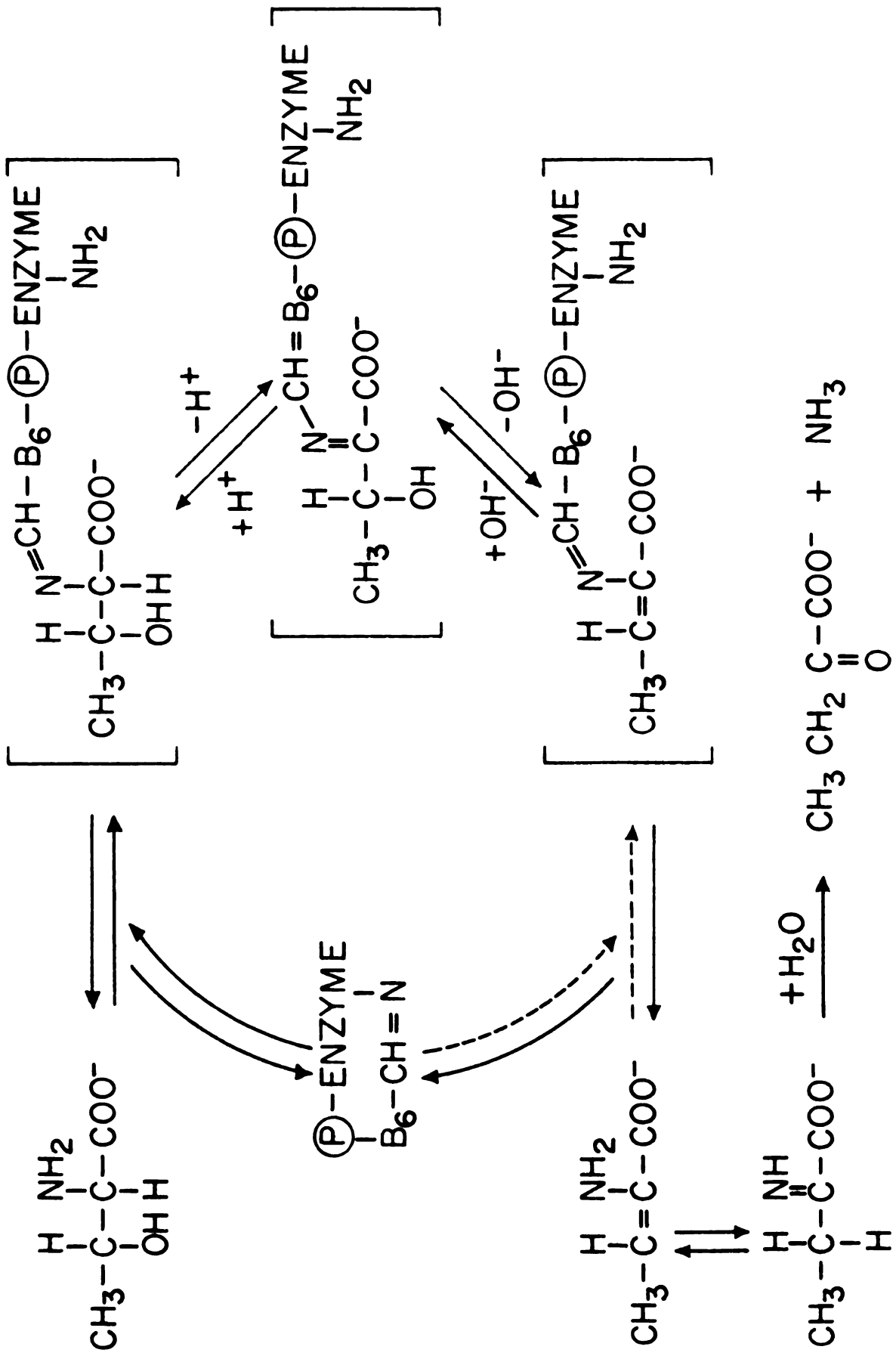
A detailed mechanism incorporating all these facts is presented in Figure 8. The unprotonated amino group of threonine presents the second substrate for a transaldimination reaction from the enzyme-pyridoxal phosphate Schiff base; support for this assumption comes by analogy to threonine dehydration in sheep liver (41, 42), where kinetic studies showed that the reactive species has an uncharged amino group.

The existence of the azomethines shown in brackets on Figure 8 is somewhat speculative but such structures are postulated as logical intermediates in the removal of an α-hydrogen and β-hydroxyl by a pyridoxal enzyme. Because all these azomethines should be present during dehydration and all contain bonds reducible by borohydride, it seems reasonable to expect that borohydride treatment of reaction mixtures might allow the isolation of these enzyme-bound intermediates in their stable reduced state. This would provide definitive evidence for the existence of such structures.

The quite similar rates of back-incorporation of D and O^{18} into threonine suggest that a stepwise elimination of proton and hydroxyl group may occur in which the initial slow formation of a carbanion is followed by a rapid conversion to an unsaturated intermediate. Although this possibility is hardly distinguishable from a concerted elimination process and has been of quite limited importance in elimination reactions (94), it is an attractive mechanism for the pyridoxal phosphate-catalyzed enzymatic elimination. A concerted mechanism has never been proposed for the pyridoxal phosphate type of elimination and a stepwise elimination is in basic agreement with the generally accepted mechanism for pyridoxal catalysis (31).

The same intermediates as shown in Figure 8 were proposed for the non-enzymatic dehydrations catalyzed by pyridoxal (31), and emphasize the virtual identity of the enzymatic and non-enzymatic pyridoxal mechanisms for dehydration. Because of this similarity of

Figure 8. The mechanism of enzymatic threonine dehydration.


-H+
+H+
+OH-
-OH-
+H2O
CH3 CH2 C-COO- + NH3
O

mechanisms, the role of the enzyme must be that of conferring specificity toward reactants and of increasing the rate of the pyridoxal-facilitated dehydration. This latter role may be accomplished in at least three ways: (a) by enzyme-promoted specific orientation effects which serve to bring all reactants together in the most favorable positions for reaction; (b) by catalysis of the transaldimination reaction, both in the formation of a pyridoxal-substrate Schiff base and in the cleavage of a pyridoxal-product Schiff base; and (c) by facilitating the loss of H^+ and OH^- from the substrate through an attraction of these groups by various amino acid residues in the enzyme active site.

The effect which AMP exerts on threonine dehydrase catalysis is not indicated in mechanistic studies of the dehydration reaction, because the results presented are in keeping with a normal pyridoxal phosphate-catalyzed dehydration mechanism. But since it is evident from kinetic studies that the binding of threonine is markedly enhanced by AMP, and that V_{max} is increased, one must conclude that AMP functions as an allosteric effector in controlling threonine dehydrase action; that is, AMP binds at a site somewhat removed from that for substrate but still exerts an effect on substrate binding and on the velocity of catalysis.

The apparent dimerization of the enzyme protein in the presence of AMP, under conditions which simulate those existing in an enzymatic assay, strongly suggests that the increased catalytic effectiveness produced by dimerization in the presence of AMP may be due to an assembling of additional groups which further facilitate substrate binding and catalysis, or in other words furnishes a more effective active site than is present in the monomer dehydrase.

The manner in which activation is brought about is uncertain, principally because the nature of the protein-AMP interaction is unclear.

It is clear, however, that AMP is not a completely specific agent. X-ray diffraction studies (95) indicate a close spatial relationship between the phosphate and primary amino group of 5'-AMP. Since several related compounds possessing these same groups in roughly the same spatial relationship are also capable of activating threonine dehydrase (CMP, dAMP, 3'-AMP) and since CMP, at least, can also induce a dimerization and activate, these two groups must play an important role in activation as well as in determining the specificity of the protein-nucleotide interaction.

The requirement for a reducing agent such as GSH or 2-mercaptoethanol has long been known for threonine dehydrase (5, 11). The present work indicates that one or more SH groups are essential for AMP activation, and therefore that reducing agent functions to maintain the integrity of these groups. A direct test of this hypothesis would be to show that dimerization cannot take place in the absence of these SH groups.

The role of SH groups in threonine dehydrase is strengthened by the demonstration that lanthionine is formed by incubation of serine with the dehydrase. This lanthionine is presumably formed by the addition of the SH group of a cysteinyl residue to the double bond of α-aminoacrylate, an intermediate in serine dehydration. Since it is likely that the particular SH groups involved are near the site (or sites) where α-aminoacrylate is released from the protein, it would be interesting to see whether these groups are also essential for AMP activation. That this could be the case comes from observations that serine inactivation is most efficient when the AMP concentration is relatively low (see protocol for serine inactivation experiments), indicating that excess AMP may partially protect these groups from α-aminoacrylate addition.

The model for threonine dehydrase activation and activity, illustrated in Figure 7, is consistent with all the data obtained, but only illustrates one of the active sites. It also features several details which have not been experimentally established. While there is evidence bearing on the number of binding sites for substrate, there is no indication of the number of binding sites for AMP. Also, because the monomeric form exhibits a small degree of enzymatic activity, a threonine binding site on each monomer is illustrated. Whether this same active site is merely modified in some way in the dimer (as shown in Figure 7) or whether a new and different active site is brought into being in the dimer cannot be decided on the basis of the present data.

It must be mentioned that the present data suggest but do not conclusively establish that a monomer-dimer relationship exists in threonine dehydrase. The observed S_{20} changes could well be a reflection of only extensive intramolecular conformational changes. This possibility will remain unchallenged until such time as more direct molecular weight measurements are made.

Attempts to induce biodegradative threonine dehydrase in resting cells grown under non-inducing conditions have consistently failed. Despite testing of a variety of carbon and nitrogen sources for growth and several different possible inducers (serine, threonine, casein hydrolyzate, tryptone), no conclusive induction was obtained. Although this failure is not completely understood, the difficulty may lie in the failure to remove the repression of this enzyme which occurs in cultures of E. coli grown aerobically on a tryptone-yeast extract medium.

An additional point remaining to be clarified is the in vivo significance of AMP activation of threonine dehydrase. This question will remain unanswered until the exact fate of α-ketobutyrate is known. When the pathway for α-ketobutyrate catabolism is established, it should

be possible to explain how the rate of utilization of threonine affects the AMP:ATP ratio.

It should be emphasized that the demonstration of adenylate kinase in both E. coli and C. tetanomorphum does not invalidate the control mechanism proposed by Tokushige et al. (23) but does make it difficult to understand how different nucleotides (i.e., ADP and AMP) could regulate threonine catabolism in these two organisms by the same mechanism.

SUMMARY

Fifty-fold purified threonine dehydrase from E. coli grown anaerobically on an amino acid medium has been found to require pyridoxal phosphate for activity. In the native enzyme, pyridoxal phosphate is bound as a Schiff base to the ϵ-amino group of a lysyl residue.

Studies with deuterium oxide, α-tritiothreonine and H_2O^{18} have confirmed that dehydration proceeds through unsaturated intermediates such as α-aminocrotonate and α-iminobutyrate. Further evidence for these intermediates was the production of α-aminobutyrate from α-iminobutyrate upon borohydride treatment of dehydrase reaction mixtures.

Adenosine monophosphate (AMP) increases the catalytic effectiveness of threonine dehydrase by decreasing the K_m for threonine and increasing the maximum velocity. Furthermore, threonine dehydrase undergoes an increase in sedimentation coefficient, corresponding roughly to a dimerization, in the presence of AMP.

The nature of serine inactivation of this enzyme has been investigated and found to result from the addition of α-aminoacrylate, the unstable product of serine dehydration, to an essential SH group near the active site of threonine dehydrase.

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