ABSTRACT

A STUDY OF AEROBIC THREONINE CATABOLISM IN ESCHERICHIA COLI K12 MUTANTS

Terrence Chan

This thesis concerns the reactions involved in threonine catabolism by Escherichia coli using threonine as its sole source of carbon and energy. The pathway of carbon from threonine to the general metabolic pool was identified using genetic and biochemical methods. Threonine is first converted to α-amino-β-keto butyrate by NAD-dependent threonine dehydrogenase. Next by the action of α-amino-β-keto butyrate Co-enzyme A ligase, the four-carbon compound is split into two two-carbon compounds, glycine and acetyl Co-enzyme A. In the serine transhydroxymethylase-deficient mutant studied here, glycine is excreted by the bacteria into the culture filtrate, and acetyl Co-enzyme A is used as a source of carbon and energy. Two enzymes that are involved in threonine catabolism, threonine dehydrogenase and α-amino-β-keto butyrate Co-enzyme A ligase, were partially purified from the E.coli K12 mutant, strain JEV 73R, and characterized. This is

the first report of an *E.coli* mutant that can use threonine as its sole carbon and energy source and of glycine excretion in *E.coli*. It is also the first documentation of the existence of the enzyme, α-amino-β-keto butyrate Co-enzyme A ligase in *E.coli* cell-free extracts.

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INTRODUCTION

This thesis is concerned with the identification of the pathway by which threonine serves as the sole carbon and energy source in aerobically grown E.coli K12 mutants.

Threonine is a very stable amino acid. It is a four carbon compound with an amino group bound to the α-carbon. Therefore potentially, threonine can be the carbon or nitrogen or both carbon and nitrogen sources for micro-organisms that have the enzymes needed to degrade it. In micro-organisms, four enzymes have been shown to be involved with threonine catabolism. They are biosynthetic and biodegradative threonine deaminases (E.C. 4, 2, 1, 16), threonine aldolase (E.C. 4, 1, 2, 2) and NAD-dependent threonine dehydrogenase (E.C. 1, 1, 1, 103).

A recent study (Bell, Turner, Collins & Gray, 1972; Bell & Turner, 1976, 1977a, 1977b) suggests that threonine degradative enzymes are common in the microbial world. Bell and Turner surveyed threonine catabolism in 75 micro-organisms that can grow on threonine as the major source of carbon and nitrogen. They found that the enzyme which most frequently initiates threonine catabolism was NAD-dependent

threonine dehydrogenase (TDH) (46 out of 75 strains studied). The occurrence of threonine aldolase (E.C. 4, 1, 2, 2) was not only rare (6 out of 75 strains studied) but also restricted to species of Pseudomonas. The initiation of threonine catabolism by threonine deaminase (TD) was uncommon among micro-organisms capable of growth on the amino acid, but has been described in a species of Corynebacterium (Bell & Turner, 1977a).

In this introduction, the reactions catalyzed by the four threonine catabolic enzymes will be briefly described and some evidence as to their regulation will be surveyed. The further metabolism of possible intermediates in threonine catabolism including α-amino-β-keto butyrate, amino acetone, glycine and acetate will then be reviewed.

The Enzymatic Reaction Catalyzed by Threonine Deaminase (E.C. 4, 2, 1, 16)

The enzymatic reaction of threonine deaminase is shown in Figure 1, reaction 1. Threonine deaminase catalyzes the oxidative deamination of threonine in a one-step reaction producing α-keto butyrate. In this reaction, the amino group is removed from threonine and made available for general cell metabolism. This

enzyme alone suffices to make nitrogen available from threonine. However, if threonine is to be the sole carbon and energy source, further metabolism of α-keto butyrate is essential.

The Enzymatic Reaction Catalyzed by Threonine Aldolase (E.C. 4, 1, 2, 2)

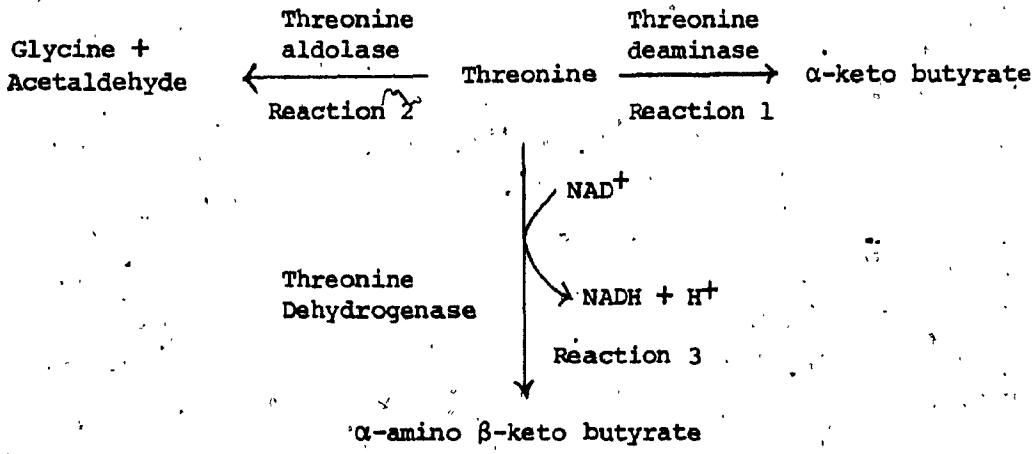
The enzymatic reaction of threonine aldolase is shown in Figure 1, reaction 2. Threonine aldolase catalyzes the cleavage of threonine, a four carbon compound, into two two-carbon compounds, glycine and acetaldehyde. Of these, glycine is the one that contains the nitrogen atom. If threonine is to be used as the sole nitrogen source via threonine aldolase, the further metabolism of glycine is essential. If threonine is used as the sole carbon source, further metabolism of glycine and/or acetylaldehyde must be involved.

Enzymatic Reaction Catalyzed by NAD-Dependent Threonine Dehydrogenase (E.C. 1, 1, 1, 103)

The enzymatic reaction of the NAD-dependent threonine dehydrogenase is shown on Figure 1, reaction 3. Threonine dehydrogenase catalyzed the oxidative conversion of threonine to α-amino-β-keto butyrate (AKB). The product is a very unstable compound with

Figure 1

Threonine Metabolism in Micro-Organisms



a half-life of about one minute in solution (Neuberger, 1959). The product of the rapid non-enzymatic decarboxylation is amino acetone. Further metabolism of AKB may occur by more than one pathway. In this work, a pathway involving the formation of glycine and acetyl Co-enzyme A, and their further metabolism, will be demonstrated.

The Regulation of Enzymes Involved in Threonine Metabolism in E.Coli

Threonine deaminases (biodegradative and biosynthetic). In order for a metabolic reaction to actually take place in the cell, the enzyme responsible for its catalysis must be present in sufficient amount. That is not, however, enough to ensure that the reaction will occur since this will also depend on the pattern of metabolic regulation. This can be seen particularly clearly in threonine catabolism. In this case, one of the enzymes whose enzymatic mechanism should allow threonine deamination is rendered inactive by end product inhibition even at a low concentration of its end product, isoleucine. The other threonine deaminating enzyme is controlled at the level of transcription such that it cannot be formed in aerobic conditions and it is not responsible

for aerobic threonine catabolism. Thus, the cell has the information for making two threonine deaminating enzymes but their control mechanisms are such that neither can be used for aerobic threonine degradation (Changeux, 1961; Wood, 1969; Shizuta & Hayaishi, 1970; Hatfield & Burns, 1970; Calhoun & Hatfield, 1973; Wasmuth & Umbarger, 1973).

Threonine aldolase. Generally speaking, the physiological function of threonine aldolase in micro-organisms has not been demonstrated (Bell & Turner, 1977a). In E.coli, in particular, it has not been reported at all and the following arguments suggest that this enzyme cannot be physiologically significant.

a) In E.coli strain AT 2046, the parental strain of strain JEV 73R and TE 111 used in this investigation, no threonine aldolase enzymatic activity could be detected in an in vitro system (Fraser, 1975).

b) In an E.coli mutant deficient in serine transhydroxymethylase activity (STHM), the biosynthetic pathway of glycine from serine is blocked (Miller, 1972). The mutant needs exogenous glycine for growth and cannot use exogeneous threonine. If it had threonine aldolase activity in physiologically significant amounts, the mutant would be able to

supply the glycine from exogeneous threonine.

NAD-dependent threonine dehydrogenase (TDH). It had been shown previously that in the presence of leucine, NAD-dependent threonine dehydrogenase is the first enzyme in the provision of glycine from threonine and in the provision of nitrogen from threonine in E.coli (Newman, Kapoor & Potter, 1977).

In this thesis, it will be shown that TDH is the first enzyme in the provision of carbon and energy from threonine in E.coli. The regulation of this enzyme is somewhat unusual. The enzyme is induced by an unrelated amino acid leucine but not by its substrate, threonine.

In summary, E.coli can produce two enzymes which act on threonine when grown aerobically on glucose as the sole source of carbon and energy. Only TDH is catabolic in function, producing AKB. In the next paragraph, the further metabolism of AKB is considered in details (Figure 2).

Further Metabolism of α-Amino-β-Keto Butyrate, the Product of the Enzymatic Catabolism of Threonine by Threonine Dehydrogenase

Due to the chemical instability of AKB, there are two possibilities for the further metabolism of the compound. The main difference between the two

possible pathways is that the first one is initiated by non-enzymatic decarboxylation of AKB to amino acetone while the other is initiated by the enzymatic splitting of the compound AKB into glycine and acetyl Co-enzyme A. This enzymatic reaction is a Co-enzyme A dependent reaction catalyzed by the enzyme α-amino-β-keto butyrate Co-enzyme A ligase[1]. In this thesis, the enzymatic metabolism of AKB is named 'Glycine route' because glycine is formed as one of the products (Figure 2).

Amino acetone cycle: the supporting evidence and its limitations. Amino acetone was once thought to be a physiologically important compound. The amino acetone cycle was suggested as a possible pathway for threonine catabolism (Elliot & Green, 1964). By this pathway, α-amino-β-keto butyrate (AKB) would be non-enzymatically decarboxylated to amino acetone. The amino acetone would then be deaminated to form methylglyoxal which would be converted to D-lactate by glyoxylase. The action of D-lactate dehydrogenase would convert D-lactate

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[1]This enzyme has also been known incorrectly as amino acetone synthetase. It was thought to be responsible for the formation of amino acetone from glycine and acetyl Co-enzyme A. It was later realized that the actual product was AKB and the formation of amino acetone was not depending on enzyme (McGiluray & Morris, 1971).

to pyruvate which would be the carbon and energy precursor for all other metabolic reactions (Figure 2).

There were some evidence to support the involvement of the amino acetone cycle in threonine catabolism. In Staphylococcus aureus, large amounts of amino acetone could be detected in the culture filtrate when threonine was the sole carbon and energy source (Elliot & Green, 1964). This is consistent with a conversion of threonine to AKB and the non-enzymatic decarboxylation of the AKB during growth. In addition, all the enzyme are essential in the amino acetone cycle have been shown in Arthrobacter (Green & Lewis, 1968).

The role of amino acetone as an intermediate during threonine catabolism is made less likely by the fact that exogeneously provided amino acetone cannot serve as the sole carbon source in most of the micro-organisms that can use theronine as the sole carbon source and nitrogen source (Bell & Turner, 1976, Komatsubra, Murata, Kisumi & Chibata, 1978).

The Glycine Route: The Supporting Evidences and the Missing Information

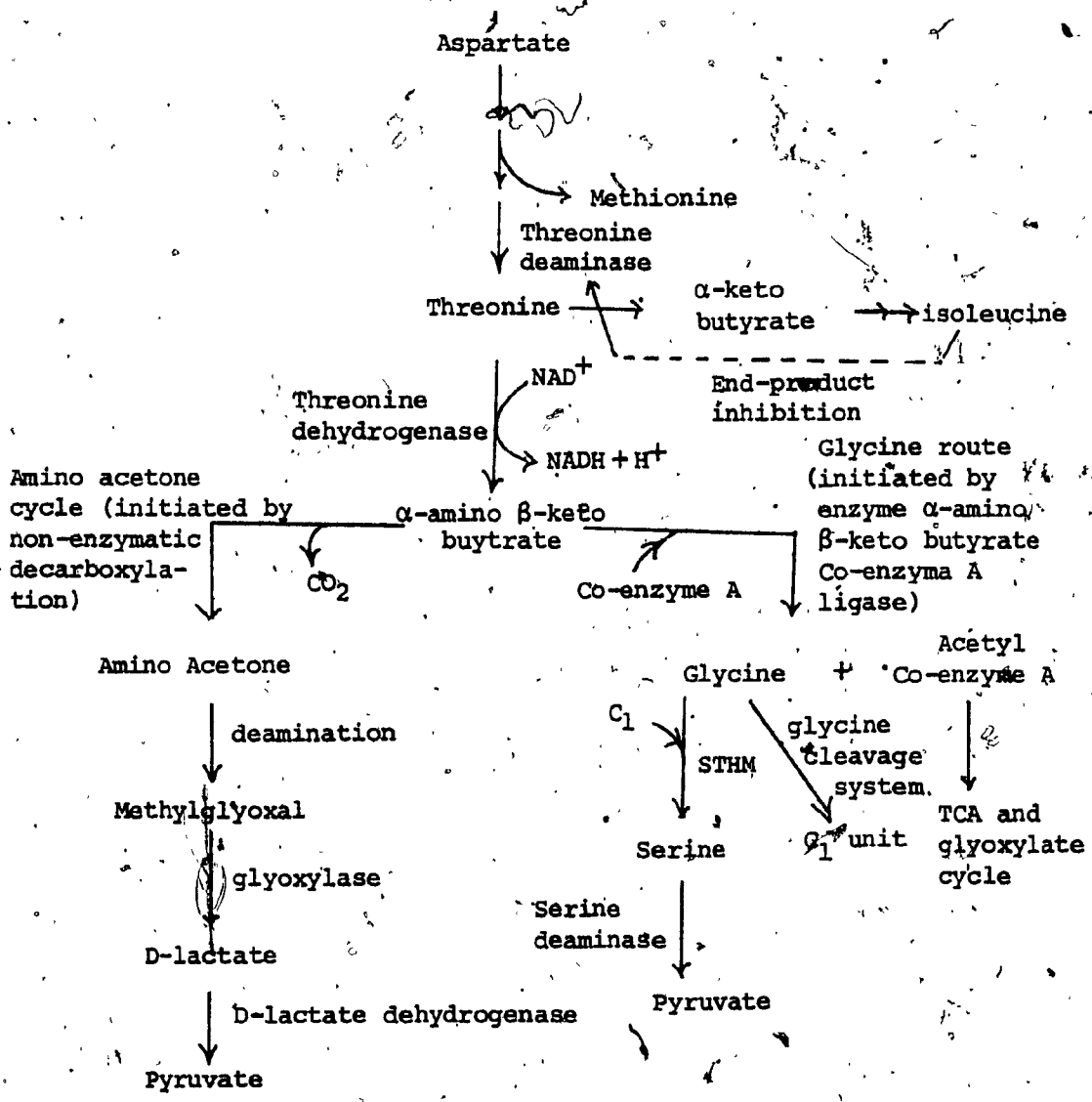
α-Amino-β-keto butyrate Co-enzyme A ligase (ligase) is a Co-enzyme A dependent enzyme that can split AKB into glycine and acetyl Co-enzyme A.

Newman, Baptist, Fraser, Isenberg, Weyman and Kapoor (1976) by using STHM-deficient mutants as well as threonine deaminase (biosynthetic) deficient mutants have already shown that the glycine route is responsible for the threonine catabolism when threonine is used as the sole nitrogen source. The nitrogen flow was considered to be as follows. Threonine is first converted to AKB by threonine dehydrogenase. Then, presumably, by the enzymatic activity ligase, the four carbon compound is split into two two-carbon compounds: glycine and acetyl Co-enzyme A. Glycine is then converted by STHM to serine by combining with a C_1 compound and serine is the actual nitrogen donor. Its amino group is released, perhaps by serine deaminase. This glycine route is also responsible for the threonine catabolism in Pseudomonas cepacia when threonine is the sole carbon source (Lessie & Wong, 1979).

However, the data supporting the glycine route in E.coli threonine catabolism when exogeneous threonine was used as the sole carbon and energy source are not sufficient. In particular, although the enzymatic activity of ligase has been shown in many micro-organisms, it has not previously been shown in E.coli. In addition, as it will be discussed in this

Figure 2

Aerobic Threonine Catabolism in E.Coli.



text glycine itself, cannot serve as carbon source for E.coli.

Glycine Metabolism in Wild Type E.Coli. and STHM-Deficient Mutants

Though glycine cannot serve as the sole carbon source in E.coli , it is a metabolic intermediate of considerable importance. Glycine is derived mainly from serine by the enzyme STHM. During the conversion, serine will transfer its β-carbon as a C_1 unit to tetrahydrofolic acid (Blukely, 1954). Therefore, this reaction fulfills two physiological functions: providing both glycine and C_1 units. Newman and Fraser (1975) described a second biosynthetic pathway for glycine this time derived from threonine. In this case, threonine is first converted to AKB and then cleaved by ligase to form glycine.

In an STHM-deficient mutant, the interconversion between serine and glycine is blocked. Glycine will have to be supplied exogeneously, as such, or as threonine. Because of the STHM deficiency, C_1 units cannot be made from serine. The chief supply of C_1 units will be from glycine via the 'Glycine Cleavage System'. 'Glycine Cleavage System' is the name given to the enzymes which form C_1 units from glycine and

have been studied in detail in D. glycinophilus (Sagers & Gunsalus, 1961). They resulted in the formation of C_1-THF from the α-carbon and carbon dioxide from the carboxyl carbon of glycine. In a STHM-deficient mutant, the main supply of C_1 units is from the Glycine Cleavage System.

C_1 units are used for many biosyntheses---- including two C_1 to make one purine molecule (Buchanan & Hartman, 1959), one C_1 for thymidine (Elwyn & Sprinson, 1950), one C_1 for histidine and one C_1 for methionine (Cohen, 1968). Since glycine is also a precursor of purine C 4,5 and N 7, in STHM-deficient mutant, it takes 3 glycine molecules to make one purine, one glycine for one thymidine, one glycine for one histidine, and one glycine for one methionine molecule.

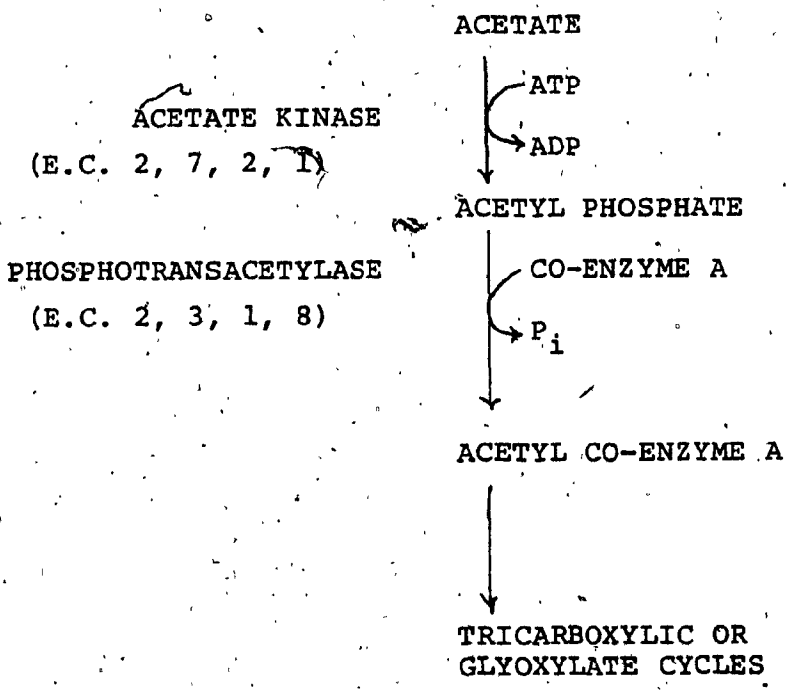
Metabolism of Acetate in E.Coli.

Threonine catabolism, if it is via the glycine route, will produce glycine and acetyl Co-enzyme A. Though E.coli does not use glycine as the sole carbon source, it does use acetyl Co-enzyme A as the carbon source. Acetate metabolism in E.coli is well understood (Brown, 1977). It starts with the conversion of acetate to acetyl Co-enzyme A via

phosphorylation including the enzymes acetate kinase (E.C. 2, 7, 2, 1) and phosphotransacetylase (E.C. 2, 3, 1, 8). Then the acetyl Co-enzyme A will enter into the well known tricarboxylic acid cycle and glyoxlate cycle (Kornberg, 1964) as shown in Figure 3.

Figure 3

Acetate Catabolism in E.Coli. (Brown, 1977)



METHODS AND MATERIALS

Media

Bacteria were grown according to the usual methods in a phosphate-based medium containing 0.54% K_2HPO_4, 1.26% KH_2PO_4, 0.2% $MgSO_4$ and 0.001% $CaCl_2$ at a pH 6.4. 0.2% $(NH_4)_2SO_4$ was used as the sole nitrogen source. When glucose or acetate was used as the sole source of energy and carbon, it was autoclaved separately and added to the minimal medium to a final concentration of 0.2%. When threonine was the sole energy and carbon source, it was added to the minimal medium before autoclaving at a concentration of 0.2%.

Cultures of all strains were kept by frequent transfer on slants of yeast tryptone agar containing 1% yeast extract, 1% tryptone, 2% agar and 0.25% K_2HPO_4. Glucose was added to a final concentration of 0.1%.

Cell Extracts Preparation

Two litres of mid-log phase cells were harvested by filtration using porcelain filtre elements (Selas Flortronic Co.). Cells were resuspended from the filter in the same medium and centrifuged at 6,000 rpm at 4°C for 15 minutes. The pellet usually about three grams of wet weight was

resuspended to 20% weight/volume in potassium phosphate 0.05 M pH 7.2. The cells were then disrupted by sonication in an ice bath using a sonifier cell disruptor (Model 350, Branson Sonic Power Co.), for two pulses of oscillation each with 15 seconds with an interval of 15 seconds. The suspension was clarified by centrifugation at 10,000 rpm at 4°C for 30 minutes. The supernatant was then used for enzyme assays.

Sources of Strains

Strain AT 2046, an STHM-deficient, glycine requiring derivative of E.coli K12 was acquired from L.I. Pizer, University of Pennsylvania.

Strain JEV 73R, an STHM-deficient prototroph was isolated by Fraser (1975). It is a derivative of strain AT 2046, and is distinguished by a high TDH activity (Newman, Kapoor & Potter, 1976).

Strain K 10, a protrophic strain of E.coli K12, was obtained from A. Garen, Yale University.

Strain TE 111, TE 111A, and TE 103 were isolated by the author from strain JEV 73R as described in the text.

Isolation of Strain TE 103

Strain TE 103 is an acetate-positive derivation of strain JEV 73R. To isolate this, strain JEV 73R was

inoculated into minimal medium using glucose as the sole carbon and energy source. 10 ml of that culture were centrifuged and resuspended in 10 ml of 0.9% saline. Then the bacteria were irradiated by ultra-violet light for one minute before plating on minimal medium using acetate as the sole carbon and energy source. After 4-5 days of incubation, acetate-positive colonies were isolated and purified. One of these acetate-positive strains was named TE 103.

Isolation of Strain TE 111

Strain TE 111 is a spontaneous threonine-positive derivative of strain JEV 73R. To isolate this, strain JEV 73R was cultured in liquid minimal medium using glucose as the sole source of energy and carbon. After the culture had reached mid-log phase, the bacteria were washed once with sterilized 0.9% saline and plated directly on minimal medium using threonine (0.2%) as the sole source of energy and carbon. The colonies that appeared after 4-5 days of incubation were isolated and repurified. One of these threonine--positive strains was named TE 111.

Isolation of Strain TE 111A

Strain TE 111A is an acetate-negative derivative of strain TE 111. To isolate it, strain TE 111 was

cultured in liquid minimal medium using acetate as the sole source of energy and carbon. After the culture had reached mid-log phase, the bacteria were irradiated with ultra-violet light followed by penicillin enrichment (Miller, 1972) to isolate acetate-negative strains. One of the acetate-negative derivatives was named TE 111A.

Threonine Dehydrogenase Enzyme Assay

Colorimetric method. This assay measures the threonine-dependent production of amino ketone (amino acetone) from threonine and NAD (Newman, Kapoor & Potter, 1976). The direct enzymatic product of TDH is AKB which is rapidly and nonenzymatically decarboxylated to amino acetone. The 3 ml incubation mixture contained 500 μM NAD, 30 mM threonine, 0.2 M Tris-HCl buffer pH 8.5. The reaction was started with the addition of enzyme, incubated for 10 minutes at 37°C and ended with the addition of 0.8 ml cold 30% TCA solution. One ml of the sample was then assayed for amino acetone by the method of Urata and Granick (Urata & Granick, 1963) with the modification of Ehrlich's reagent by Mauzerall and Granick (1956) using δ-aminolevulinic acid as a standard. The optical density of the assay solution was measured

at 540 nm. This colorimetric TDH assay was routinely applied during the purification.

Spectrophotometric method. This assay measures the threonine-dependent reduction of NAD at 340 nm. Due to the high concentration of 'NADH oxidation' in strain JEV 73R crude extracts, this enzyme assay was useful only with the partially purified preparation of TDH.

α-Amino-β-Keto Butyrate Co-Enzyme A Ligase (Ligase) Assay

This assay measured the acetyl Co-enzyme A-dependent production of amino acetone from glycine and acetyl Co-enzyme A. The direct enzymatic product of ligase is AKB which is rapidly decarboxylated to amino acetone. The one ml incubation mixture contained 40 mM glycine, 0.4 mM acetyl Co-enzyme A, 0.2 M phosphate buffer pH 7 containing 0.01 M 5,5'-dithio-bis-(2-nitrobenzoic acid) (DTNB). The reaction was started with the addition of enzyme, incubated 10 minutes at 37°C, and ended with the addition of 0.2 ml of 30% cold TCA. The content of amino acetone in the incubation mixture was measured as described previously.

Serine Transhydroxymethylase (STHM) Assay

Serine transhydroxymethylase activity was assayed by a modification of the method of Fold and Berg (1970) as described by Miller (1976).

Phosphotransacetylase (PTA) Assay

This enzyme assay measured the glycine-independent deacylation of acetyl Co-enzyme A to Co-enzyme A. The Co-enzyme A formed from PTA will convert DTNB to p-nitrothiophenol anion which can be measured at 412 nm. The two ml assay mixture contained 0.4 mM acetyl Co-enzyme A, 0.2 M phosphate buffer pH 7 and 0.01 M DTNB. The reaction was started with the addition of enzyme, incubated for 5 minutes at 37°C and stopped by chilling in an ice bath. The optical density at 412 nm was measured by Spectronic 21 (Bausch and Lomb Company).

DOWEX-50 Desalting Procedure

This is a simple method for desalting amino acid in the growth medium suggested by Karen Thitlow, University of British Columbia (Personal Communication to E.B. Newman). A DOWEX-50 (H^+) 100-200 mesh column (1.2 x 10 cm) was equilibrated with 0.1 M acetic acid before 25 ml of the culture filtrate (pH adjusted to 2 with HCl) was applied. Then the column was washed

with 10 ml of 0.1 M acetic acid before the amino acids were eluted with 0.1 M NH_4OH. The eluant was collected until it reached pH 12, and was then frozen and lyophilized. This preparation was then used for thin layer chromatography or paper chromatography as described in the text.

Protein Determination

Protein was determined by the method of Lowry et al. (1951) using BSA as a standard.

Carbon Dose and Protein Production Experiment

Various amount of carbon source was applied to strain TE 111 and the protein content of the culture was determined at the stationary phase. Threonine, acetate and glucose were in this experiment.

RESULTS

Part 1: Genetic Studies

Isolation of An E.Coli Mutant That Uses Threonine as Sole Carbon And Energy Source

The strain of E.coli K12 studied here is strain JEV 73R which has a very large amount of threonine dehydrogenase (TDH): about 100-fold more than that in usual strain of E.coli JEV 73R is serine transhydroxymethylase-(STHM)-deficient but, using this large supply of TDH, it can make glycine from endogenously formed threonine and therefore requires no addition of glycine to its growth medium. This pathway moreover must function at a high capacity. Since strain JEV 73R is STHM-deficient, it requires a great deal more glycine than would a STHM-sufficient strain which could make most of its C_1 units from serine.

Strain JEV 73R, then, is thought to produce all the glycine it requires from threonine via TDH and α-amino-β-keto butyrate Co-enzyme A ligase (ligase). If this were true, one would expect that strain JEV 73R should use threonine as the carbon and energy source because when threonine was cleaved by the glycine route, a large amount of acetyl Co-enzyme A would be

produced together with glycine. Since acetyl Co-enzyme A can be the carbon donor for the general metbolic pathways in E.coli (Kornberg, 1964), threonine should serve as the sole source of carbon and energy of strain JEV 73R.

However, when JEV 73R was tested with threonine as the sole carbon and energy source, it did not grow. This could be explained in several ways, as follows:

a) Strain JEV 73R was producing acetyl Co-enzyme A but unable to use it.

b) Threonine was catabolized by an enzyme other than TDH: the product of that enzyme cannot serve as carbon source.

c) Threonine was catabolized by the glycine route but the regulation was set in such a way that the route could not be used during the test condition.

The following experiment shows that the first of these is correct.

If the inability of strain JEV 73R to use threonine as the carbon and energy was due to the inability of the bacteria to use acetyl Co-enzyme A as the carbon source, strain JEV 73R should not grow on medium using acetate as the sole carbon and energy source. In fact, strain JEV 73R could not use acetate as the sole carbon and energy source.

One might then assume that if one could isolate an acetate-positive strain, it would also use threonine. To isolate an acetate-positive mutant of JEV 73R, the strain was exposed to ultra-violet light, subcultured and plated in large numbers (approximately 10^8/plate) on minimal acetate plates. One of the colonies which grew on this plate was purified and named TE 103. A reciprocal selection for threonine ultilizing strain TE 111 was made in the same way — on plates with threonine as sole carbon source — in this case without ultra-violet light mutagenesis.

TE 103 selected on acetate proved to be able to use threonine as the sole carbon and energy source. Reciprocally, TE 111, selected on threonine proved to be able to use acetate as the sole carbon and energy source. The parent strain JEV 73R was unable to use either. This showed clearly that a single mutation affected the ability to use both threonine and acetate as the sole carbon and energy source. Therefore, it can be concluded that what was missing in JEV 73R is in the metabolism of acetyl Co-enzyme A and not in the threonine catabolism.

Generalization of The Relationship Between Threonine Catabolism And Acetate Metabolism

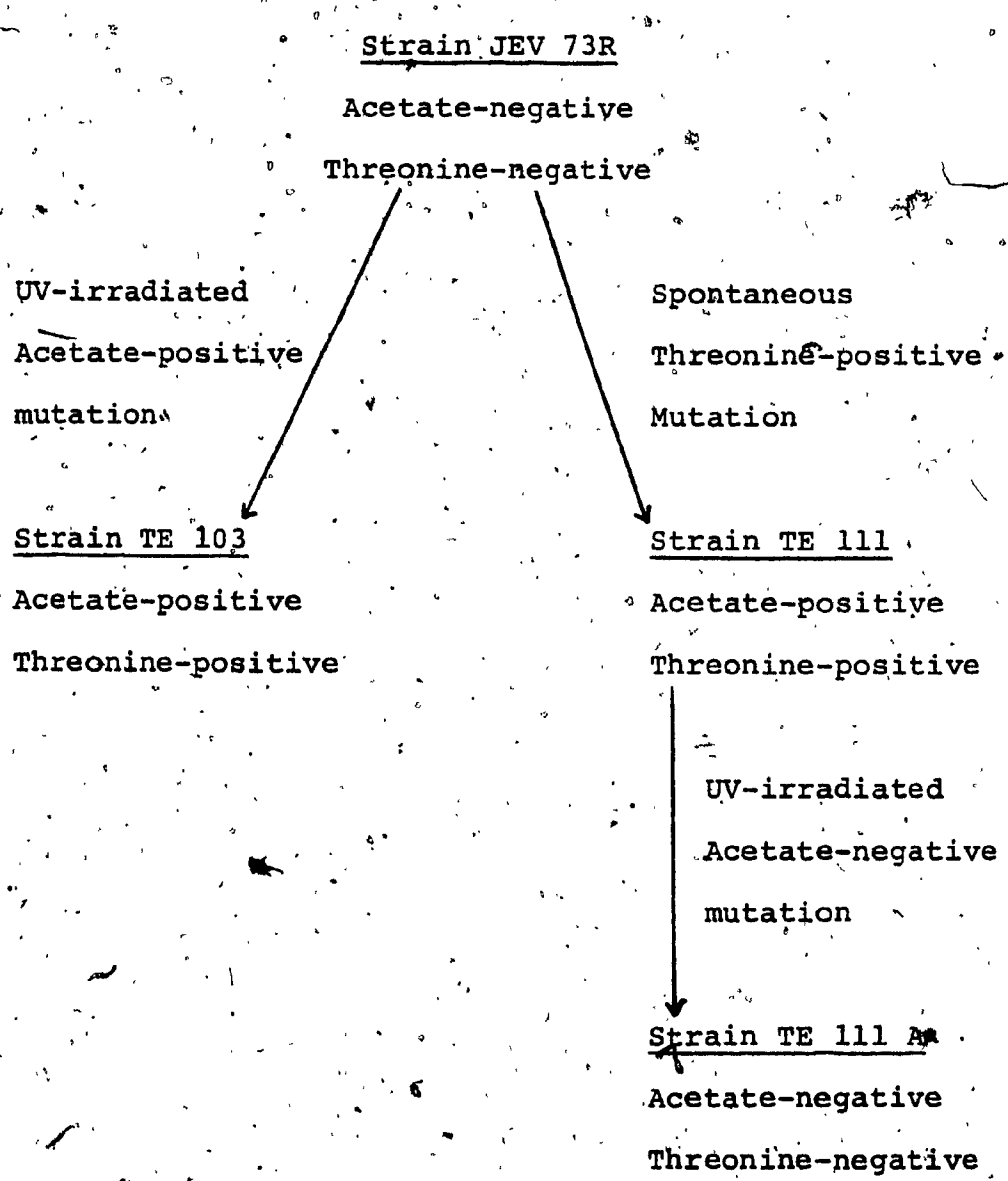
The fact that TE 103, the acetate-positive strain from strain JEV 73R can use threonine as the sole carbon and energy source and TE 111, the threonine-positive mutant can use acetate as the carbon and energy source, suggests that acetate metabolism and threonine catabolism are related. In order to generalize such a relationship, more acetate-positive mutants and threonine-positive mutants from strain JEV 73R were isolated.

Ten independent acetate-positive mutants and eight independent threonine-positive mutants were isolated and purified before reciprocal plating on threonine and acetate minimum plates respectively. All ten acetate-positive mutants could use threonine as the carbon source and seven out of eight threonine positive mutants could grow on acetate minimum plates.

Thus in all ten cases, the addition of the capacity to use acetate suffices to allow the strain to use threonine. In seven out of eight cases, the aquisition of the ability to use threonine allowed the strain to use acetate. This confirmed that threonine catabolism in E.coli involved conversion of threonine to acetate or an acetate-related compound. In one case, the

Figure 4

Genetic investigation on strain JEV 73R to determine the threonine catabolism



strain has used some other mechanism to become threonine-ultilizing. This is interesting but has not been further investigated here.

Confirmation of The Relationship Between Threonine Catabolism And Acetate Metbolism by Isolation of An Acetate-Negative Derivative of TE 111

In order to confirm the relationship between threonine catabolism and acetate metabolism, an acetate-negative strain was isolated from TE 111 using ultra-violet light as the mutagen and penicillin as the enriching agent (Miller, 1972). The purified acetate-negative mutant from TE 111 could not use threonine as the carbon source. This showed that a loss in the capacity to metabolize acetate leads to an inability to use threonine as the carbon and energy source (Figure 4).

Confirmation That Strain TE 111 Is An STHM -Deficient Mutant

TE 111 is isolated from JEV 73R and therefore it should be STHM-deficient. The confirmation that TE 111 is a STHM-deficient mutant is essential to the further experiments in this thesis for two reasons: first to be certain that it actually is a derviative of strain JEV 73R and second to exclude STHM participation in

the further metabolism of glycine in the cell.

Newman et al. (1977) showed that the use of glycine as the nitrogen source requires STHM activity. If TE 111 was STHM-deficient, it should not use glycine as the nitrogen source.

TE 111 was tested with glycine as the sole nitrogen source, using E.coli K10 as the wild type control and strain JEV 73R as the STHM-deficient control. The results proved that TE 111 was unable to use glycine as the nitrogen source. In addition, STHM activity was also assayed in extracts of TE 111. No activity could be detected.

Glycine Cannot Be The Sole Carbon And Energy for E.Coli

It seems then that strain TE 111 converts threonine to acetyl Co-enzyme A and glycine. The next experiments are designed to inquire as to the fate of this glycine.

E.coli strains K10, JEV 73R and TE 111 were all tested on glycine as the sole carbon and energy and were unable to grow. Thus even strain TE 111, which can use threonine as carbon and energy source and that by converting threonine to glycine and acetyl Co-enzyme A cannot use glycine as the sole carbon and energy source.

Plates with a large number of TE 111, K10, JEV 73R were incubated for long periods and yet no glycine-metabolizing mutant of any of the three strains could be isolated. It must be concluded that E.coli does not use glycine as the sole carbon source.

Excreton of Glycine By TE 111 Using Threonine as The Sole Carbon And Energy Source

It is clear that TE 111 uses threonine as the sole carbon and energy source by converting threonine to acetyl Co-enzyme A and glycine. But as has been just shown, glycine cannot be used as the carbon or nitrogen source and cannot be converted to serine since the strain is STHM-deficient. The cells do use acetate as the carbon and energy source. One might then predict that during threonine catabolism, glycine should accumulate in the cell, and be excreted into the medium. The next experiments prove that this is so.

In order to investigate what compounds are excreted during threonine catabolism, strain TE 111 was grown to stationary phase with limiting amounts of threonine (2 mg/ml) and the culture supernatant obtained by centrifugation, desalted in DOWEX-50 column and concentrated by lyophilization. The redissolved lyophilized powder was chromatographed in two

one-dimension systems as follows:

a) Silica gel thin layer chromatography: the redissolved lyophilisate was analyzed by silica gel thin layer chromatography using the solvent ethanol: water (7:3, v/v). As can be seen in Figure 5, only one ninhydrin reactive spot could be seen and that was coincided with the mobility of glycine.

b) Paper chromatography: the redissolved lyophilizate was analyzed by paper chromatography using the solvent; phenol; ammonium hydroxide: water (80:0.3:20, v/v). As can be seen in Figure 6, only one spot of ninhydrin reactive material could be seen and that was in the position of glycine.

In order to confirm that the lyophilizate contained glycine, two dimension silica gel thin-layer chromatography was carried out using the solvent; n-butanol: acetone: ammonium hydroxide: water (10:10:5:2, v/v) in the first dimension and isopropanol, formic acid and water (20:1:5, v/v) in the second dimension. As can be seen in Figures 7 and 8, only one ninhydrin reactive spot can be seen on the sample plate. The R_f valves of the sample and glycine standard were 0.25 and 0.27 for the first dimension; 0.66 and 0.68 for the second dimension respectively.

The results from the chromatographic analysis on

Figure 5

Qualitative analysis by one dimension TLC

NOTE: All the samples used in here are after the desalting procedure by DOWEX-50 column and then concentrated by freeze-drying method.

Physical parameters: Thickness of the silica gel: 500 micron

Solvent system used: EtOH/H_2O 7 to 3

Time: 2 hours

Solvent front: 14.1 cm

Temperature: 21°C

Colour of the spots: deep purple

Minimum detection level:
glycine 0.006 µ gm
threonine 0.1 µ gm

Sample # 1 Glycine standard 10 µ gm R_f = 0.30

2 Threonine standard 10 µ gm R_f = 0.44

3 Glycine standard + threonine standard, 10 µ gm each R_f = 0.32, 0.44

4 Sample 2 µl R_f = 0.30

5 Sample 1 µl R_f = 0.31

6 Sample 3 µl R_f = 0.30

7 Sample 4 µl R_f = 0.31

Sample

7 6 5 4 3 2 1

Figure 6

Qualitative analysis by one dimension paper chromatography

NOTE: All the samples used in the chromatography are after the desalting procedure by DOWEX-50 column and freeze drying concentration.

Physical parameters:

Paper: Whatman #1 paper

Solvent system used: Phenol/ammonium hydroxide/H_2O
80 / 0.3 / 20

Time: 24 hours

Solvent front: 34 cm

Temperature: 21°C

Colour of the spots: deep purple

Minimum detection level: Glycine 0.1 µg
Threonine 2 µg

Sample # 1 Threonine standard 10 µ gm R_f = 0.43

2 Threonine + Glycine standard 10 µ gm each R_f = 0.36, 0.45

3 Sample 5 µl R_f = 0.36

4 Sample 5 µl R_f = 0.36

5 Glycine standard 10 µ gm R_f = 0.34

Sample

5 4 3 2 1

Direction of the solvent

Figures 7 and 8

Qualitative analysis by two dimension TLC

NOTE: All the samples used in here are after the desalting procedure by DOWEX-50 column and concentrated by freeze drying method.

Physical parameters:

Thickness of the silica gel 500 micron

Solvent systems used:

1st dimension — n-butane/acetone/NH_4OH/H_2O
10 / 10 / 5 / 2

Time: 4 hours

Solvent front: 16.5 cm

R_f Glycine standard 0.27
Sample 0.25

2nd dimension — isopropanol/formic acid/H_2O
20 / 1 / 5

Time: 6 hours

Solvent front: 16 cm

R_f Glycine standard 0.68
Sample 0.66

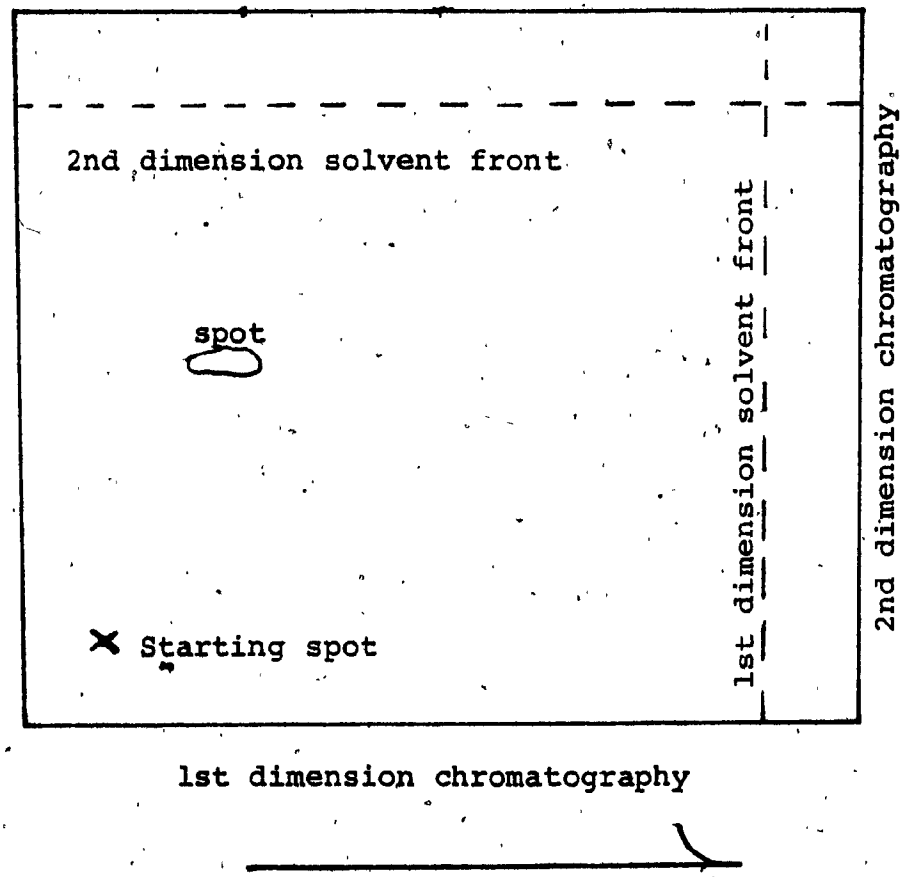
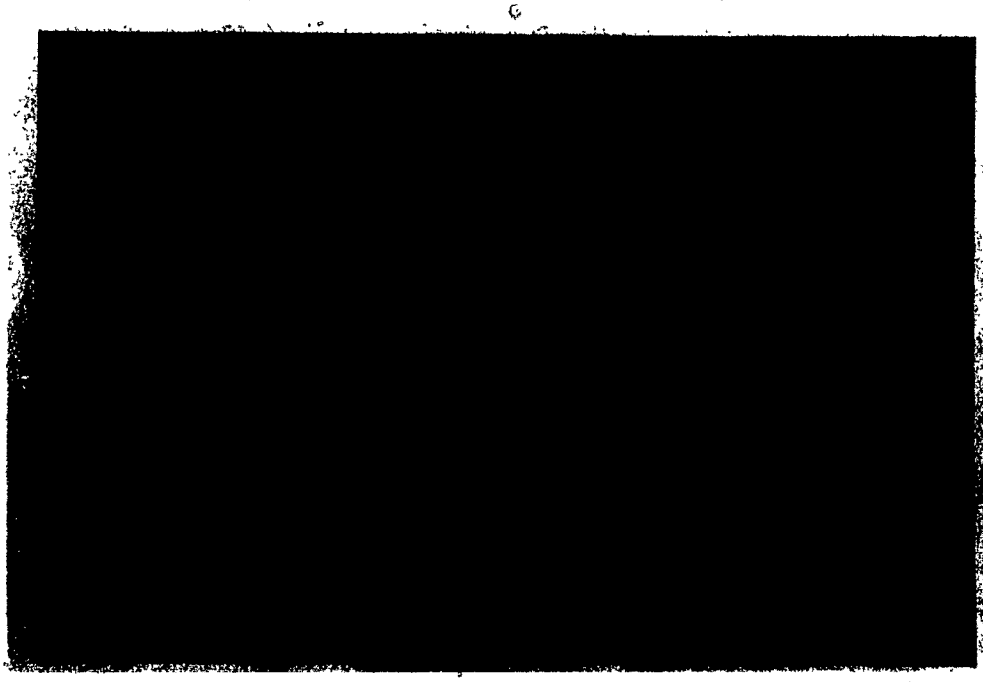

2nd dimension solvent front
spot
Starting spot
1st dimension solvent front
2nd dimension chromatography
1st dimension chromatography

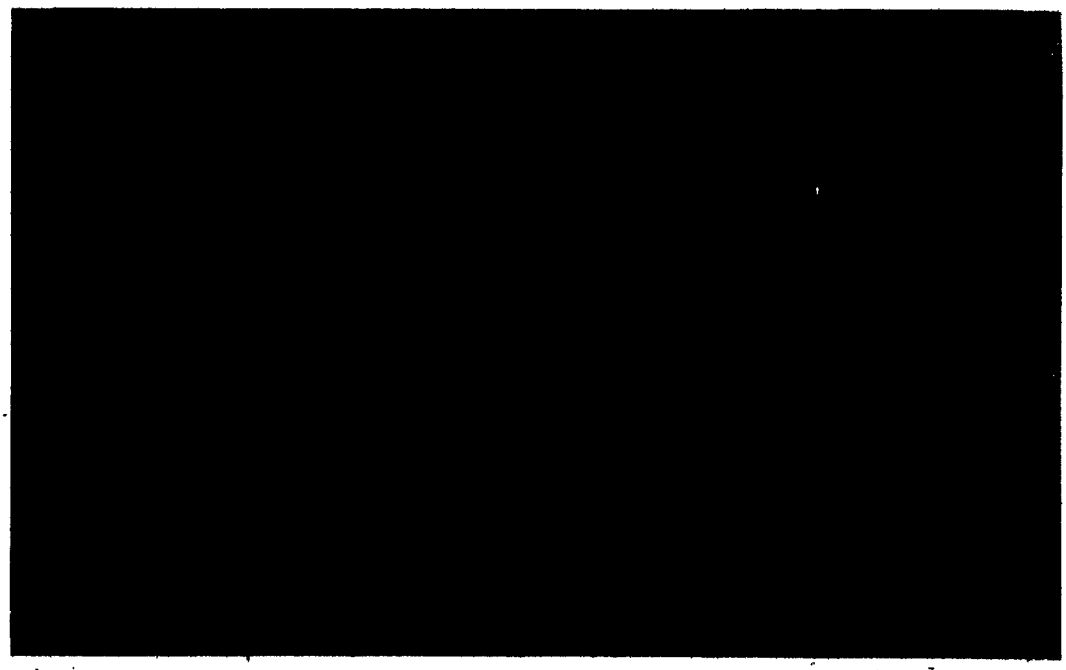
Figure 7

Glycine Standard

Figure 8

Sample





the lyophilizate confirmed that glycine was actually excreted by the strain TE 111 when the bacteria were using threonine as the sole carbon and energy source.

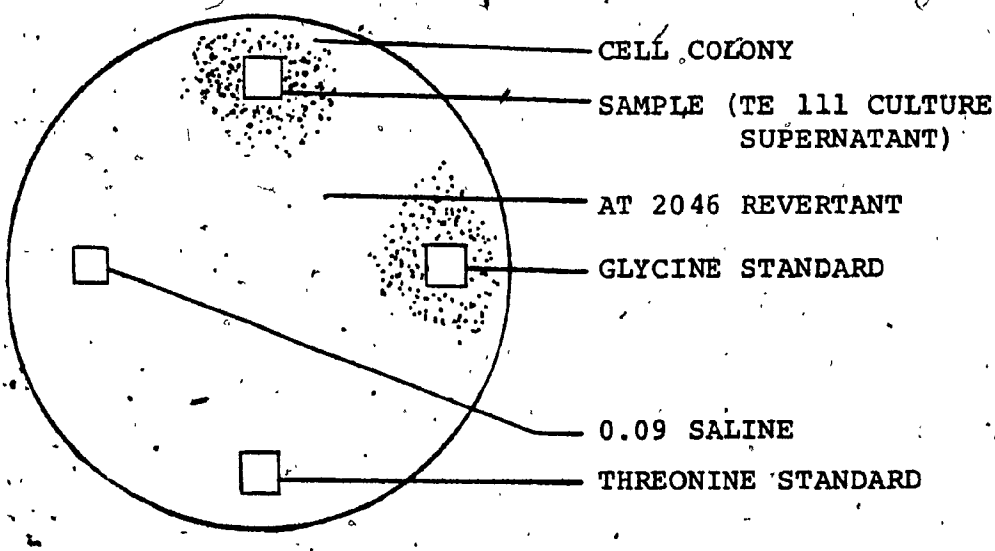
Besides the chemical chromatography analysis, a biological measurement can be used to confirm the existence of glycine in the culture filtrate. If the compound is in fact glycine, it should support the growth of a glycine-requiring mutant of E.coli. To test this, strain AT 2046, a strain which cannot grow without glycine, was plated on minimal medium agar and an aliquot of the sterilized supernatant was dropped upon it. Aliquots of standards glycine and threonine and of a saline control were tested in the same way. As can be seen in Figure 9, strain AT 2046 was only able to grow on the supernatant and glycine control but not on the threonine and saline control. This confirmed that the compound excreted by strain TE 111 when threonine was the sole carbon and energy source is indeed glycine.

The Gradual Accumulation of Glycine During Growth in Threonine

If the use of threonine as the sole carbon source involves the obligatory formation of glycine, glycine should accumulate in the medium at a rate comparable

Figure 9

Growth of glycine requiring strain AT 2046 in TE 111 culture medium.



with that of cell growth.

To test this, samples were taken from a culture of strain TE 111 using threonine as the sole source of carbon and energy at three different points in the growth cycle: at the early mid-log phase (O.D. 15 kletts) at the mid-log phase (O.D. 102 kletts) and at the stationary phase (O.D. 195 kletts). The samples were centrifuged and the supernatants were prepared and chromatographed as described earlier.

Photographs of chromatograms of this experiment (Figure 10) showed that by mid-log, there were substantial quantities of glycine excreted and by the stationary phase, no detectable threonine remained in the culture filtrate. This is consistent with the idea that glycine is excreted continuously during growth.

Analysis of The Threonine Catabolism in TE 111; Using The Ratio between The Carbon Source Input And The Protein Produced As The Comparison Parameter

The preceeding experiments have shown that for one molecule of threonine used, only two out of its four carbons will be used as the carbon source, while the other two will be excreted mainly in the form of glycine. If this is true, one would expect TE 111 would use the same number of molecules of threonine

Figure 10

Qualitative analysis of the glycine excretion by TE 111 using threonine as the sole carbon source.

Physical parameter:

Thickness of the silica gel 500 micron
Solvent system used: $EtOH/H_2O$ 7 / 3
Solvent front: 16.2 cm
Time: 2 hours

Sample # 1 Glycine standard 10 µ gm

2 Sample 1 O.D. 15 K.U. Early mid-log phase 3 µl

3 Glycine + threonine standard 10 µ gm each

4 Sample 2 O.D. 102 K.U. mig log phase 3 µl

5 Threonine standard 10 µ gm

6 Sample 3 O.D. 195 K.U. stationary phase 3 µl

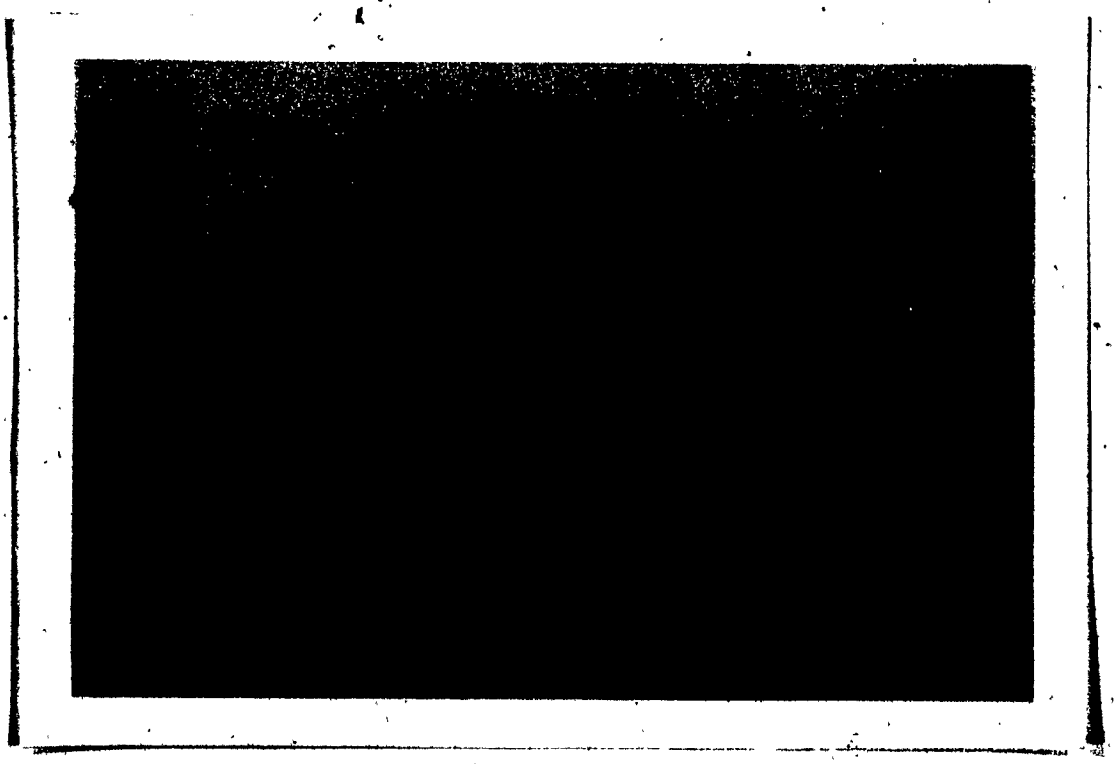
7 Glycine standard 10 µ gm

8 Threonine standard + Glycine standard 10 µ gm each

9 Sample 2 mid-log phase 3 µl

Sample

9 8 7 6 5 4 3 2 1



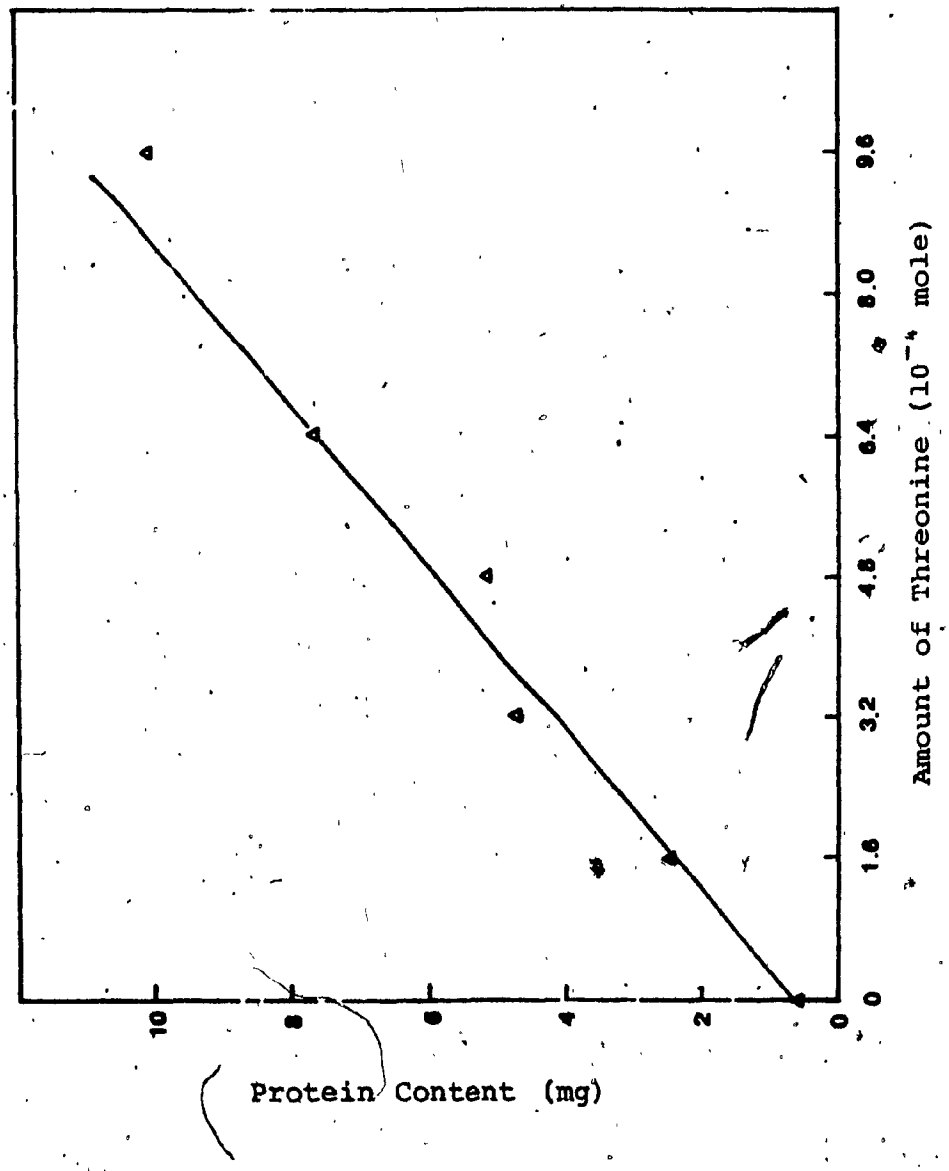
or acetate to make a given amount of cell material although threonine is a four carbon compound and acetate is a two carbon compound. Thus the efficiency of using threonine as the carbon source should be equal to that using acetate as the carbon source when expressed as milligram cell protein formed per micro mole of the substrate. The next experiment will show that this is true.

Threonine (concentration range from zero to 3 mg/ml), was used as the sole carbon and energy source for TE 111 cell cultures. After the cultures had reached stationary phase, the cultures were centrifuged and the protein content of the pellets was determined. Similarly, sodium acetate (concentration range from zero to 0.4 mg/ml) and glucose (concentration range from zero to 0.5 mg/ml) were used as the sole carbon and energy source in strain TE 111 bacterial culture. The experimental results are shown in Figure 11.

When threonine was supplied in the range from zero to 3 mg/ml, the protein content of the cells at stationary phase was proportional to the amount of the threonine provided. From this relationship, it could be calculated that strain TE 111 used 93 μ mole of threonine to produce one mg of protein. Similarly,

Figure 11

By culturing strain TE 111 in limiting amount of threonine, this was found that strain TE 111 needed 93 μ mole of threonine to produce one mg of protein.


Amount of Threonine (10^{-4} mole)
0
1.6
3.2
4.8
6.4
8.0
9.6
Protein Content (mg)
10
8
6
4
2
0

the cell growth was proportional to acetate added in the range zero to 0.4 mg/ml, and glucose added in the range zero to 0.5 mg/ml. From these relationships, it could be calculated that strain TE 111 used 95 μ mole of acetate or 40 μ mole of glucose to produce one mg of protein (Figures 12 and 13).

The carbon dose and protein yield experiment showed that strain TE 111 needed equal number of moles of threonine and acetate to produce an equal amount of protein even though threonine is a four carbon compound and acetate is a two carbon compound. This is consistent with the idea that half of the threonine carbon is not metabolically useful.

Analysis of Threonine Catabolism in Strain TE 111 Using Uniformly Labelled ^{14}C Threonine

In order to study quantitatively the relationship between threonine catabolism and glycine excretion, uniformly labelled ^{14}C threonine was added as the sole carbon source in strain TE 111. The distribution of ^{14}C at the stationary phase in the culture medium was examined.

In this experiment, strain TE 111 was provided with 6.91 μ Ci of uniformly labelled ^{14}C threonine, 2 mg/ml. At this concentration, the only limiting

Figure 12

By culturing strain TE 111 in limiting amount of acetate, this was found that strain TE 111 needed 95 μ mole of acetate to produce one mg of protein.

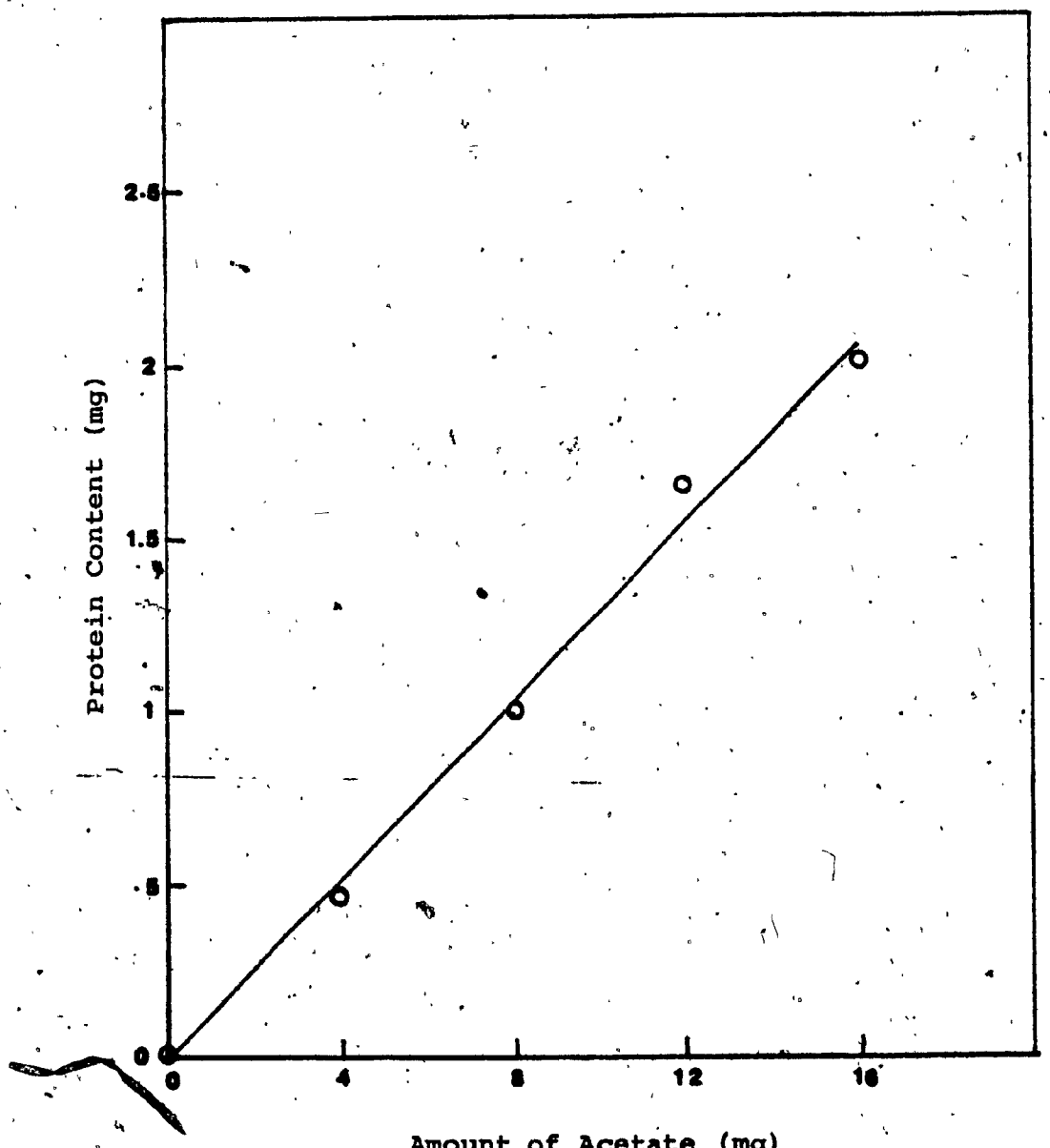
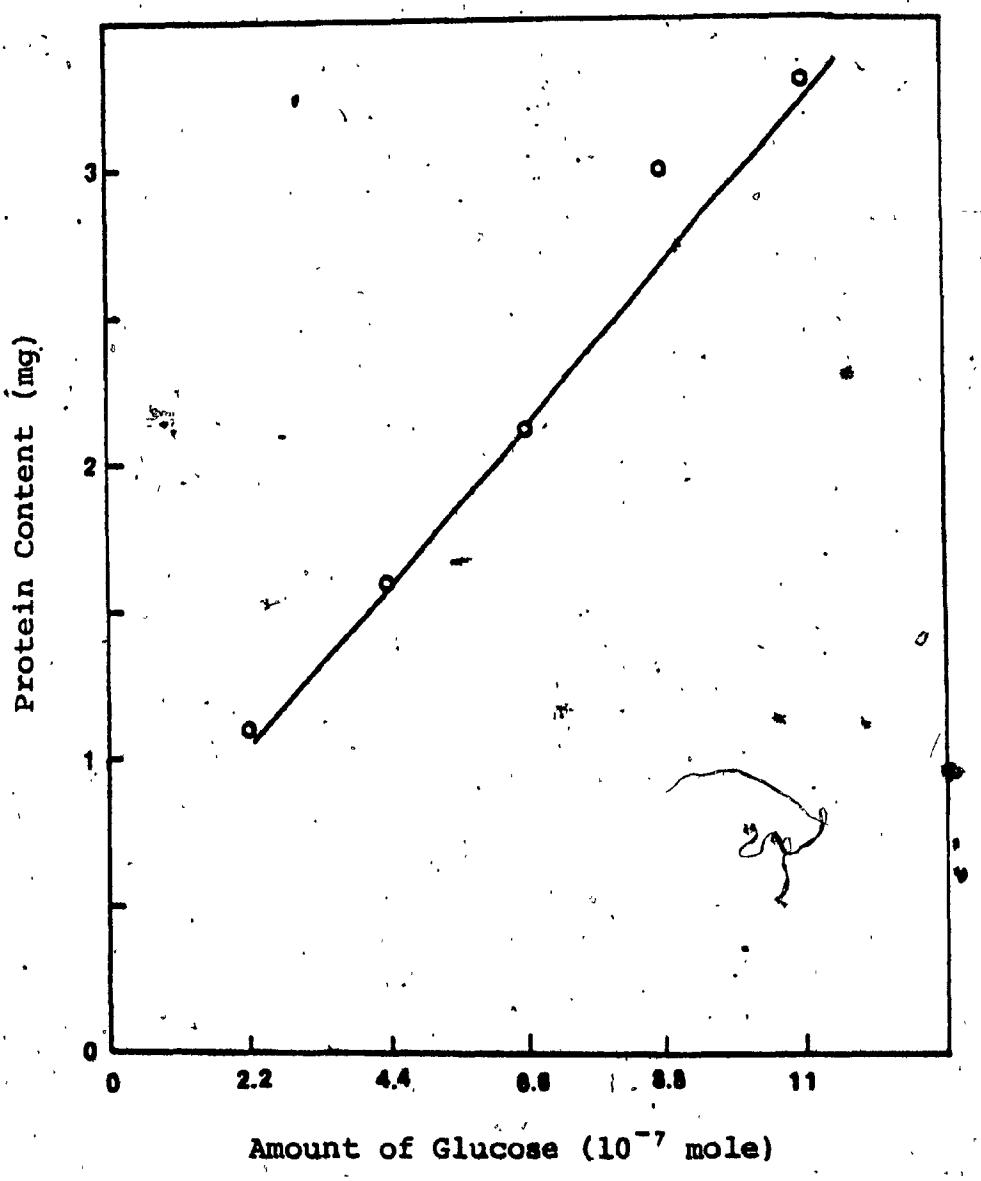

Protein Content (mg)
2.5
2
1.5
1
.5
0
0
4
8
12
16
Amount of Acetate (mg)

Figure 13

By culturing strain TE 111 in limiting amount of glucose, this was found that strain TE 111 needed 40 µ mole of glucose to produce one mg of protein.


Protein Content (mg)
0
1
2
3
0
2.2
4.4
6.6
8.8
11
Amount of Glucose (10^{-7} mole)

factor for the bacterial growth should be the carbon source, threonine. At stationary phase, 1.2 μ Ci could be found in the cell pellet and 3.78 μ Ci in the supernatant. This corresponds to 73.5% recovery of total ^{14}C added. The supernatant was desalted in DOWEX-50 column with 88% recovery of ^{14}C, of which 0.28 μ Ci (8%) did not adhere to DOWEX-50 at low pH used, and 3.06 μ Ci (92%) adhered and were eluted with ammonium hydroxide. This eluant was lyophilized (recovery was 86%), 2.65 μ Ci and the lyophilized powder was analyzed by paper chromatography. The total recovery of ^{14}C on the glycine and threonine zones 83% of which 88% was in the glycine spot and 12% in the threonine spot.

Although only 73.5% of the ^{14}C threonine added was recovery in the pellet and the culture supernatant, the experiment indicated that much of the threonine added to the bacteria cultures as the sole carbon and energy source did end up in the form of glycine excreted by the bacteria.

Figure 14

Analysis of threonine catabolism in strain TE 111 using uniformly labelled ^{14}C-threonine

Total ^{14}C-threonine input = 6.91 μ Ci

↓

Growth until stationary phase
Recovery of radioactivity = 73.5%
Radioactivity in pellet = 1.3 μ Ci
Radioactivity in supernatant = 3.78 μ Ci

↓

DOWEX-50, desalting process
Total radioactivity recovery = 88%
Radioactivity in the washing acetic acid = 0.28 μ Ci
Radioactivity in the NH_4OH eluant = 3.06 μ Ci

↓

Lyophilization
Total radioactivity recovery = 86%, 2.65 μ Ci

↓

One dimension paper chromatography
Total recovery in radioactivity = 83%
In glycine zone = 1.85 μ Ci
In threonine zone = 0.26 μ Ci

Part 2: Enzyme Purification

The experiments described in the first section of this thesis showed that aerobic threonine catabolism in E.coli K12 is initiated by threonine dehydrogenase, and that the further metabolism of AKB is via the 'glycine route' in which ligase cleaves this four-carbon unstable intermediate into glycine and acetyl Co-enzyme A. Acetyl Co-enzyme A is then the actual carbon donor to the general metabolic pathways. Thus, two enzymes, TDH and ligase are responsible for threonine metabolism. In the experiments described next, an attempt was made to purify both of the enzymes and TDH was characterized in some details.

A Comparison of Two Assays for NAD-Dependent Threonine Dehydrogenase Activity

Two methods for the assay of TDH have been described. One is a colorimetric method working on the principle of detecting amino acetone in the assay mixture (Urata & Granick, 1962). The actual product of the oxidative reaction is AKB which is unstable in solution and, by non-enzymatic decarboxylation yields amino acetone. The second is a spectrophotometric method working on the principle of monitoring at 340 nm, the change in the optical density of the assay

mixture due to the reduction of NAD to NADH. However, due to the high NADH oxidase activity in the crude extracts, the spectrophotometric method could not be used. It could be used on partially purified preparations, presumably because TDH was separated from NADH oxidizing enzyme(s) in the first purification procedure.

The colorimetric enzyme assay method was used routinely during the purification procedures and the spectrophotometric enzyme assay method was used to characterize the enzyme. The estimations of enzymatic activity, given by the colorimetric and the spectrophotometric methods were generally consistent (Elliot & Green, 1964 and by the author). Both of the enzyme assays were very sensitive and can detect the enzymatic products at the level of 10^{-7} M.

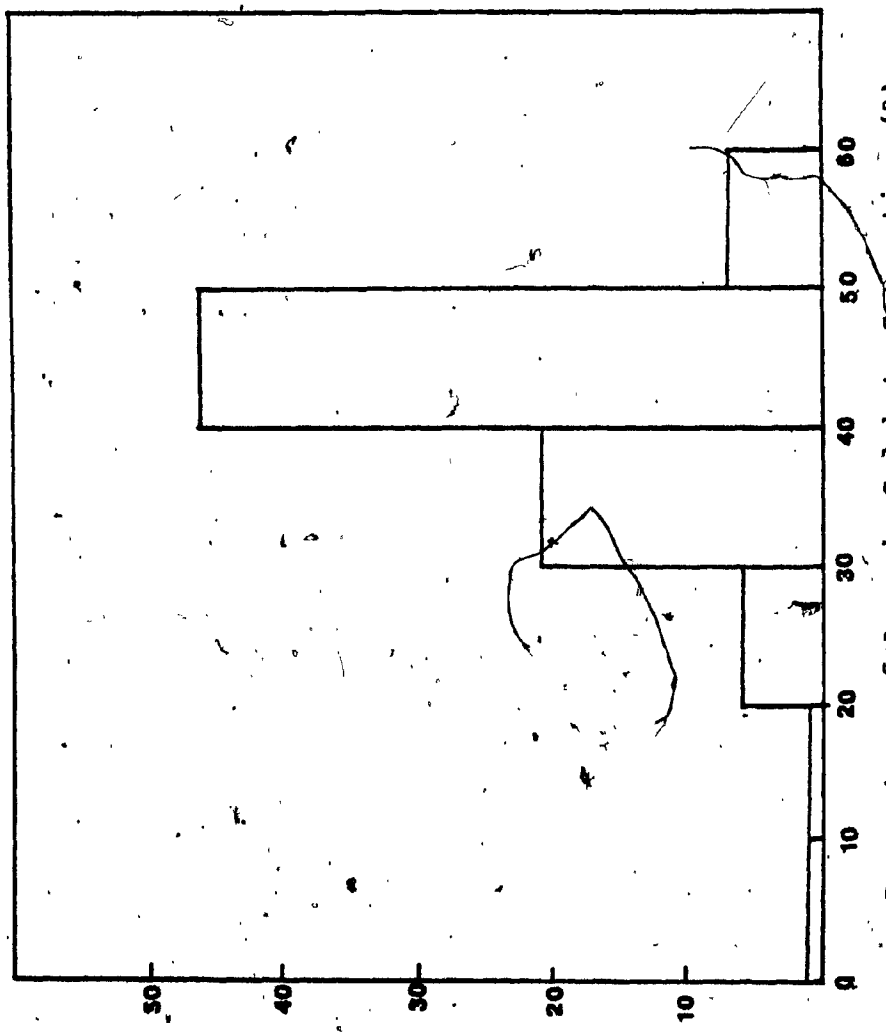
Attempt to Purify TDH in E.Coli. Strain JEV 73R by Ammonium Sulphate Precipitation

Threonine dehydrogenase was very stable in crude extracts. The crude extracts could be stored at $4^{\circ}C$ for one week or dialyzed against distilled water for 24 hours without losing more than 15% of its activity.

The first attempt to purify TDH was by ammonium sulphate precipitation. Crude extracts were

Figure 15

The activity of threonine dehydrogenase (TDH) during fractionation of crude extracts by ammonium sulphate. TDH activity was determined at 20%, 30%, 40%, 50% and 60% of ammonium sulphate at each addition stage. TDH remained soluble up to 20% and it started to precipitate between 40% and 50% ammonium sulphate. No TDH activity remained in the 60% ammonium sulphate supernatant.


Percentage of Ammonia Sulphate Saturation (%)
0
10
20
30
40
50
60
50
40
30
20
10
TDH Enzyme Activity (10^{-5} mole/min)

fractionated by slow addition of ammonium sulphate crystals. Although ammonium sulphate interfered with the colour forming reaction between the amino ketone and the Ehrlich's reagent in the TDH assay, the degree of inhibition was not a function of the ammonium sulphate concentration in the assay higher than 20%.

TDH activity was determined on pellets precipitated at 20%, 30%, 40%, 50%, and 60% of ammonium sulphate saturation at each addition stage. As can be seen in Figure 15, TDH remained soluble up to 20% and it started to precipitate between 30% to 40% with the peak of the precipitation between 40% and 50% ammonium sulphate. No TDH activity remained in the 60% ammonium sulphate supernatant. In later experiments, TDH was found to precipitate mainly between 35% to 50% ammonium sulphate concentration and this range was used routinely as the first step of the purification procedure.

Attempt to Purify Threonine Dehydrogenase in E.Coli Strain JEV 73R by The Sephadex G100 Gel Filtration

After the ammonium sulphate precipitation, the pellet needed desalting before further purification. This is particularly important since ammonium sulphate interferes with the determination of amino acetone.

One would think it was logical to use sephadex G100 gel filtration to do the desalting thus purifying TDH further by separating proteins by molecular weight at the same time as salt is being removed.

Several unsuccessful attempts were made to follow TDH activity by chromatography crude extracts on sephadex G100. These gave very low recovery distributed in two peaks in the collected fractions. Both the composition of the eluant buffer and the rate of elution influenced recovery. If the eluting buffer was 0.05 M phosphate buffer pH 7.2, no threonine dehydrogenase activity was detected in any of the fractions tested. If the eluting buffer was 0.05 M phosphate buffer pH 7.2 with 0.2 M KCl, about 14% of threonine dehydrogenase activity was recovered. The most successful formulation of the eluting buffer was phosphate buffer 0.05 M pH 7.2 with 0.1 M KCl which resulted in no less than 75% and usually 100% recovery, all in one band. In addition, the speed of the elution was the other important factor in this process. According to the instruction from the supplier (Phamacia), the maximum flow rate for sephadex G100 at the optimal operating pressure was 72 ml/hr for a column with diameter 2.5 cm and 234 ml/hr for a column with diameter 5.0 cm. The column actually used in

the gel filtration experiment was 3.6 cm in diameter. When the correct buffer was used with a flow rate about 63 ml/hr, two peaks with a total activity recovery of about 18% were detected. When the speed of elution was reduced to about 10 ml/hr, TDH activity was found in one peak with a recovery of above 75%.

Attempts to Purify Threonine Dehydrogenase in E.Coli Strain JEV 73R by The Combination of Ammonium Sulphate Precipitation And Sephadex G100 Gel Filtration

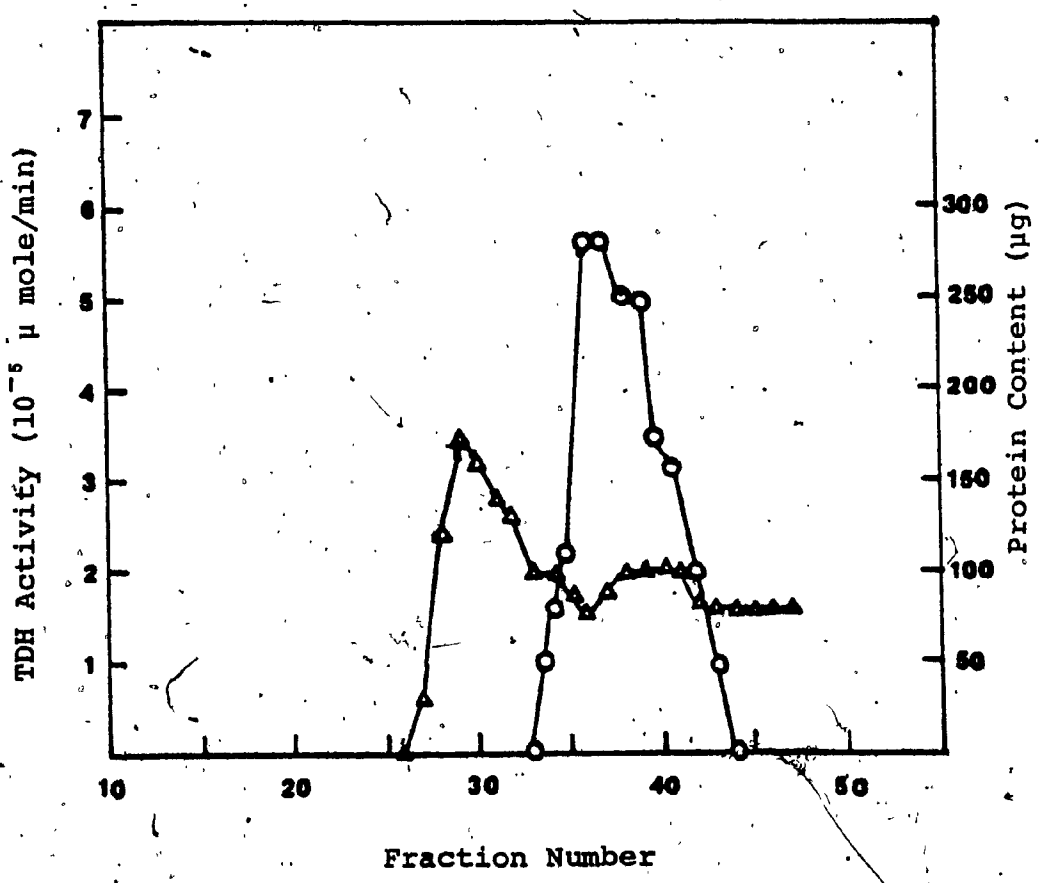
The pellet obtained between 35% to 50% ammonium sulphate precipitation was redissolved in 2 ml of phosphate buffer and applied to Sephadex G100 column (3.6 x 64 cm). The eluting pattern is shown in Figure 16. As can be seen, TDH was retained by Sephadex G100 and eluted from it in fractions just following standard adolase, M.W. 139,000. From this, the molecular weight of TDH was estimated to be around 141,000 daltons (Figure 17). The specific activity of TDH was increased by a factor of 10 to 12 (specific activity increased from 0.3 to 3.5 mM/min/mg of protein) after these combined purification steps with usually 100% recovery of TDH activity.

Figure 16

Elution pattern of threonine dehydrogenase (TDH) on Sephadex G100 gel filtration after 35%-50% ammonium sulphate precipitation treatment. 10 ml of strain JEV 73R crude extract were treated with 35%-50% ammonium sulphate crystals, stirred for 30 minutes before the centrifugation at 10,000 rpm for 30 minutes. Then the pellet was redissolved in 3 ml of phosphate buffer before added to the Sephadex G100 gel filtration column (3.6 x 64 cm). The column was eluted with 0.05 M phosphate buffer pH 7.2 with 0.1 M KCl at 4°C with the elution rate of 10 ml/hr (approximately 3 fractions/hr).

—△— Protein content

—○— TDH activity


TDH Activity (10^{-5} μ mole/min)
Protein Content (μg)
Fraction Number
1
2
3
4
5
6
7
50
100
150
200
250
300
10
20
30
40
50

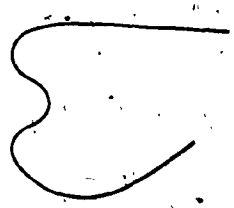
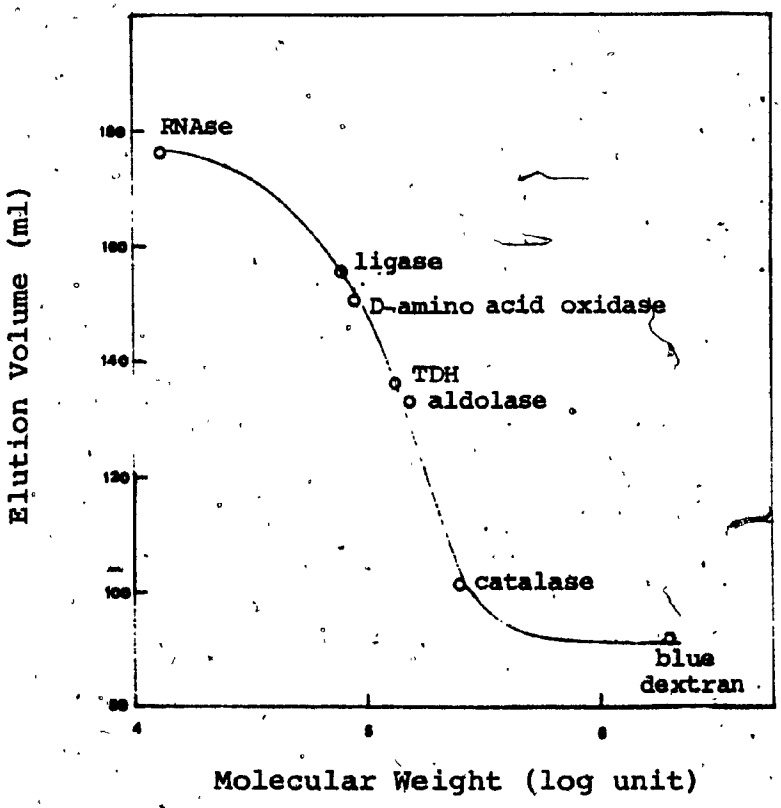


Figure 17

Calibration curve of Sephadex G100 column (3.6 x 64 cm) using blue dextran; M.W. 2,000,000, catalase; M.W. 250,000, aldolase; M.W. 159,000, D-amino acid oxidase; M.W. 90,000, and RNAse; M.W. 13,700 as the standard proteins. The molecular weights of threonine dehydrogenase (TDH) and α-amino β-keto butyrate Co-enzyme A ligase (ligase) found were 141,000 daltons and 79,000 daltons respectively.


RNAse
ligase
D-amino acid oxidase
TDH
aldolase
catalase
blue dextran
Elution Volume (ml)
Molecular Weight (log unit)

Characteristics of The Partially Purified TDH in E.Coli. Strain JEV 73R

After the purification step of ammonium sulphate precipitation and Sephadex G100 gel filtration, TDH was sufficiently free from NADH reductase to be assayed by the spectrophotometric method. By this assay, the Michaelis constant for NAD was measured as 63 μM (average of 56, 58, 77 μM). Michaelis constant for threonine was measured as 3.18 mM (average of 2.7, 2.77, and 3.08 mM). The curves of both NAD and threonine concentration against velocity showed typical Michaelis curve with no indication of sigmoidal character (Figures 18, 19, 20 and 21).

Inhibition Studies on Partially Purified TDH

Various compounds related to threonine catabolic pathways or to projected attempts at affinity chromatography were tested as possible effectors of TDH activity. Those were tested with 30 mM threonine and 0.5 mM NAD and also with 10 mM threonine and 0.33 mM NAD. Inhibition studies were routinely carried out by putting the inhibitor into the assay mixture two minutes after the enzymatic reaction had started and observing the change of the enzymatic reaction rate for another two minutes. The compounds chosen for

Figure 18

Plotting threonine dehydrogenase (TDH) enzymatic rate against NAD concentration showing a standard Michaelis curve. This indicated that there was no homotropic co-operation between the NAD binding sites.

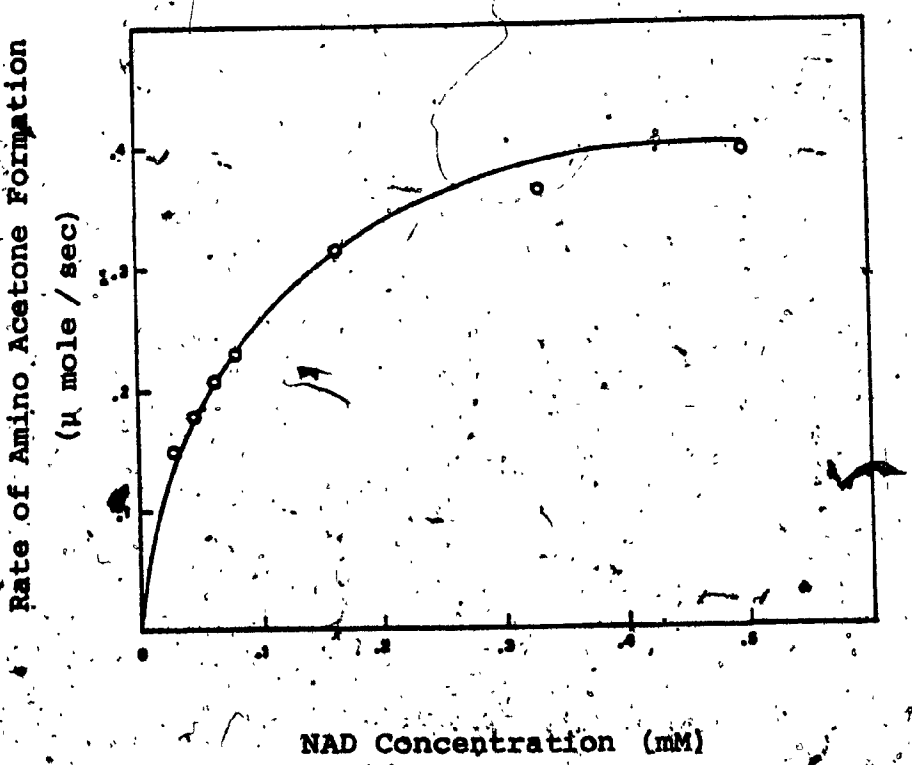

Rate of Amino Acetone Formation
(μ mole / sec)
NAD Concentration (mM)

Figure 19

A Lineweaver-Burk plot to show the Michaelis constant of NAD for threonine dehydrogenase (TDH). The K_m found in this plotting was 77 μM.

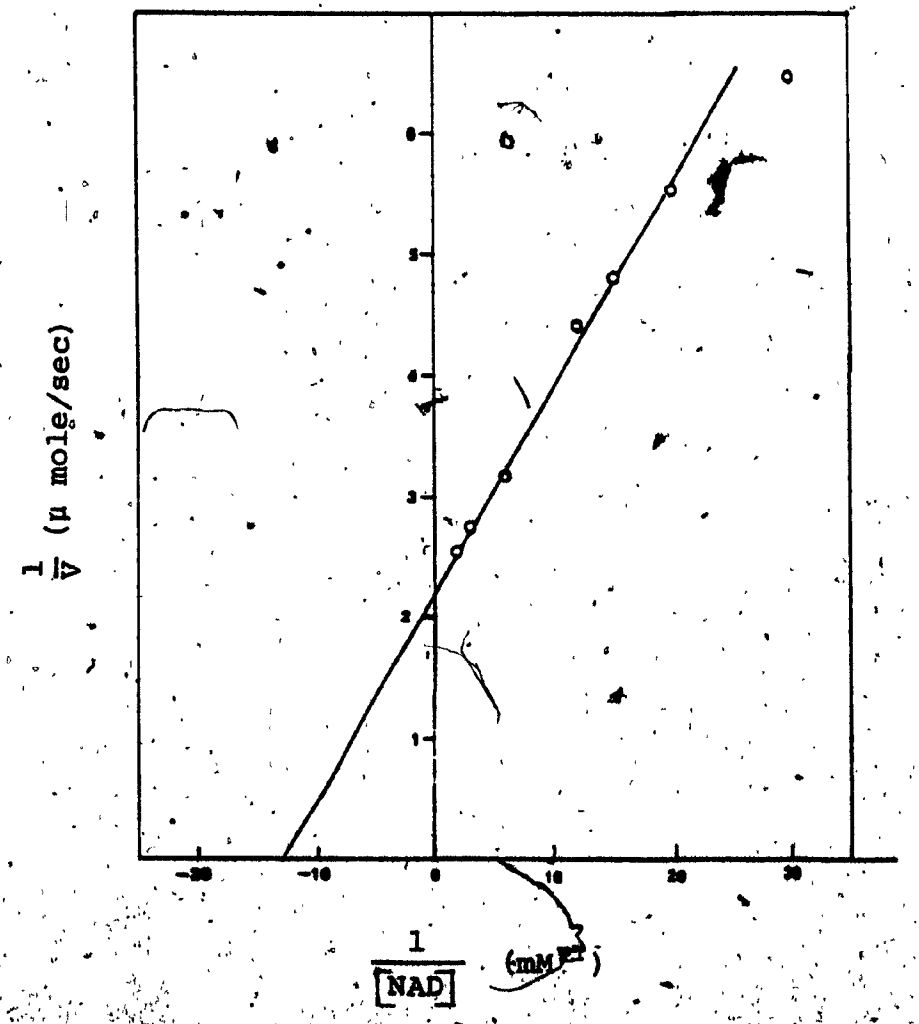

$\frac{1}{V}$ (μ mole/sec)
$\frac{1}{[NAD]}$ (mM^{-1})

Figure 20

Plotting threonine dehydrogenase (TDH) enzymatic rate against threonine concentration showing a standard Michaelis curve. This indicated that there was no homotropic co-operation between the threonine binding sites.

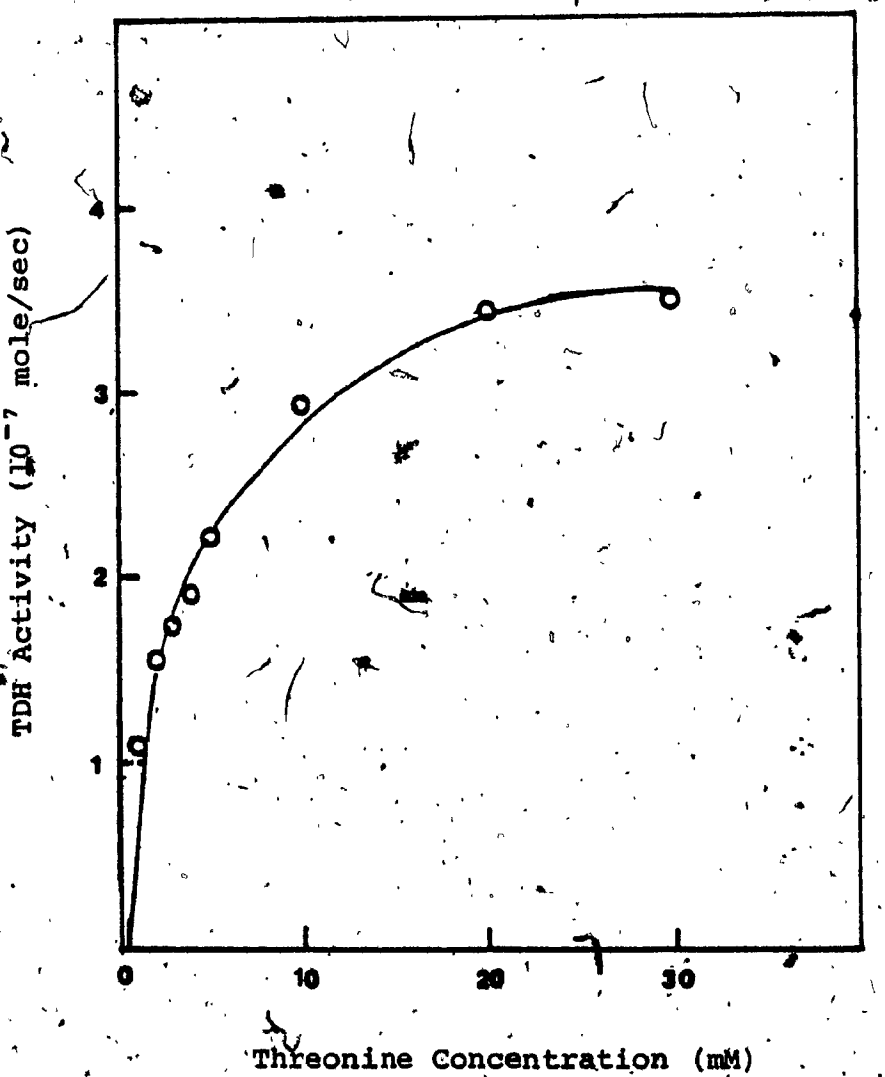
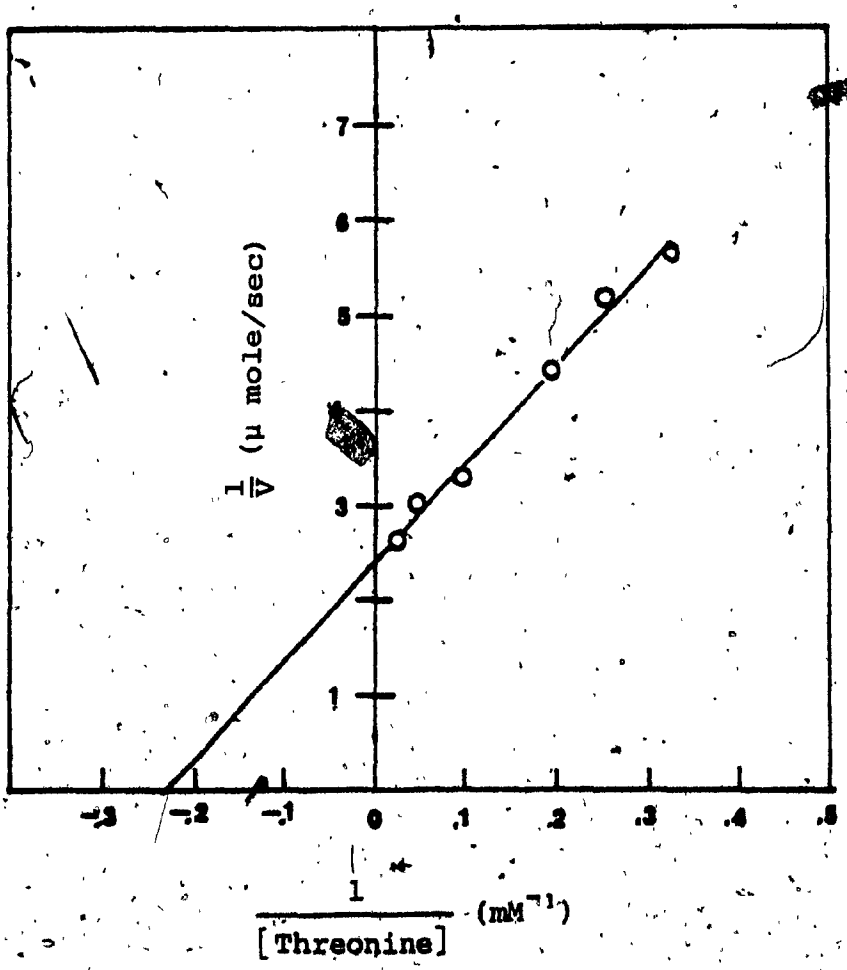

TDH Activity (10^{-7} mole/sec)
1
2
3
0
10
20
30
Threonine Concentration (mM)

Figure 21

A Lineweaver-Burk plot to show the Michaelis constant of threonine for threonine dehydrogenase (TDH). The K_m found in this plotting was 4 mM.


7
6
5
3
1
$\frac{1}{V}$ (μ mole/sec)
-.3
-.2
-.1
0
.1
.2
.3
.4
.5
$\frac{1}{[\text{Threonine}]}$ (mM^{-1})

study were L-serine (3.17 mM), glycine (8.8 mM), L-leucine (2.54 mM), Co-enzyme A (1.3 mM), blue dextran (0.16, 0.8, 2.72 μM), and 5' AMP (1.9 mM). No inhibition or activation of TDH activity was observed in any of these cases.

Other Attempts to Purify TDH from Strain JEV 73R Crude Extracts

Several attempts were made in order to purify TDH from the crude extracts other than ammonium sulphate precipitation and Sephadex G100 filtration. They included two methods which actually separate proteins; ion-exchange chromatography and affinity chromatography, and also attempts to concentrate the proteins by ultra-filtration.

Attempts to purify TDH by ion-exchange methods. Basically, there are two kinds of ion-exchangers; anion exchanger and cation exchanger. In this work, CM-Sephadex was used as a cation exchanger and DEAE-Sephadex and DEAE-cellulose were used as anion-exchangers.

Like the majority of proteins, TDH did not stick to CM-Sephadex at 0.05 M phosphate buffer pH 6.0. Therefore, the cation exchanger, CM-Sephadex, could not be used to purify TDH. TDH did adhere to both

DEAE-Sephadex and DEAE-cellulose columns but could not be removed. In each case, the column was equilibriated with 0.01 M Tris-HCl pH 7.2. Two gradients of increasing ionic strength were tested with each anion exchanger; one was a buffer gradient (Tris-HCl 0.01-0.5 M, pH 7.2) and the other was a salt gradient of Tris-HCl buffer 0-1 M KCl in 0.01 M Tris-HCl, pH 7.2. No more than 15% of TDH activity was recovered in any case.

One might propose three possibilities to account for the low activity recovered in the column eluants,

1) TDH was still in the column and not yet eluted even at 1 M KCl of 0.5 M Tris-HCl buffer at pH 7.2.

2) TDH did elute from the column. However, the enzyme was in a high salt environment which might interfere with enzyme assay.

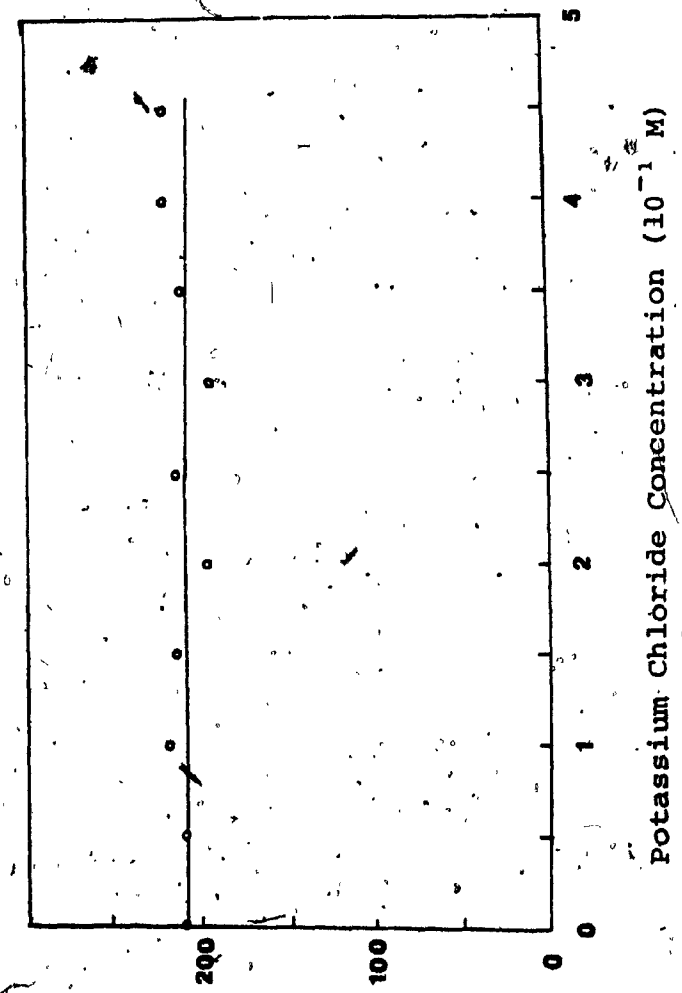
3) TDH was labile in high salt solution.

The first possibility was eliminated by eluting the column further with higher concentrations of eluants (1 M Tris-HCl or 2 M KCl in 0.01 M Tris-HCl buffer) with no TDH detected in the eluant.

The second possibility was eliminated by adding KCl to the conventional colorimetric enzyme assay. As can be seen in Figure 22, the enzyme assay is not altered by the addition of zero to 0.45 M KCl.

Figure 22

Activity of threonine dehydrogenase (TDH) in different salt concentrations. By using colorimetric enzyme assay, TDH enzymatic activity was assayed in different salt concentrations ranged from 0 to 0.45 M. There was no indication that TDH assay was affected by the salt.

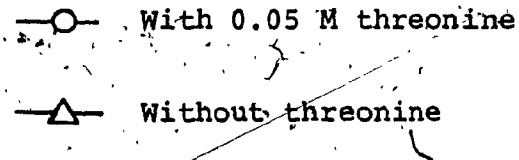

Potassium Chloride Concentration (10^{-1} M)
0
1
2
3
4
5
200
100
0
TDH Activity
(O.D. 540 nm, Klett)

The third possibility was investigated by diluting the crude extracts ten-fold and exposing the diluted solution to various concentrations of KCl, ranging from 0 - 1 M. Threonine (0.05 M) was added to test whether it could stabilize TDH in high salt solution. The diluted enzyme solution was incubated at 4°C and TDH activity was tested at various times (0, 24, 71, 94, and 144 hours) after the exposure. As can be seen in Figures 23 to 28, TDH was labile in high salt solution and could be stabilized by 0.05 M threonine. In addition, the stability effect of threonine on TDH was shown very clearly in Figure 29. After 71 hours of high salt exposure, no TDH was detected in 1 M KCl solution while nearly 100% of TDH activity was protected by 0.05 M threonine at the same salt content. In addition, as can be seen in Figure 30, the half-life of threonine dehydrogenase was inversely proportional to the salt concentration of the solution.

Threonine protection of TDH could be demonstrated further by using threonine as the protecting agent in the purification of TDH on DEAE-Sephadex. Three ml of E.coli strain JEV 73R crude extracts were added to DEAE-Sephadex column (3.6 x 32 cm) which was equilibrated with 0.01 M Tris-HCl pH 7.2 containing 0.05 M threonine. After about 50 ml of the same

Figure 29

Protection of TDH by threonine after 71 hr of salt exposure.



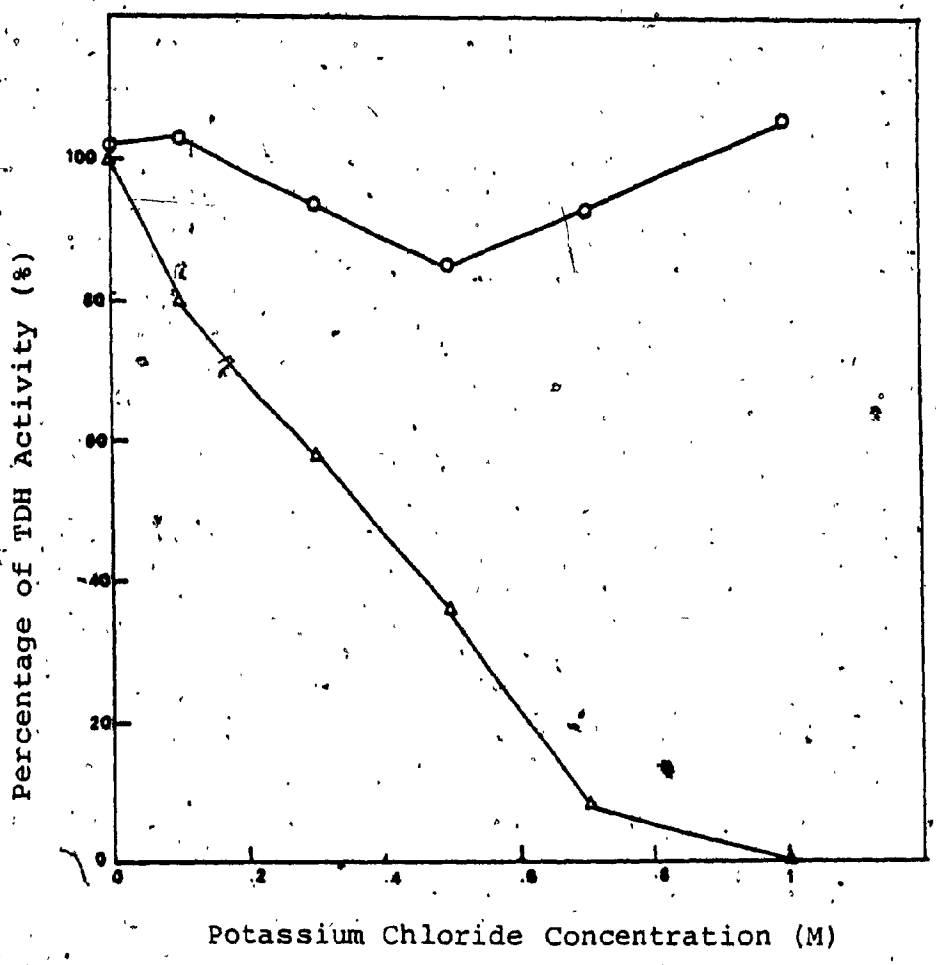
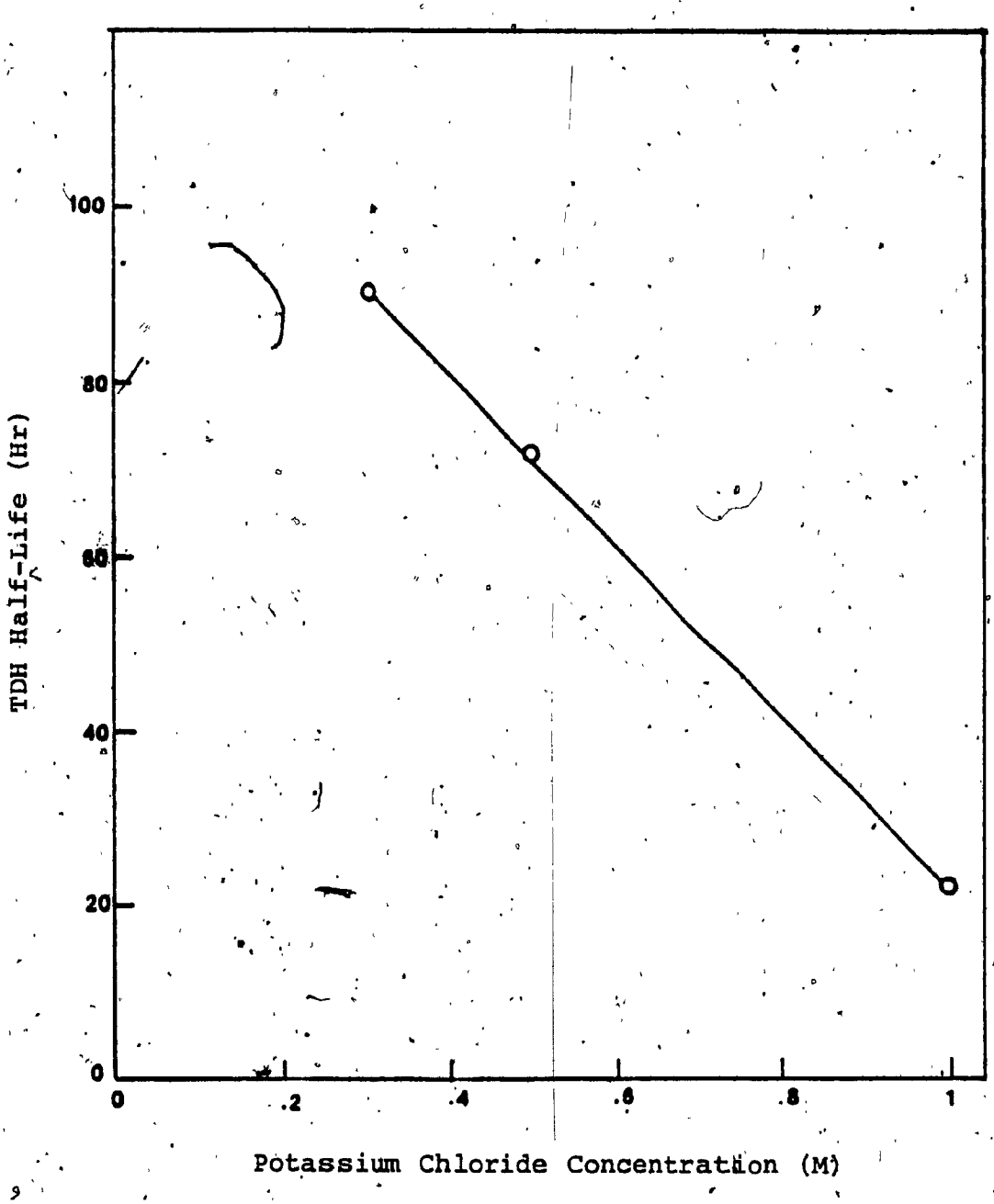

Percentage of TDH Activity (%)
100
80
60
40
20
0
0
.2
.4
.6
.8
1
Potassium Chloride Concentration (M)

Figure 30

Plotting the threonine dehydrogenase (TDH) half-life against the corresponding salt concentration showing the stability of TDH is inversely proportional to the salt concentration in the solution.


TDH Half-Life (Hr)
100
80
60
40
20
0
0
.2
.4
.6
.8
1
Potassium Chloride Concentration (M)

buffer was added to wash out those proteins which did not adhere, the column was eluted with the buffer gradient at pH 7.2 Tris-HCl 0.01-0.5 M containing 0.05 M threonine as the protecting agent. The elution pattern was shown in Figure 31. The total TDH activity recovered was 74%. TDH specific activity had increased 60-fold from 0.3 to 18 mM/min/mg of protein in the most purified fractions. Thus, TDH could be purified in DEAE-Sephadex using threonine as the protecting agent. However, the purified TDH was labile and no desalting procedure could be successfully applied afterward to lower the salt as well as the threonine content. The desalting procedures tried were ultra-filtration and dialysis against 0.01 M Tris-HCl buffer. When the same ultra-filtration desalting procedure was tried with crude extracts, TDH was not labile. A XM 100A membrane retained TDH. No TDH was found to adhere to the ultra-filter membrane.

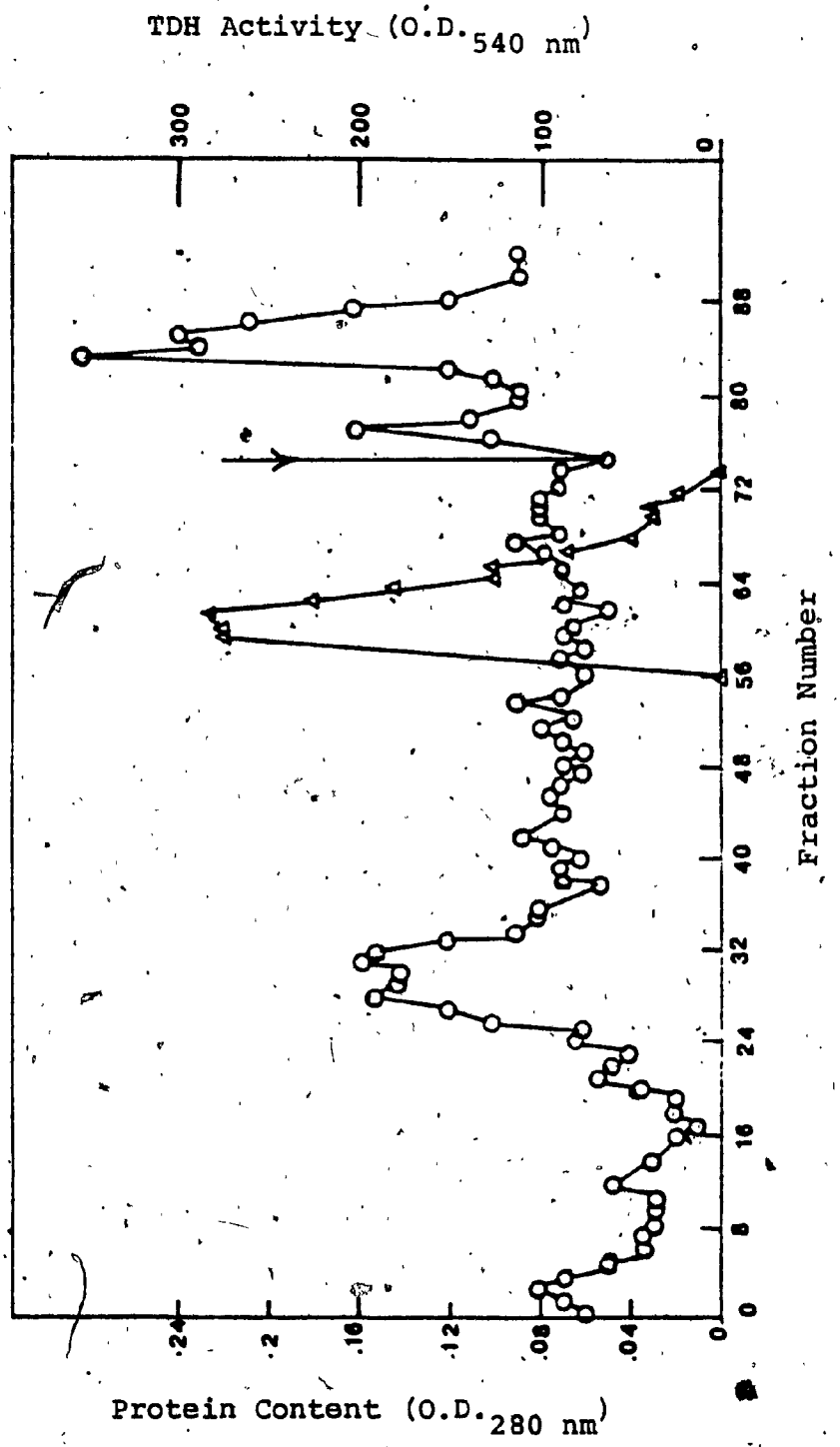
Attempts to purify TDH by affinity chromatography. Various attempts have been made to purify TDH by affinity chromatography, though with limited success (Lowe, Harvey, Gaven, Herfoot, Hollows & Dean, 1973). Since TDH needs both threonine and NAD as its substrates, it is possible to use either one of these substrates or their analogs as the ligand.

Figure 31

The elution of TDH from DEAE-Sephadex using threonine as protective agent. Three ml of E.coli. strain JEV 73R crude estracts were added to the column (3.6 x 32 cm) which was equilibrated with 0.01 M Tris-HCl pH 7.2 containing 0.05 M threonine. After about 50 ml of the same buffer added to wash out those proteins which did not adhere, the column was eluted with the buffer gradient at pH 7.2 Tris-HCl 0.01-0.5 M containing 0.05 M threonine as the protecting agent. TDH specific activity had increased 60-fold in the purified fractions. The total TDH activity recovered was 74%.

—○— Protein content

—△— TDH activity


TDH Activity (O.D. 540 nm)
300
200
100
0
88
80
72
64
56
48
40
32
24
16
8
0
Fraction Number
.24
.2
.16
.12
.08
.04
0
Protein Content (O.D. 280 nm)

As can be seen in the previous experiments, the Michaelis constant of TDH for threonine is 3.16 mM and for NAD is 63 μM. Therefore, TDH has a higher affinity for NAD than that of threonine. As suggested by Wu and Graves (1975), the critical Ki between the ligand and the enzyme should not be higher than 10^{-3} M in order to apply affinity chromatography. Therefore, both threonine and NAD are capable for using in affinity chromatography, but NAD can serve the purpose better because of the lower K_m. Attempts to use threonine-agarose and NAD-hexane-agarose are described here.

Threonine-agarose (Sigma Company, stock number T 0387) was packed in a Pasteur pipette and equilibrated with 0.01 M Tris-HCl pH 7.2. One ml of strain JEV 73R crude extract was added to the column, which was then washed with 10 ml of the Tris buffer at a rate of 0.3 ml/min. TDH did not adhere to the column under this condition. All the enzyme activity could be detected in the washing buffer.

NAD-hexane-agarose (P-L Biochemicals Inc., AGNAD Type I) was packed in a column (1.5 x 10 cm) equilibrated with 0.01 M Tris-HCl pH 7.2. Two ml of strain JEV 73R crude extract were added to the column, which was then washed with 20 ml of the Tris buffer.

Then the column was eluted with two additions of buffer, 20 ml each, containing 200 µM and 500 µM of NAD. respectively. About 14% of the TDH activity was discovered in the 500 µM NAD buffer. The protein content in the washing buffer and those in the NAD-containing buffers suggested that nearly all the proteins had been eluted out from the column. The experiment was repeated using 40% glycerol 0.01 M Tris pH 7.2 to equilibrate the column as well as the eluting buffer. The recovery of TDH activity was still low. It appears that TDH is unstable in these conditions.

Difficulties in Further Purification of TDH

In these studies, TDH was partially purified from the crude extracts 10 - 12-fold by the combination of ammonium precipitation between 35% to 50% and Sephadex G100 gel filtration. On the other hand, TDH was also purified 60-fold by DEAE-Sephadex ion-exchange column using threonine as the protecting agent. However, when the partially purified TDH from the Sephadex G100 column was applied to the DEAE-Sephadex column equilibrated with threonine containing eluant, no TDH activity was detected in the eluted fractions. When the partially purified TDH from the DEAE-Sephadex

ion-exchange column was applied to the Sephadex G100 column equilibrated with or without threonine as the protecting agent, no TDH activity was detected in any of the fractions collected. The reason for this was not known. In any case, it showed that threonine alone was not enough to protect TDH in extensive protein purification steps.

Attempts to Demonstrate The Existence of Ligase in Extracts of E.Coli Strain JEV 73R

In the previous investigation on threonine catabolism, TDH and ligase were considered to be responsible for the conversion of threonine to acetyl Co-enzyme A and glycine (Newman & Fraser, 1975). However, ligase was not actually assayed in E.coli - in vitro. In this work, ligase enzymatic activity was shown, for the first time, in a partially purified preparation of extract of E.coli strain JEV 73R.

Theoretically, ligase can be assayed in both directions. Due to the instability of AKB, it is more convenient to assay ligase using acetyl Co-enzyme A and glycine as the substrates and to determine the amino acetone content in the incubation mixture. In all the assay mixtures, 5,5'-dithio-bis(2-nitrobenzoic acid) was added as the coupling reagent of Co-enzyme

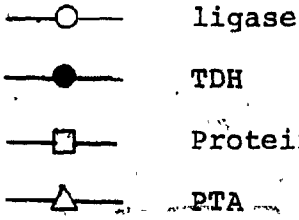
A which is formed as the product of the reaction. Co-enzyme A is an inhibitor of ligase in Arthrobacter (McGilvary & Morris, 1971) and might therefore interfere with the assay as it accumulated.

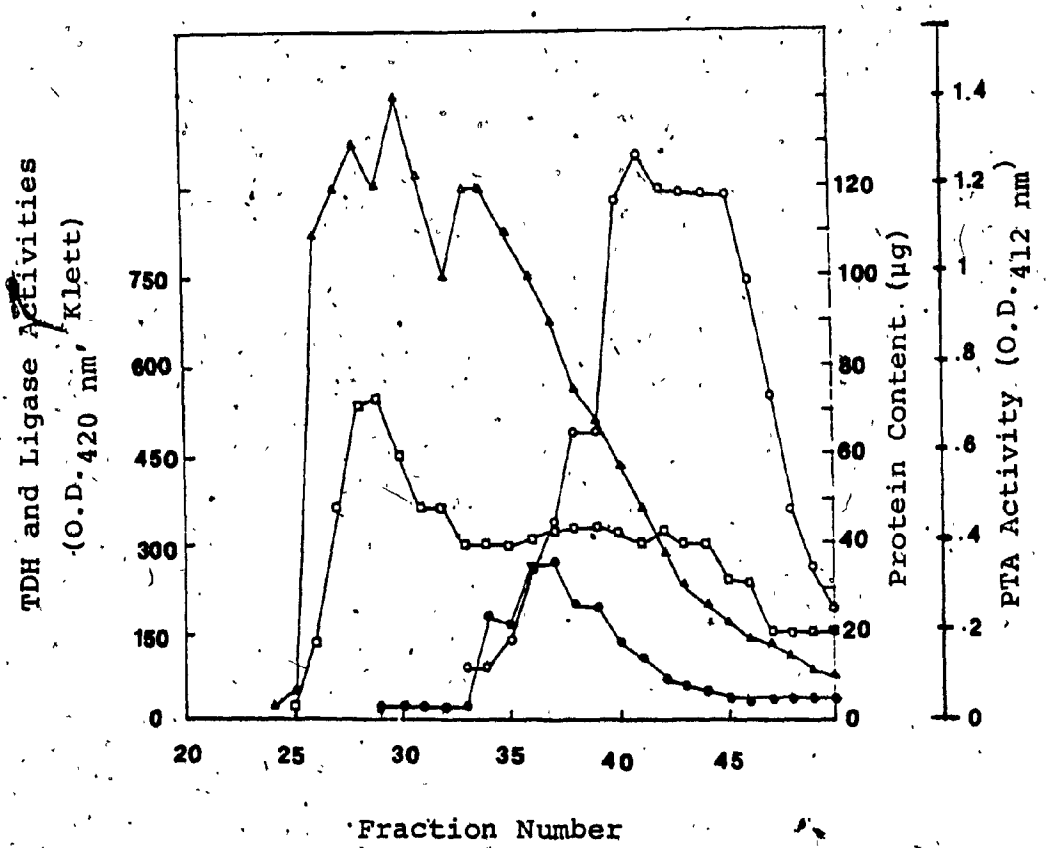
After several attempts to show ligase activity in crude extracts, it was concluded that ligase enzymatic activity could not be demonstrated in the crude extracts due to the fact that the deacylation of acetyl Co-enzyme A was very rapid even when glycine was not present. Therefore, ligase had to compete with another acetyl Co-enzyme A utilizing enzyme, phosphotransacetylase (PTA), for the substrate. If this were so, in order to demonstrate ligase activity, the enzyme should be separated from PTA or inhibitor had to be added to the assay mixture to check the action of PTA. In this work, the enzymes were separated by gel filtration.

To do this, 3 ml of the crude extracts were added to a Sephadex G100 column (3.6 x 64 cm) which was equilibrated with 0.05 M PO_4 buffer, pH 7.2 containing 0.1 M KCl before elution with the same buffer. The collected fractions were assayed for TDH, PTA, and ligase (Figure 32). Ligase activity was detected in one single band between fractions 40-50 corresponding to M.W. 79,000 daltons. PTA appeared in the void volume of the elution while TDH was detected in

Figure 32

Elution pattern of ligase in aSephadex G100 column (3.6 x 64 cm). Three ml of strain JEV 73R crude extracts were added to the column and eluted by 0.05 M PO_4 buffer pH 7.2 containing 0.1 M KCl. Threonine dehydrogenase (TDH), α-amino β-keto butyrate Co-enzyme A ligase (ligase) and phosphotransacetylase (PTA) activities in the collected fractions were assayed and their corresponding protein content were measured.




TDH and Ligase Activities
(O.D. 420 nm, Klett)
750
600
450
300
150
0
Protein Content (µg)
120
100
80
60
40
20
0
PTA Activity (O.D. 412 nm)
1.4
1.2
1
.8
.6
.4
.2
0
20
25
30
35
40
45
Fraction Number

fractions 35 to 40.

For the preliminary characterizion of ligase, fraction number 43 was used. It was shown that the amount of amino acetone formed by the assay was actually dependent on the amount ligase used in the assay (Figure 33). The Michaelis constant of acetyl Co-enzyme A was estimated by assaying amino acetone providing the enzyme with a constant amount of glycine (40 mM) and varying the concentration of acetyle Co-enzyme A. This gave a value of 3.1×10^{-5} M (Figures 34 and 35). This must be taken as a preliminary estimation since the experiment was carried out once only. The Michaelis constant of glycine could be determined in the same assay. However, all the preparations of glycine tested produced color in the amino acetone assay. This makes an accurate estimation of the Michaelis constant for glycine very difficult.

Figure 33

Production of amino acetone as a function of amount of enzyme added. Fraction number 43 from the Sephadex G100 column was used in this assay.

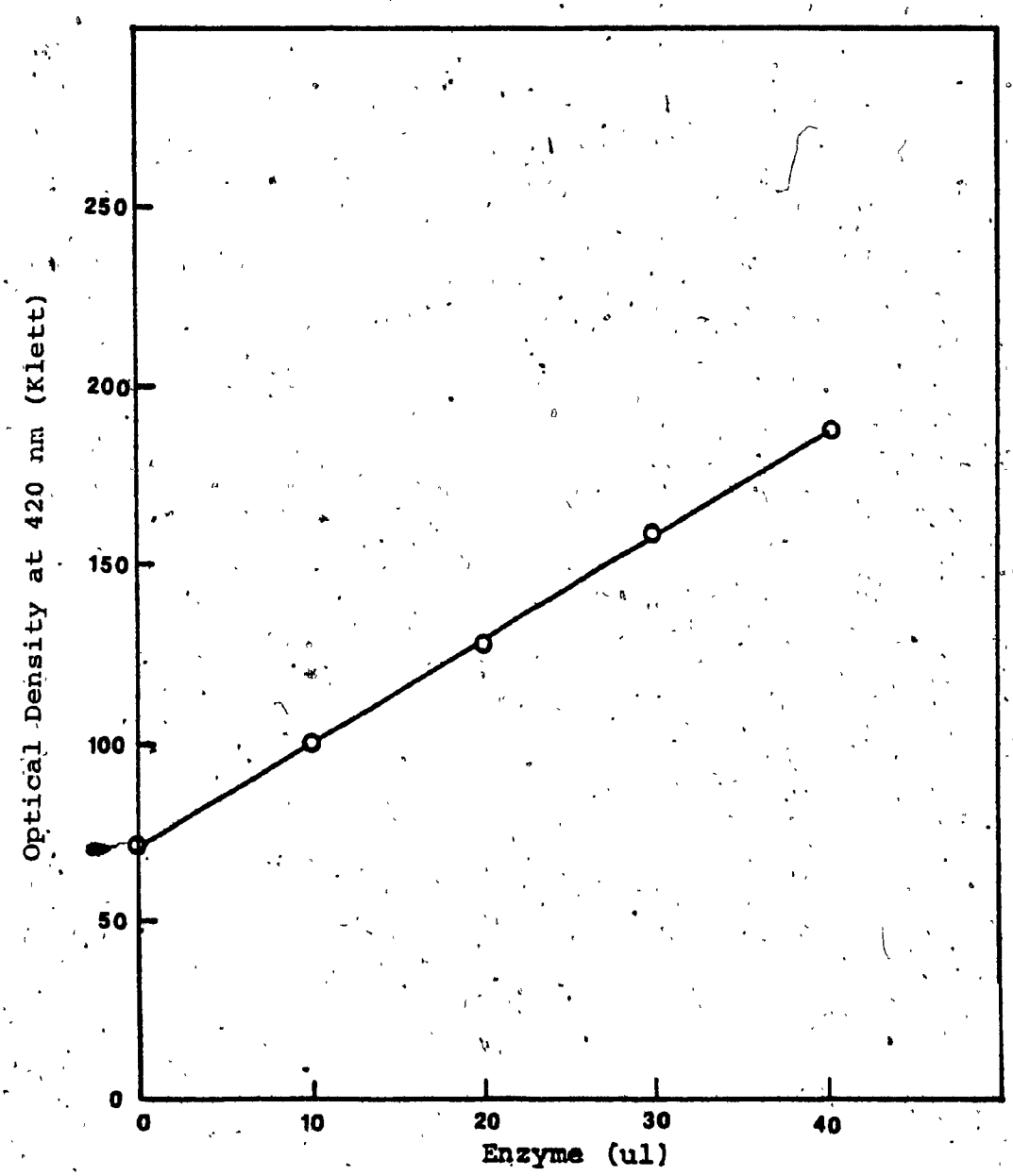

Optical Density at 420 nm (Klett)
250
200
150
100
50
0
0
10
20
30
40
Enzyme (ul)

Figure 34

Plot α-amino β-keto butyrate Co-enzyme A ligase enzymatic rate against acetyl-Co-enzyme A concentration showing a standard Michaelis curve.

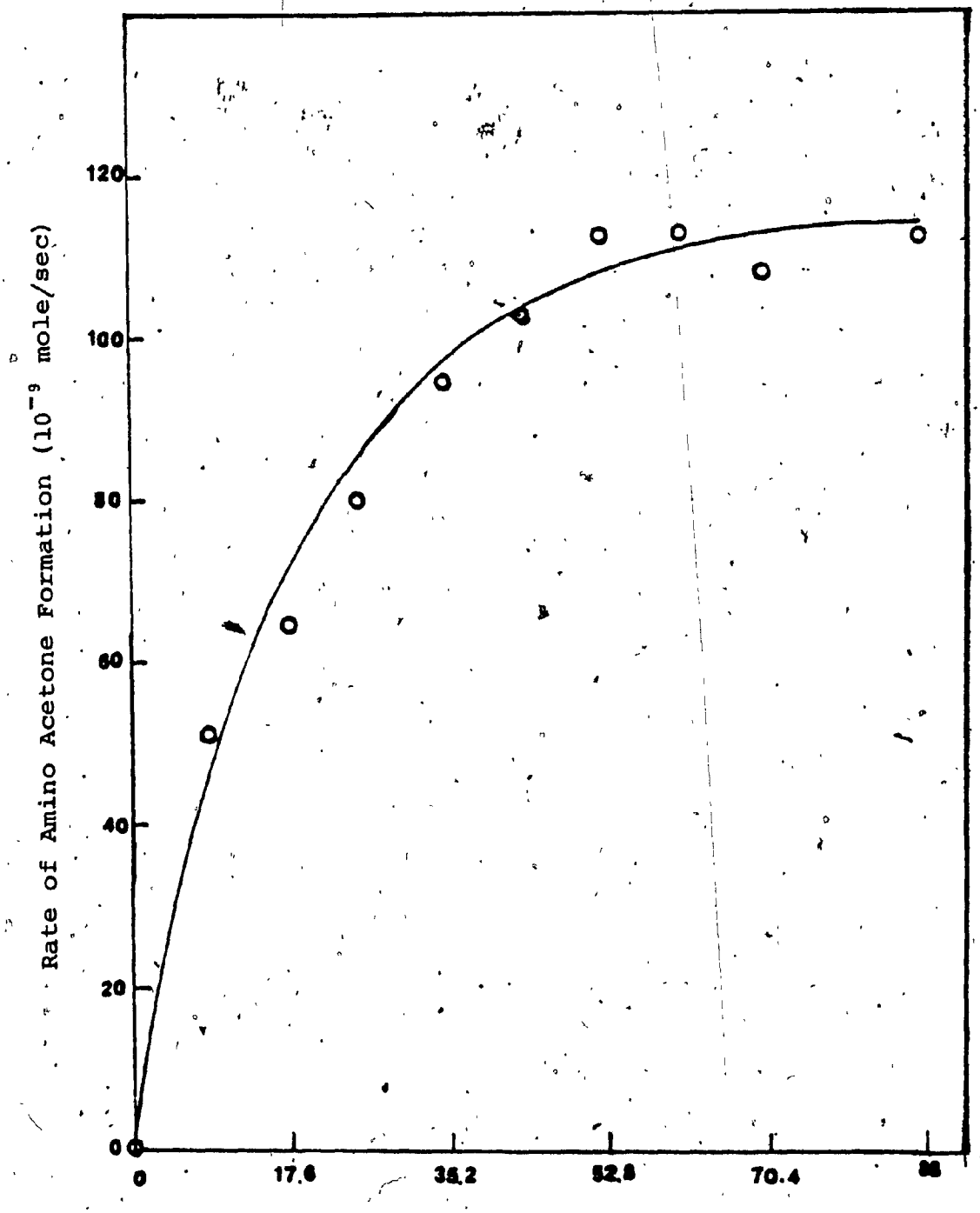
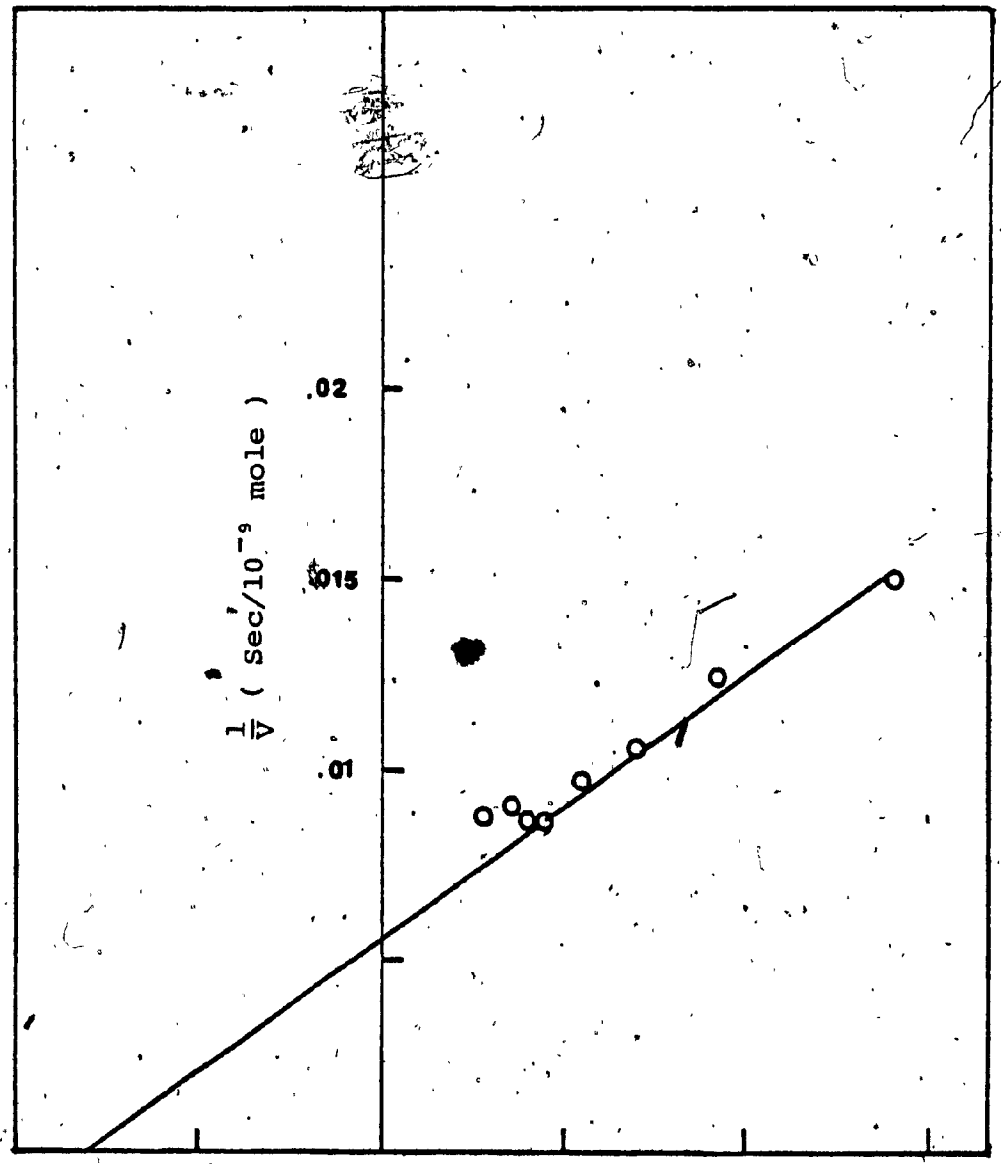

Rate of Amino Acetone Formation (10^{-9} mole/sec)
120
100
80
60
40
20
0
0
17.6
35.2
52.8
70.4
88
Concentration of Acetyl Co-enzyma A (μM)

Figure 35

A Lineweaver-Burk plot to show the Michaelis constant of acetyl Co-enzyme A for α-amino β-keto butyrate Co-enzyme A ligase. The K_m found in this plotting was 3.1×10^{-5} M.


.02
.015
.01
$\frac{1}{V}$ (Sec/10^{-9} mole)
-0.02
0
0.02
0.04
0.06
$\frac{1}{[\text{Acetyl Co-enzyme A}]}$ (μ M^{-1})

DISCUSSION

> It is characteristic of Science and Progress that they continually open new fields to our vision.
>
> Pasteur

Can E.coli use threonine as its sole carbon and energy source? That is the starting question of the thesis. That this is not a far fetched idea in indicated by previous experiments which showed that E.coli is capable of threonine catabolism at least at a low rate sufficient to supply the glycine requirement of an E. coli serine transhydroxymethylase (STHM)-deficient mutant AT 2046T (Newman & Fraser, 1975). It was then shown that the threonine could be catabolized to a more quantitatively significant extent so that the amino group of threonine could be used as the sole nitrogen source of the bacteria (Newman, Kapoor & Potter, 1977). Use of threonine as the sole carbon and energy source might involve an even higher rate of threonine catabolism. This experiment in this thesis address the question of how E.coli might deal with this problem. Two enzymes related to threonine catabolism were partially purified and in vitro

characteristics of one of them, NAD-dependent threonine dehydrogenase (TDH) were determined. By in vivo studies of strains which use threonine as the sole source of carbon and energy and in vitro studies of the enzymes involved in the catabolism, a clear picture of aerobic threonine catabolism was made.

Part 1: Enzymology

Demonstration of Ligase Activity

The previous experiments on threonine degradation led to the proposal that the use of threonine as the source of either nitrogen or glycine depends on the reaction of two enzymes: TDH and α-amino-β-keto butyrate Co-enzyme A ligase (ligase). TDH has been directly assayed (Newman & Fraser, 1975). However, until this work described here the role of ligase was inferred but ligase activity had not been demonstrated.

In this work, ligase activity has been conclusively demonstrated in vitro in Strain JEV 73R. However, this could be accomplished only in partially purified preparations. The assay used involved providing acetyl Co-enzyme A and glycine as substrates, so that α-amino-β-keto butyrate (AKB) and Co-enzyme A could be produced. However, Co-enzyme A is known to inhibit

ligase in other organisms as Arthrobacter (Mcgiluray, Morris, 1971). To avoid the inhibition by one product of the reaction, 5,5' dietho-bis(2-nitrobenzoic acid) (DTNB) is also provided. The DTNB reacts with the Co-enzyme A produced, releasing a yellow p-nitrothiophenol anion. Thus the reaction produces two assayable compounds: the p-nitrothiophenol anion which absorbs light at 412 nm and the amino acetone which can be assayed by Ehrlich's reagent.

Attempts to show amino acetone produced by crude extracts from glycine and acetyl Co-enzyme A were all unsuccessful. However, the reaction mixtures could be seen to turn yellow - even though no glycine was added as substrate. It seemed then that the extracts contained an activity which deacetylated acetyl Co-enzyme A without carrying out the ligase reaction. This could effectively block the ligase reaction by rapidly decreasing the amount of acetyl Co-enzyme A available as substrate.

However, when the crude extracts were eluted from a Sephadex G100 column, amino acetone production (i.e. ligase activity) could be detected in fractions 40-50 collected from the column. In these fractions, production of the yellow p-nitrothiophenol anion was

dependent on the addition of glycine. It seems clear then that the ligase chromatographs as a peak corresponding to a molecular weight of about 79,000 daltons.

Earlier fractions from the column, at about the void volumn of the column, catalyzed the deacetylation of acetyl Co-enzyme A. In these fractions, the production of the 412 nm absorbing compound, n-nitrophenol anion, was independent of the presence of glycine. This activity appears to be phosphotransacetylase (PTA), which forms acetyl-phosphate and Co-enzyme A. It is clear that in crude extracts both the activities must exist. However, the activity of PTA is much higher than that of ligase. Therefore, acetyl Co-enzyme A is destroyed so that no Co-enzyme A ligase activity can be observed.

It is clear then that an extract of strain JEV 73R contains ligase activity, and catalyses the formation of amino acetone (decarboxylated AKB) from glycine and acetyl Co-enzyme A. This reaction was shown to be dependent on the amount of enzyme added and on the presence of both substrates. The enzyme was not further studied to any great extent, except to make a preliminary estimation of its K_m for substrate.

From the Sephadex G100 column described earlier,

it can be seen that the TDH forms a peak which is different from either PTA or Co-enzyme A ligase. Therefore, the TDH and ligase reactions are clearly catalyzed by different molecules. Since AKB will rapidly decarboxylate to amino acetone, this is of interest to know whether TDH and ligase are closely associated. This experiment seems to exclude a close physical association (e.g. multienzyme complex). Still, no attempts to avoid protease action were made and this subject is opened to further investigation.

The Michaelis constant for acetyl Co-enzyme A was estimated by assaying amino acetone providing the enzyme with a constant amount of glycine and varying the concentration of acetyl Co-enzyme A. This gave a value of 3.1×10^{-5} M. This must be taken as a preliminary estimation since the experiment was carried out once only.

The Michaelis constant for glycine could be determined in the same assay. However, all the preparations of glycine tested produced colour in the amino acetone assay. This makes an accurate estimation of the Michaelis constant of glycine very difficult. In order to achieve a satisfactory estimation of this Michaelis constant, the enzyme should be further purified and assayed by

the spectrophotometric method of estimation at 412 nm of the amount of p-nitrothiophenol anion produced.

The role of TDH in threonine catabolism was well established prior to this work (Newman, Kapoor & Potter, 1976; Newman, Baptist, Fraser, Isenberg, Weyman & Kapoor, 1976; Newman, Kapoor & Potter, 1977). However, at the start of this work, nothing was known about the physical characteristics of the enzyme. During our study, some characteristics of the enzyme were described by another laboratory (Dekker & Boylan, 1980). The present study was therefore limited to a partial purification and characterization of the enzyme.

TDH Stability As Influenced by Small Molecules

The purification of TDH is aided by the general stability of the enzyme. Crude extracts could be dialysed against distilled water for 24 hours without loss of activity. This seems to rule out a role of small cations or small organic co-factors in the TDH reaction. This is different from the enzyme in Tetracoccus soyae and Staphylococcus aureus, in which potassium ion stimulates TDH activity (Omata, Tokirua, Nishimura, Kamasabi, Veno & Sakai, 1974; Green, 1964).

This stability is much lessened in the presence of potassium chloride. However, it was possible to

protect the enzyme against even 1 M KCl by adding its substrate, threonine. Elution of TDH from DEAE-Sephadex column therefore was done in the presence of threonine using a Tris-gradient. This allowed 74% recovery with a 60-fold increase in specific activity. When the elution was made with the same Tris gradient but in the absence of threonine added, no TDH activity was recovered.

Successful chromatography of TDH on Sephadex G100 column actually depended on the presence of salt — in this case 0.1 M KCl. When the extracts were chromatographed without KCl, no activity could be recovered. With 0.1 M KCl, TDH was eluted in one band with 75%-100% recovery. This KCl concentration, however, was critical. At 0.2 M KCl TDH eluted in two bands with total recovery of 14%. It would be interesting to know the effect of threonine on the behavior of TDH on the Sephadex G100 column at the relevant salt concentration. However, these studies were not done.

TDH is clearly strongly affected by its environment, stablized by threonine and destablized by high salt concentration. There is some indication that TDH can be isolated in two forms. This may suggest that the enzyme has subunits and tends to

remain associated in the presence of threonine and of a critical concentration of salt. Further experiments would be needed to determine whether the enzyme actually has subunits.

Purification of TDH

In order to purify an enzyme, a reliable enzyme assay is important in the sense that the assay should correctly differentiate the enzyme from other proteins and estimate the specific enzymatic activity accurately. In many conventional purification steps, i.e. ammonium sulphate precipitation of proteins, ion-exchanging chromatography and affinity chromatography, the ionic strength of the composition of the solution that the enzyme exposed would change. In order to determine accurately the fractions in which the enzyme is located, and the specific activities of the enzyme, the enzyme assay should not be affected by the components used during the purification steps, or else, such effects should be accounted for. The purification of an enzyme requires the routine assay of many fractions. In this work, TDH was assayed in two ways: One relies on the use of Ehrlich's reagent to measure the amount of amino acetone which is the indirect product of the TDH enzymatic activity; the other method depends on

monitoring the change in the NADH concentration at 340 nm, since TDH converts NAD to NADH.

Theoretically, both enzyme assays could be used in locating fractions containing TDH activity, estimating the TDH specific activity. However, the spectrophotometric method is not applicable in crude extracts because of the presence of NADH-reductase. During the purification of TDH in this thesis, the colorimetric enzyme assay was used to locate TDH and estimate its specific activity. Besides ammonium sulphate interference, the colorimetric enzyme assay was not affected by high salt content in the assay mixtures. Spectrophotometric enzyme assay was used to characterize TDH as well as to carry out the inhibition study. The estimation of enzymatic activity, given by colorimetric and spectrophotometric method were generally consistent (Elliot & Green, 1963; and by the author).

Ammonium sulphate precipitation. By stepwise increase of ammonium sulphate added to crude extracts, TDH was found to precipitate between 30%-50% (Figure 15). Later experiments showed that ammonium sulphate range could be reduced to 35% to 50%. It should be noted that high ammonium sulphate concentration inhibited the detection of the amino acetone by

Ehrlich's reagent. Therefore, the percentage of enzyme recovery after ammonium sulphate could not be accurately estimated.

Gel filtration. The pellet precipitation between 35%-50% ammonium sulphate was redissolved and placed on a Sephadex G100 gel filtration column. The column was eluted with 0.05 M PO_4 buffer, 0.1 M KCl pH 7.2 with the rate 10 ml/hr. All TDH activity, usually more than 75% of total input, was discovered in a single band with the corresponding molecular weight of 141,000 daltons. The specific activity increases 10 - 12 folds, e.g. from 0.3 to 3.5 mM/min/mg of protein (Figure 16). This partially purified TDH was free from NADH-reductase and therefore the spectrophotometric enzyme assay could be applied to characterize the enzyme.

Characteristics of TDH. Partially purified TDH was characterized by using the spectrophotometric enzyme assay: monitoring the threonine dependent increase in optical density of the assay mixture at 340 nm. A Michaelis constant of 63 μM for NAD was measured by fixing the concentration of threonine at 30 mM. When the rate of NADH production was plotted against the concentration of NAD, the shape of the curve was that of the standard Michaelis curve,

eliminating the possibility of homotropic co-operative effects of NAD. A Michaelis constant of 3.18 mM for threonine was measured by fixing the concentration NAD at 500 μM. Again no evidence of homotropic co-operative effect of threonine was seen.

Inhibition studies were carried out in order to facilitate the affinity chromatography and to clarify whether TDH showed end product inhibition by glycine or serine or stimulation by leucine. Using the condition with threonine 30 mM and NAD 0.5 mM and also threonine 10 mM and NAD 0.33 mM, TDH was not affected by 3.17 mM serine, 8.8 mM glycine, 1.9 mM 5' AMP, 0.16, 0.8, and 2.72 μM blue dextran, 1.3 mM Co-enzyme A or 2.54 mM leucine.

Glycine and serine are the end products of aerobic threonine catabolism. In this study, they had no in vitro effect on TDH. The fact that leucine which increased TDH activity in vivo did not stimulate TDH in vitro ruled out the possibility that leucine allosterically activates TDH by direct binding. Since there was no indication that 5' AMP and blue dextran would bind TDH, 5' AMP-agarose and blue dextran-agarose were not used in the affinity chromatography. In a personal communication, Dekker E.E. said that he had used blue dextran-agarose to purify TDH. In this

study, the failure of using blue dextran as the TDH inhibitor may be due to low blue dextran concentration which is limited by its low aqueous solubility.

Purification attempts using ion-exchange and affinity chromatography. By using threonine as the protecting agent, TDH could be isolated in one band with a total recovery of 74% and 60-fold increase in specific activity (Figure 31). However, this purified TDH was labile in the desalting procedures (e.g. dialysis and ultrafiltration), attempts were made to precipitate this purified TDH with 60% ammonium sulphate and put the pellet into a Sephadex G100 column. No TDH enzyme activity was detected in any of the fractions collected.

Threonine-agarose and NAD-hexane-agarose were used as stationary ligands in the affinity chromatography of TDH. By using the crude extracts TDH did not bind to threonine agarose at 0.01 M Tris-HCl pH 7.2. Although TDH adhered to NAD-hexane-agarose, the low recovery of the enzymatic activity in the pulse elutions with 200 μM and 500 μM NAD in buffer suggested that the enzyme could not be purified in this way. The change of the elution conditions to include 10% glycerol did not improve the percentage of enzymatic activity recovered.

Difficulty in TDH purification. In this work, TDH has been purified 12-fold by a combination steps with ammonium sulphate precipitation and gel filtration, and 60-fold by DEAE-Sephadex —— starting in each case with a crude extract. However, attempts to combine these two purified TDH seems to be unstable for further purifications. The reason for this instability is not known but may relate to the dissociation of the enzyme into subunits. However, this was not extensively studied in this work.

Comparison between The Data Reported in This Thesis with Those Reported by Dekker E.E.

TDH was extensively purified by Dekker and Boylan (1980). The Michaelis constants for threonine and NAD were reported to be 1.4 mM and 190 μM respectively. The molecular weight reported was 140,000 daltons. In this study, the Michaelis constants for threonine and NAD were reported to be 3.18 mM and 63 μM respectively. The molecular weight was 141,000 daltons. By comparing the reported data, this is clear that both labs reported the similar molecular weight and K_m for threonine but different in the K_m for NAD.

By Sephadex G100 gel filtration and DEAE-Sephadex ion-exchange chromatography, only one band of TDH activity was detected in the fractions collected suggesting that there was only one kind of TDH in strain JEV 73R.

The difference in the Michaelis constants reported in two labs may be due to the fact that each laboratory used a different strain of E.coli from which to isolate the mutant studied. Dekker and Boylan used nitrosoguanidine as the mutagen and selected directly using threonine as the sole carbon source. In this study, strain JEV 73R was derived from strain AT 2046 via several spontaneous mutations. Both of the selections resulted in elevation of TDH activity, about 20 folds in Dekker's case and 100-folds in JEV 73R. The enzymes thus produced may or may not be identical.

Part 2: Genetic Studies

In this first part of this discussion, the nature of two enzymes involved in threonine catabolism has been reviewed. These two enzymes constitute a pathway converting threonine to glycine and acetyl Co-enzyme A. That this is really the pathway of

threonine catabolism is indicated by several kinds of experiments. An obligatory connection between threonine and acetate metabolism is indicated by mutation studies. Threonine catabolism is shown to be accompanied by glycine excretion. As one would expect from this, the cell uses only half of the carbon atoms in building all materials and excretes the rest in the form of glycine. This pathway is further confirmed by studies using U-^{14}C-threonine and finding ^{14}C glycine in the medium. Therefore, it is concluded that aerobic threonine catabolism in strain TE 111 involves the conversion to glycine and acetate and the further catabolism of acetate only.

Mutant Studies

The genetic investigations described in this work suggested that threonine and acetate utilization are connected in such a way that if the acetate utilization is lost by mutation, the bacteria will lose the threonine-utilizing ability as result of the same mutation. The connection between threonine and acetate metabolism is indicated by the following data:

a) A strain which could not use aceate could not use threonine even though its TDH specific activity is high (strain JEV 73R).

b) An acetate-positive derivative (strain TE 103) could use threonine.

c) A threonine-positive derivative (strain TE 111) could use acetate.

d) An acetate-negative derivative (strain TE 111A) of the preceeding acetate-positive strain TE 111 could not use threonine.

e) By using more acetate- and threonine-positive derivatives, a connection between threonine and acetate utilization was generalized.

This catabolic connection can easily be explained. Threonine catabolism is first initiated by TDH by which threonine is converted to AKB. By the enzymatic reaction of ligase, AKB is split into glycine and acetyl Co-enzyme A. Then acetyl Co-enzyme A is the actual carbon donor to the general metabolic cycles. The connection between threonine and acetate catabolism is that both involve acetyl Co-enzyme A as an intermediate.

Strain TE 111 is an STHM-deficient mutant in which serine cannot be converted to glycine and vice versa. The further metabolism of glycine is limited to its incorporation into proteins, glycine and its conversion to C_1 units. In threonine catabolism, glycine and acetyl Co-enzyme A will be produced simultaneously.

Therefore, as the bacteria metabolize acetyl Co-enzyme A, the bacteria have to deal with the problem of glycine accumulation. In this work, the bacteria seem to solve this problem by excreting glycine into the medium.

Glycine Excretion in The Threonine Catabolism in Strain TE 111

When strain TE 111 cultured in threonine as the sole carbon source, glycine was detected in the culture filtrate. This was done with both biological assay and chemical methods (Figures 5 to 8). This excretion of glycine appeared to be continuous during growth as indicated by assay of samples of culture filtrate collected at different points of the growth cycle. This is the first report of glycine excretion in E.coli. No attempt to investigate the mechanism of glycine excretion was made. This may be an interesting topic for those interested in amino acid transport in E.coli.

Efficiency of Derivation of Cell Materials from Various Carbon Sources

Since threonine is catabolized to glycine and acetyl Co-enzyme A and glycine is excreted, one would expect strain TE 111 to only use half of the carbon atoms derived from threonine to build up the cell

material and excrete the other half in the form of glycine. Thus the cell would use equal numbers of threonine or acetate molecules to build up equal amount of cell materials even though the number of carbon atoms in threonine is twice that of acetate. By using a limiting amount of carbon source in strain TE 111 cultures, it was found that, strain TE 111 needed 40 μ moles of glucose, or 93 μ moles of threonine or 95 μ moles of acetate to build up one mg of protein (Figures 11 to 13). Glucose is a six-carbon compound. If glucose was used as efficiently as acetate, one would expect to read about 31 μ moles of glucose to build up 1 mg of protein. However, glucose is not completely oxidized by E.coli which uses its TCA cycle only sluggishly. Therefore, it is not surprising that strain TE 111 shows lower efficiency in using glucose than acetate to build up an equal amount of cell material. On the other hand, the efficiency in using threonine is half of that using acetate, which is consistent with the idea that threonine is degraded to acetyl Co-enzyme A and glycine which is actually excreted from the cell. The fact that the derivation of cell material from threonine and acetate comes out so close to the expected ratio also suggested, perhaps, that threonine and acetate are

metabolized by the same route.

Part 3: An Overall Summary Threonine Utilization in Strain TE 111 (Figure 36)

In order to use threonine as the sole carbon and energy source, a bacteria must carry out a series of metabolic reactions. These include the transport of threonine into the cell, the degradation of threonine into metabolic intermediates, the generation of energy from these metabolic intermediates, replenishing the metabolic intermediates that are needed to build up the cell materials, and the excretion of metabolic wastes that may be toxic to the bacteria. Since strain TE 111 can grow on threonine as the sole carbon and energy source, it should carry out all these reactions.

Uptake of threonine is carried out, presumably, by the threonine transport system. In this study, there is no attempt to investigate this system and it is assumed that during growth on threonine, threonine uptake system must function satisfactorily.

TDH is the enzyme responsible for threonine catabolism. In strain TE 111, TDH specific activity is much higher than that of wild type E.coli. Besides threonine, TDH needs NAD as the Co-enzyme. Therefore,

enough NAD should be present in the cytoplasm to support TDH enzyme activity and furthermore, NADH will be produced as one of the products of threonine degradation. Since the bacteria is grown aerobically, NADH will eventually be oxidized again using oxygen as the ultimate electron acceptor. If the bacteria were grown anaerobically, they might have problems due to a limited supply of electron acceptors. However, under the anaerobic situation, another threonine degradation enzyme, biodegradative threonine deaminase is produced and the direct enzymatic product α-keto butyrate is itself the electron acceptor (Magasanik, 1961).

The direct enzymatic product of TDH is AKB. This is a labile compound in the sense that AKB rapidly decarboxylates with the formation of amino acetone. There are two possibilities for the further metabolism of AKB. One is this nonenzymatic decarboxylation from which amino acetone is formed. The second possibility is an enzymatic reaction catalyzed by ligase. In this work, the ligase was shown to be present in E.coli and thus it seems likely that AKB will be further degraded by ligase.

If ligase is actually the enzyme that degraded AKB, there is a possibility that TDH and ligase have a physical orientation such that the AKB formed by

TDH is channelled to ligase. The elution pattern of these enzymes in the gel filtration did not support the idea that they exist as an enzyme complex. However, no precautions were taken to avoid disruption of such complex, and this question is clearly unresolved.

Ligase cleaves AKB forming glycine and acetyl Co-enzyme A. In this study, it has been demonstrated that acetyl Co-enzyme A is the carbon donor for the general metabolic pathways in the threonine growing cells, and glycine is excreted by the bacteria continuously during growth. Glycine is required by E.coli for incorporation into proteins and purines. Especially in strain TE 111 which is a STHM-deficient mutant, a certain amount of glycine will be degraded by glycine cleavage system to yield C_1 units. During the cleavage, the carboxylate carbon of glycine will be released in the form of CO_2 and the α-carbon will be transferred to tetrahydrofolic acid. When glycine is incorporated into proteins and purines all its carbon atoms will be retained in the cell. When glycine is cleaved to yield C_1 units, half of its carbon will be lost. Since strain TE 111 is a STHM-deficient mutant, the interconversion between glycine and serine is blocked. From the inhibition studies, glycine and

serine do not inhibit TDH *in vitro*. This suggested that there is actually no end-product inhibition in aerobic threonine catabolism. This lack of feedback control facilitates threonine catabolism but faces the bacteria with a glycine accumulation problem. In this work, glycine is detected in the culture filtrate and this suggests the idea that the bacteria solve the problem by excreting glycine.

No attempt to investigate the mechanism of this glycine excretion was made. This is the first report of glycine excretion in *E.coli*. To the extent that glycine excretion is passive, one must assume that the metabolism of *E.coli* will function apparently normally at a high glycine concentration. In other words, glycine does not affect metabolism in *E.coli*. However, preliminary results, which are not presented in this thesis, indicate that glycine may be toxic to *E.coli* under some conditions. This is a subject which must be further investigated.

Acetyl Co-enzyme A serves as the carbon and energy source in aerobic threonine catabolism. Acetyl Co-enzyme A will enter into two different metabolic pathways, one to generate energy and the other to serve as the precursor of all cell materials. A portion of the acetyl Co-enzyme A formed from the threonine catabolism will

enter into the TCA cycle to generate the energy that is required for the growth. Acetyl Co-enzyme A will first bind to oxaloacetate to form a six-carbon compound, citrate. By various interconversion steps in the TCA cycle, the six-carbon compound will end up in the form of oxaloacetate, a four-carbon compound. During the TCA cycle, all the carbon atoms come from acetate will be decarboxylated to produce energy. The rest of the acetyl Co-enzyme A formed from the threonine will enter into the glyoxlate cycle. This constitutes a by-pass of the decarboxylation steps in the TCA cycle. The two-carbon from acetate that are incorporated into citrate are not lost by decarboxylation but retained in glyoxylate and succinate. The glyoxylate then combine with another acetyl Co-enzyme A so that the cycle will end up with a net gain of a four-carbon compound from two two-carbon compounds. This allows the accumulation of new cell materials from acetyl Co-enzyme A. This four-carbon compound, succinate is converted to oxaloacetate. The anabolic pathway starts with oxaloacetate from which phosphoenolpyruvate (PEP) will be formed. PEP is regarded as the end product of the anaplerotic sequence of acetate metabolism in E.coli (Kornberg, 1966). PEP will then be the precursor of many cell materials. During the conversion of

oxaloacetate to PEP, one of the carbon atoms of oxaloacetate will be lost in the form of CO_2.

The distribution of ^{14}C in cultures derived from U-^{14}C-threonine is roughly in agreement with this formulation of metabolism. 6.9 μ Ci of threonine were supplied as the sole source of carbon and energy. According to our formulation these 6.9 μ Ci of threonine should give 3.45 μ Ci of acetyl Co-enzyme A and 3.45 μ Ci of ^{14}C present as glycine in the culture filtrate. However, some of this glycine must be incorporated into proteins and into purines, some glycine must be oxidized to give C_1 units. A small amount of threonine could be detected in the culture filtrate indicating that some of the radioactivity in the culture filtrate was actually in the form of threonine. Therefore, the finding of 3.06 μ Ci after the DOWEX-50 desalting procedure in the culture filtrate is roughly in accord with expection.

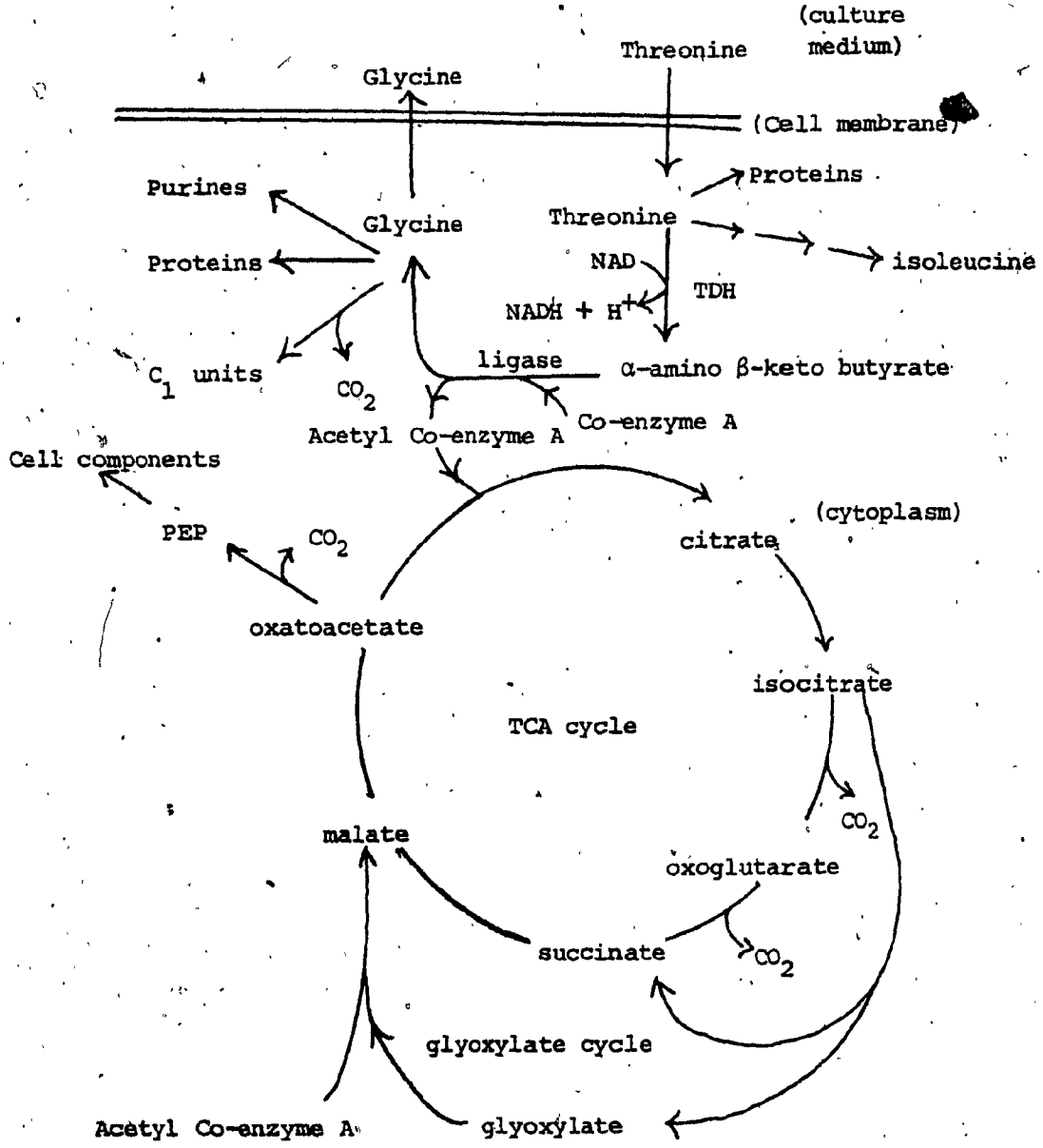
What then happens to the 3.45 μ Ci which originated in acetyl Co-enzyme A. As mentioned before, the metabolism of acetyl Co-enzyme A is conducted by two cycles, TCA and glyoxylate cycles. In the TCA cycle, energy will be generated but all the carbon atoms of acetyl Co-enzyme A will be decarboxylated and lost as CO_2. In the glyoxylate cycle, all the carbon atoms

from acetyl Co-enzyme A will be retained in the formation of oxaloacetate. However, when oxaloacetate is converted to PEP, one out of four carbon atoms will be lost.

If it is assumed that half of the acetyl Co-enzyme A (1.725 μ Ci) is used as the energy source and half of it is used in the glyoxylate cycle (1.725 μ Ci), by the above formulation, only 1.725 μ Ci would be incorporated in oxaloacetate. The portion of those 1.725 μ Ci actually found in cell materials, however, would depend on how much oxaloacetate is converted to PEP, what other decarboxylation reactions occur, and the extent to which CO_2 may be recycled. The actual number of 1.2 μ Ci that is incorporated in the cell materials appears somewhat low. However, this could be further investigated by comparing the fate of acetate U-^{14}C. Since the purpose of this thesis was to show that aerobic threonine catabolism is via the 'glycine route', this matter was not further investigated.

Figure 36

Threonine Utilization in E.coli. strain TE 111 - An overall summary.



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