

ENZYMOLOGICAL AND REGULATORY ASPECTS OF  
ISOLEUCINE, VALINE AND LEUCINE BIOSYNTHESIS  
IN SCHIZOSACCHAROMYCES POMBE

by

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A thesis submitted in partial fulfilment of the  
requirements for the degree of

Doctor of Philosophy

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November 1971

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ABSTRACT

Studies were undertaken on the synthesis and regulation of the branched-chain amino acid biosynthetic enzymes in the fission yeast, Schizosaccharomyces pombe. Enzymological studies have shown that this yeast possesses all the enzymes known to be involved in the biosynthesis of isoleucine and valine. Threonine deaminase (TD), the first enzyme of the isoleucine pathway, is strongly inhibited by L-isoleucine, and this inhibition is relieved by L-valine. Threonine saturation kinetics at pH 9 (the optimum for activity) are Michaelian; the substrate curves become increasingly sigmoidal as the pH is lowered to 7, demonstrating homotropic interactions of L-threonine. At these lower pH's, valine activates TD and normalizes the substrate curve. Isoleucine increases the homotropic effects of threonine. TD could not be desensitized by the usual chemical means, but at pH 10, the enzyme is insensitive to isoleucine while still retaining catalytic activity. A model which accounts for these and other findings is presented. Acetohydroxy acid synthetase (AHAS), the first enzyme in valine biosynthesis, has a pH optimum of 6.5, in contrast to the pH optimum of 7.4-8.0 found in other systems. It is sensitive to feedback inhibition by L-valine, which effector shows homotropic cooperative effects. Substrate saturation of AHAS from glycerol-grown cells, or partially purified from glucose-grown cells, is Michaelian, while that of the crude extract from the latter is strongly sigmoidal. This effect was shown to be due to competition for pyruvate between AHAS and pyruvate decarboxylase, which is present in large amounts in cells fermenting glucose. The first enzyme of the leucine pathway, isopropylmalate synthetase, was shown to be sensitive to L-leucine. Of all the ilva enzymes, only AHAS and isomeroreductase appear to be subject to multivalent repression.

RESUME

Ce travail avait pour but d'étudier l'activité et la régulation des enzymes de la chaîne de biosynthèse des acides aminés l'isoleucine, la valine et la leucine, chez la levure Schizosaccharomyces pombe. Les études enzymatiques ont démontré que cette levure possède tous les enzymes impliqués dans la synthèse de l'isoleucine et de la valine. Le thréonine désaminase (TD) premier enzyme de la chaîne de l'isoleucine, est très sensible à cet acide aminé, et cette inhibition est antagonisée par la L-valine. La cinétique de la TD en fonction de la concentration en thréonine est Michaelienne à son pH optimum de 9; les courbes deviennent de plus en plus sigmoïdales lorsque le pH est baissé à 7, ce qui démontre des effets homotropiques coopératifs de la thréonine. Sous ces conditions, la valine stimule l'activité de l'enzyme et régularise la courbe; l'isoleucine augmente les effets homotropiques du substrat. On n'a pas pu désensibiliser la TD par des méthodes chimiques, mais à pH 10, l'enzyme n'est plus sensible à l'isoleucine, tout en retenant son activité enzymatique. Un modèle qui tient compte de ces propriétés est présenté. L'acétohydroxy acide synthétase (AHAS), qui assure la première étape dans la synthèse de la valine, a un pH optimum de 6.5, par contraste avec le pH optimum de 7.4-8.0 caractéristique de l'enzyme de tous les autres organismes. Il est sensible à la rétroinhibition par la L-valine, qui exerce des effets homotropiques. La courbe de saturation de l'enzyme prélevé des cellules cultivées sur glycerol, ou partiellement purifié à partir de cellules poussées sur glucose, est hyperbolique, tandis que celle de l'enzyme de l'extrait brut obtenu des cellules poussées sur le glucose est de caractère fortement sigmoïdale. La présence du

pyruvate décarboxylase dans l'extrait brut de ces cellules qui concurrence l'AHAS pour leur substrat commun, le pyruvate, semble expliquer ce phénomène. Le premier enzyme de la chaîne de la leucine, l'isopropylmalate synthétase, est sensible à la rétroinhibition par la L-leucine. De tous les enzymes des chaînes de biosynthèse de l'isoleucine et de la valine, seuls le AHAS et l'isoméroréductase semblent être soumis à la répression multivalente.

#### ACKNOWLEDGEMENTS

Sincere thanks are due firstly to Dr. J. Gordin Kaplan, in whose laboratory I carried out this work. I am greatly indebted to him for his direction, motivation and encouragement in shaping my career as a scientist. He provided a fine intellectual atmosphere, stimulating discussions, and the best of material conditions in which to work.

I give special thanks to Dr. P.T. Magee, who received me so amiably and generously in his lab, and to Dr. E. Juni, both of whom offered many helpful and fruitful ideas. I am grateful to Dr. T. Satyanarayana for many discussions and assistance throughout this investigation.

I wish to thank W. Lim for the expert preparation of the graphs, and G. Ben for the photographic work.

Finally, I thank the National Research Council of Canada for the award of a post-graduate fellowship during the course of this project.

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List of Abbreviations

<u>A. aerogenes</u>	<u>Aerobacter aerogenes</u>
AB	$\alpha$ -aminobutyrate
ACB	$\alpha$ -amino- $\beta$ -chlorobutyrate
AcCoA	acetyl coenzyme A
AD	acetolactate decarboxylase
ADH	alcohol dehydrogenase
AHAS	acetohydroxy acid synthetase
AHB	acetohydroxybutyrate
AL	acetolactate
AMP	adenylic acid
ATCase	aspartate transcarbamylase
<u>B. licheniformis</u>	<u>Bacillus licheniformis</u>
<u>B. subtilis</u>	<u>Bacillus subtilis</u>
CAT	dichloroacetyl-DL-threonine
CE	crude extract
CoA	coenzyme A
CPSase	carbamyl phosphate synthetase
<u>C. tetanomorphum</u>	<u>Clostridium tetanomorphum</u>
CTP	cytidine triphosphate
dCDP	deoxycytidine diphosphate
DH	dihydroxyacid dehydratase
DHIV	$\alpha,\beta$ -dihydroxyisovalerate
DHMV	$\alpha,\beta$ -dihydroxy- $\beta$ -methylvalerate
DNPH	2,4-dinitrophenyl hydrazine
DTNB	5,5'-dithiobis-(2-nitrobenzoic acid)
DTT	dithiothreitol (Cleland's reagent)

$E_A$	energy of activation
<u>E. coli</u>	<u>Escherichia coli</u>
EDTA	ethylenediaminetetraacetic acid
FAD	flavin adenine dinucleotide
HETDP	hydroxyethylthiamine diphosphate
HM	Halvorson (minimal) medium
ile	L-isoleucine
<u>ilv</u> , <u>ilva</u>	isoleucine-leucine-valine
<u>ilv A</u>	gene specifying TD
<u>ilv B</u>	gene specifying AHAS
<u>ilv C</u>	gene specifying IR
<u>ilv D</u>	gene specifying DH
<u>ilv E</u>	gene specifying TrB
IPM	$\alpha$ -isopropylmalate
IPMS	$\alpha$ -isopropylmalate synthetase
IR	acetohydroxy acid isomeroreductase
KB, $\alpha$ -KB	$\alpha$ -ketobutyrate
KIC	$\alpha$ -ketoisocaproate
KIV	$\alpha$ -ketoisovalerate
KMV	$\alpha$ -keto- $\beta$ -methylvalerate
KNF	Koshland-Nemethy-Filmer
leu	L-leucine
MWC	Monod-Wyman-Changeux
NADH	nicotinamide adenine dinucleotide (reduced)
NADPH	nicotinamide adenine dinucleotide phosphate (reduced)
NAV	N-acetyl-DL-valine
<u>N. crassa</u>	<u>Neurospora crassa</u>

<u>P. aeruginosa</u>	<u>Pseudomonas aeruginosa</u>
P-G buffer	phosphate-glycerol buffer
PLP	pyridoxal-5'-phosphate
<u>P. radiatus</u>	<u>Phaseolus radiatus</u>
PRPP	phosphoribosylpyrophosphate
PS	protamine sulfate
R	relaxed
<u>R. spheroides</u>	<u>Rhodopseudomonas spheroides</u>
<u>S. cerevisiae</u>	<u>Saccharomyces cerevisiae</u>
<u>S. marcescens</u>	<u>Serratia marcescens</u>
<u>S. pombe</u>	<u>Schizosaccharomyces pombe</u>
<u>S. typhimurium</u>	<u>Salmonella typhimurium</u>
T	taut, tight
TCA	trichloroacetic acid
TD	threonine deaminase
TFI	trifluoroisoleucine
thr	threonine
TIL	thiaisoleucine
TME	L-threonine methyl ester
TPP	thiamine pyrophosphate
TrB	transaminase B
tRNA	transfer ribonucleic acid
UTP	uridine triphosphate
val	L-valine
XMP	xanthosine monophosphate

## 1. INTRODUCTION

This study involves the biosynthesis and regulation of the branched-chain amino acids isoleucine, valine and leucine, in the fission yeast Schizosaccharomyces pombe. Isoleucine is considered a member of the aspartic acid family of amino acids (fig. 1.1) because it obtains its carbon chain in part from threonine, which is itself derived from aspartate. Valine and leucine both derive their carbons from pyruvate. An intriguing feature of isoleucine and valine synthesis is the fact that four of the steps involved are catalyzed by a common set of enzymes. Leucine arises from the keto acid precursor of valine (ketoisovalerate), creating a branch point. These aspects of the pathways present an interesting system for the study of the regulation of carbon flow over the sequences to the end-product amino acids. The controls mediated by feedback inhibition and repression have thus been investigated in S. pombe in order to obtain a more complete picture of amino acid biosynthesis in this eucaryote.

The introduction will consist of a general discussion of the mechanisms of feedback inhibition and of repression, a description of the isoleucine-valine-leucine pathways, and the regulatory mechanisms which have been investigated. A detailed analysis of TD from Salmonella typhimurium and Saccharomyces cerevisiae will follow, in preparation for the experimental findings to be reported here.

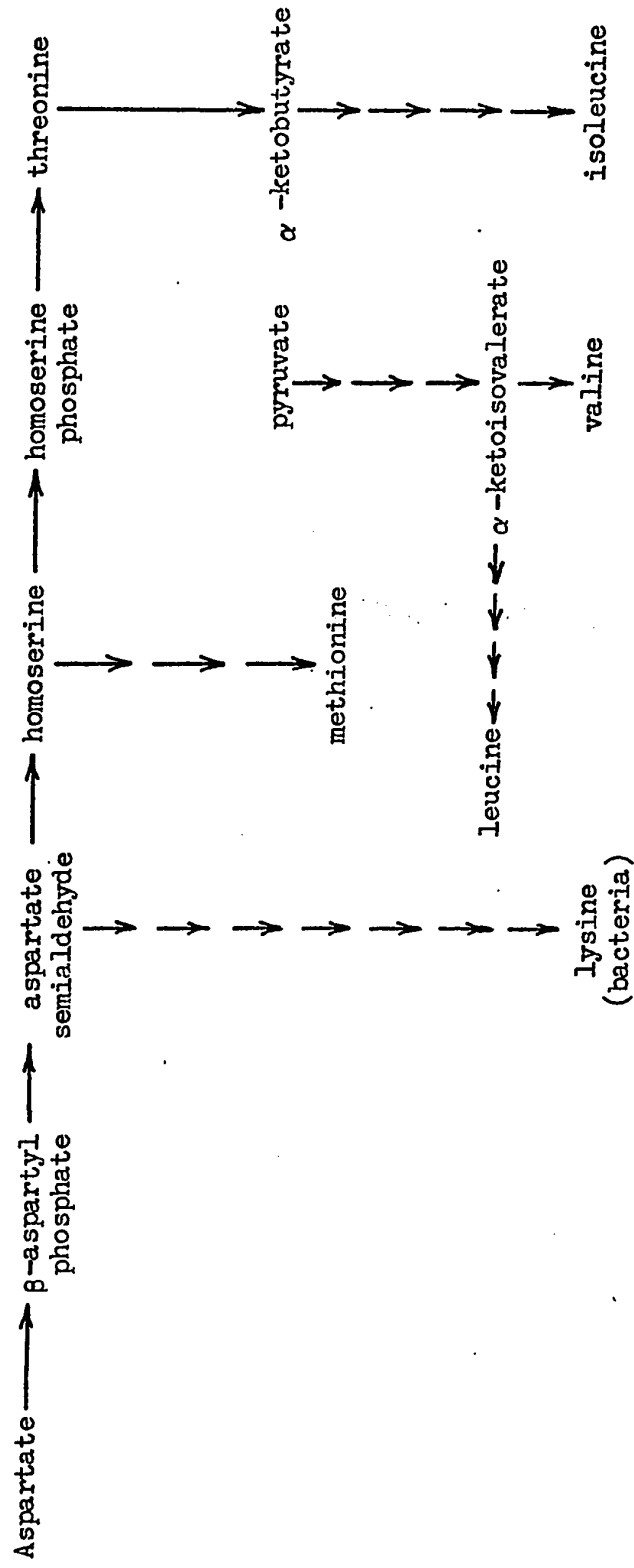


Fig. 1.1. The aspartic acid family of amino acids

## 1.1. ENZYME REGULATION

The living cell may be considered as a highly ordered system of biosynthetic and catabolic pathways. The fact that under normal conditions a living cell seldom synthesizes or degrades more materials than it requires for metabolism and growth implies the existence of a system of highly organized and efficient regulatory mechanisms. As all metabolic sequences are catalyzed by highly effective and specific catalysts (enzymes), it is necessary that "key" enzymatic reaction rates are controlled in order to maintain the fine balance between various metabolic functions.

### 1.1.1. Regulation of enzyme activity

One of the main regulatory mechanisms of cell metabolism occurs at the enzyme level, and is characterized by an inhibition of the first enzyme reaction in a metabolic sequence by its own end-product (feedback or retroinhibition).

This type of regulation was apparently first reported by Dische (as cited by Cohen, 1965) who found that the phosphorylation of glucose in erythrocytes was inhibited by phosphoglyceric acid. This discovery went almost unnoticed, although Dische appears to have been aware of the importance of his observations: he is quoted by Cohen, "the inhibition of phosphorylation of glucose by diphosphoglyceric acid leads to the automatic regulation of the formation of the latter". It was not until 15 years later that experimental results with cell-free extracts of bacteria showed that an end-product did inhibit the activity of an early enzyme of

its own pathway. Umbarger (1956) demonstrated that threonine deaminase (TD) isolated from E. coli was strongly and specifically inhibited by L-isoleucine. About the same time, Yates and Pardee (1956) showed that the first enzyme unique to pyrimidine biosynthesis in E. coli was inhibited by the end-product cytidine triphosphate (CTP). These results pointed to the apparent advantage for the organism of such regulation at the molecular level, and since then, numerous examples (cited by Cohen, 1965) have shown that end-product inhibition is the rule rather than the exception in biosynthetic pathways.

#### 1.1.2. Regulation of enzyme synthesis

A second operationally well-defined regulatory mechanism in the living cell is repression, a mechanism which acts at the genetic level and affects the formation of enzymes. Microorganisms often synthesize the products they need only when they are not available in the medium. If the product is supplied in the growth medium, the entire sequence of enzymes involved in the synthesis of that product is present only at a very low level in the cell. The term "repression" was coined by Vogel (1957), who showed that when a mutant of E. coli (which required arginine or N-acetylornithine for growth) was grown in the presence of arginine, it did not produce acetylornithinase, an enzyme involved in arginine biosynthesis. However, cells grown in the presence of acetylornithine synthesized the enzyme, a finding which led to the conclusion that acetylornithinase was induced by its substrate. Growth experiments in the presence of mixtures of the two metabolites revealed that arginine had an inhibiting effect on the synthesis of acetylornithinase

(repression), and that the inductive effect of acetylornithine was an artifact.

It can be seen that interplay of these two basic mechanisms, end-product inhibition and repression, can endow the cell with a high degree of control over the synthesis of the compounds it needs for growth. Retroinhibition serves as an immediate and temporary control on biosynthesis, while repression is a more slowly-acting mechanism which allows the cells to shut down a pathway for a longer term effect (economy of protein synthesis).

Induction also affects the formation of enzymes involved in both anabolic and catabolic sequences. For example, in the biosynthesis of leucine in Neurospora crassa,  $\alpha$ -isopropylmalate, the product of the first enzyme of the pathway, appears to induce the formation of isomerase and dehydrogenase, the next two enzymes in the sequence (Gross, 1965). A similar induction of intermediate enzymes in the biosynthesis of pyrimidines in yeast by ureidosuccinate and dihydroorotate was reported by Iacroute (1968). The enzymes responsible for the catabolism of a carbon and energy source are often formed (induced) only when that particular energy source is available for utilization.

### 1.1.3. Molecular control mechanisms

The ability of an end-product to inhibit the activity of the enzyme catalyzing the first step in its synthesis was of special interest because this compound bore little steric or structural relationship to the substrate of the reaction. In his first report on the subject, Umbarger (1956) showed that isoleucine was a strong, apparently

competitive, inhibitor of threonine deaminase. Studies on many such enzymes subject to feedback control often exhibited substrate saturation curves which were sigmoidal, in contrast to the hyperbolic curve for the binding of substrate obtained with a "classical" enzyme. Such differences in kinetics and the structural dissimilarity between the effector molecule and substrate led to the concept of allosterism and the elaboration of models relating the quaternary structure of the enzyme protein to its kinetics and binding characteristics.

The term "allosteric" was introduced by Monod and Jacob (1961) to describe the binding of dissimilar substrate and effector to a regulatory enzyme at separate sites, in order to distinguish this effect from classical competitive, or isosteric, inhibition where a steric analog of the substrate competes with it for the same active site. The notion of the effector binding at another site distinct from the substrate (active) site was supported by the observation of Changeux (1961) that the sensitivity of E. coli TD to isoleucine could be selectively removed without affecting the enzyme's catalytic activity.

Allosteric effects are defined as indirect interactions between distinct and separate sites on the enzyme molecule, which are mediated through conformational changes of the enzyme protein. They are of two types: homotropic effects are interactions brought about through binding of identical ligands; such effects are cooperative and may be reflected by a sigmoidal binding curve for that ligand. Heterotropic interactions are those occurring when dissimilar ligands are bound by the enzyme. The resulting effect can be positive (activation of enzyme activity) or negative (inhibition). In the latter case, the substrate curve can be made sigmoidal, or, if it is sigmoidal in the absence of

any effectors, made increasingly sigmoidal with increasing concentration of inhibitor. Positive heterotropic effects activate enzyme activity and normalize the substrate curve if it was sigmoidal in the absence of effectors (Changeux, 1964). Examples of these phenomena will be presented later in the discussion of the TD's from S. typhimurium and S. cerevisiae.

#### 1.1.4. Models for allosteric control mechanisms

Two models for allosteric effects of regulatory enzymes are most popular; both require enzyme molecules composed of two or more subunits, and the interactions between the subunits are mediated by the binding of ligands. Such binding results in variations of the overall enzymic activity of the protein.

##### 1.1.4.1. The concerted model

The model proposed by Monod, Wyman and Changeux (1968) (MWC model) assumes that the enzyme has two or more identical subunits, each having a site for substrate and a site for modifier ligand. The enzyme can exist in at least two conformational states (tight or T, and relaxed or R), in equilibrium with each other; cooperativity results from the differential binding of ligands to these two states of the enzyme. A third assumption is that the conformational changes of all subunits occur in a concerted manner, for reasons of symmetry, thus precluding the existence of hybrid states. In the absence of ligands, the enzyme exists mainly in the T state, which has low

affinity for substrate. Cooperative effects of substrate occur as it "pulls" the enzyme towards the more active R state to which form the substrate has higher affinity. Allosteric inhibitors and activators bind preferentially to the T or R forms, respectively, enhancing or reducing sigmoidicity in the substrate saturation curve.

#### 1.1.4.2. The sequential model

A second model, more generalized than the concerted MWC model, is that of Koshland, Nemethy and Filmer (1966) (KNF model). It also involves three assumptions: a) the substrate induces a conformational change which may be limited to a single subunit of the enzyme; b) the conformational changes in the individual subunits occur sequentially rather than simultaneously; and c) a differential binding affinity for ligands exists between one conformational state and the other of the protein. Thus, hybrid forms of the enzyme may exist, where one subunit is in the R state and another is in the T state.

Other models in which sigmoidal velocity responses are not due exclusively to subunit interactions have been proposed; these are based on the idea that binding of a ligand to an enzyme is modified by events at other sites. For instance, Sweeney and Fisher (1968) demonstrated mathematically for a number of models that changes in rate constants could alter the shape of the substrate saturation curve. This means that in models in which cooperativity is based on other than subunit interactions or multiple sites, a change in the conformation of the enzyme could change the relative magnitude of rate constants and thereby change its kinetic characteristics.

Kinetic data do not provide sufficient evidence to choose between one model or another for a particular enzyme. Subunit isomerizations can only be detected by physicochemical studies such as equilibrium dialysis, ultracentrifugation, sulfhydryl reactivity or nuclear magnetic resonance.

## 1.2. THE BRANCHED-CHAIN AMINO ACIDS

### 1.2.1. Historical

The branched-chain amino acids isoleucine, valine and leucine, have long been grouped together mainly because of their structural similarity. It was only in the 1950's, with the advent of isotopic tracer techniques and the development of assay procedures for relevant enzymes, that the rationale for this grouping was demonstrated to be sound, with respect to the biosynthesis of these amino acids.

One of the earliest observations pointing to possible metabolic interrelationships between these amino acids was the ability of isoleucine, valine or leucine, added singly to a synthetic medium, to inhibit the growth of anthrax bacillus (Gladstone, 1939). A mixture of all three stimulated growth. Gladstone suggested two possibilities to explain this antagonism: an excess of one amino acid might interfere with the utilization of the other two in making bacterial protein. The second might be an excess of one interfering with the synthesis of the other two.

Further indication of metabolic relationship between these amino acids was obtained by Bonner et al. (1943), who isolated a single-step mutant of Neurospora crassa which required both isoleucine and valine. At the time, evidence was accumulating in support of the "one gene-one enzyme" hypothesis (Beadle and Tatum, 1941), and this result appeared to be an exception to the general rule that a single gene mutation resulted in the impairment or failure of a single chemical reaction. Bonner (1946) subsequently showed that the mutation prevented this

strain from aminating ketoisoleucine (  $\alpha$ -keto- $\beta$ -methylvaleric acid, the immediate precursor of isoleucine) to isoleucine. He suggested that accumulated ketoisoleucine in the mycelium would then inhibit the amination of  $\alpha$ -ketoisovaleric acid to valine, thereby creating a secondary valine requirement as a natural consequence; this suggestion, while ingenious, is not correct as will be seen below.

### 1.2.2. Biosynthesis of isoleucine and valine

The demonstration of the pathways leading to isoleucine, valine and leucine was achieved through isotope tracer techniques, mutant methodology and enzyme analysis applied to several microorganisms, namely, E. coli, N. crassa, S. cerevisiae and Torulopsis utilis. The experimental evidence derived from these studies is well-summarized in two recent, comprehensive reviews (Greenberg, 1961 and Umbarger and Davis, 1962), and more recently by Umbarger (1969, 1969a). The general pattern which has emerged indicates that isoleucine and valine are synthesized along parallel pathways mediated by a common set of enzymes; the general pathways involved are presented in fig. 1.2.1.

The deamination-dehydration of threonine, catalyzed by threonine deaminase (L-threonine hydro-lyase (deaminating), EC 4.2.1.16), is the first step proper to the isoleucine pathway. The product,  $\alpha$ -ketobutyrate, is condensed with pyruvate to yield  $\alpha$ -aceto- $\alpha$ -hydroxybutyrate (AHB). The enzyme involved in this step, acetohydroxy acid synthetase, EC 4.1.3.12, (AHAS), also catalyzes the condensation of two molecules of pyruvate to yield  $\alpha$ -acetolactate (AL). This latter reaction is the first step specifically in the pathway to valine.  $\alpha$ -Acetohydroxy acid

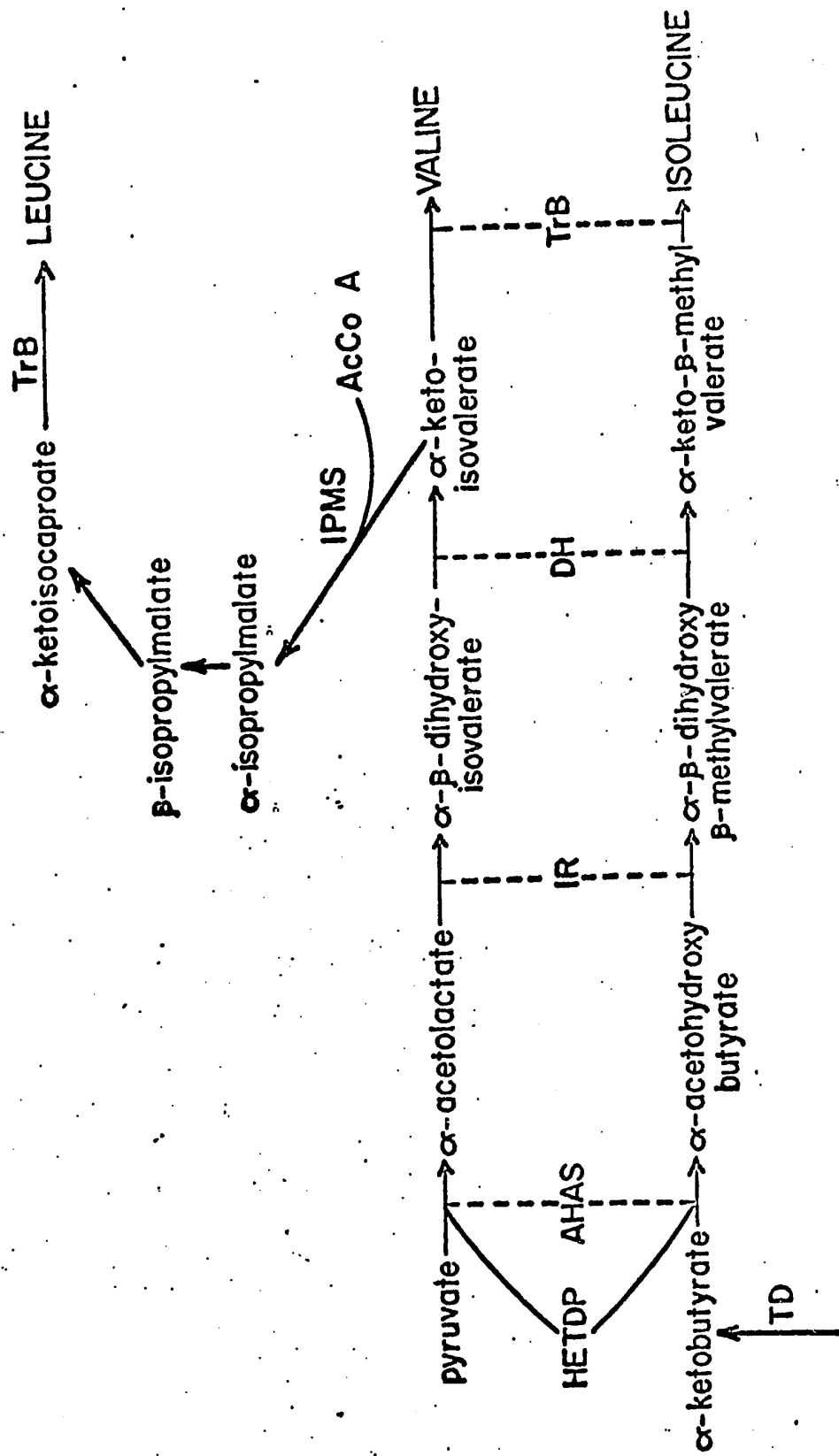


Fig. 1.2.1. Biosynthetic pathways of isoleucine, valine and leucine

isomeroreductase, EC 1.1.1.78, (IR) catalyzes the conversion of acetolactate to  $\alpha, \beta$ -dihydroxyisovalerate (DHIV), step two in the valine pathway, and acetohydroxybutyrate to  $\alpha, \beta$ -dihydroxy- $\beta$ -methylvalerate (DHMV). This conversion most likely involves a rearrangement of the acetohydroxy acids to the  $\alpha$ -keto- $\beta$ -hydroxy acids, followed by an NADPH-dependent reduction of these (enzyme-bound) intermediates to the dihydroxy acid analogs of valine and isoleucine (Arfin and Umbarger, 1969). The penultimate step in both pathways involves the conversion of DHIV and DHMV to  $\alpha$ -ketoisovalerate (KIV) and  $\alpha$ -keto- $\beta$ -methylvalerate (KMV), respectively; the enzyme concerned is dihydroxy acid dehydrase (EC 4.2.1.9) (DH). The keto acid precursors KIV and KMV are transaminated to their respective amino acids, valine and isoleucine; the amino donor is probably glutamate. The reaction is mediated by transaminase B (L-leucine: 2-oxoglutarate aminotransferase, EC 2.6.1.6, TrB). A second unnamed transaminase reacting with valine, alanine and  $\alpha$ -aminobutyrate was shown in extracts of E. coli (Rudman and Meister, 1953).

It is generally accepted that the steps in isoleucine and valine synthesis from the condensation reactions which form AHB and AL, respectively, are catalyzed by a single sequence of enzymes. The strongest evidence for this is the fact that mutants blocked at any one of these steps require both isoleucine and valine, or the appropriate precursors. Thus, a mutant of A. aerogenes lacking IR cannot form the dihydroxy acids, but can utilize them for growth (Umbarger et al., 1960); mutants of E. coli and N. Crassa having little or no DH activity require both isoleucine and valine for growth, and accumulate the dihydroxy acids (Myers and Adelberg, 1954). A mutant of E. coli blocked in the transaminase B step requires isoleucine and valine; however, the valine

requirement can be satisfied by alanine or  $\alpha$ -aminobutyrate, indicating the presence of the second transaminase (Rudman and Meister, 1953).

In addition to this mutant evidence, the fact that when these enzymes are purified from appropriate organisms, only one protein is observed in ultracentrifugal and electrophoretic studies, and the activity towards each substrate is constant at all stages of purification (IR, Arfin and Umbarger, 1969; DH, Kiritani et al., 1966; transaminase B, Coleman and Armstrong, 1971).

### 1.2.3. Biosynthesis of leucine

Leucine biosynthesis has only more recently been elucidated. Again, isotope tracer techniques were invaluable in discovering the flow of carbon over the pathway (Jungwirth et al., 1963; Gross et al., 1963; Burns et al., 1963). The initial reaction involves a condensation of acetyl coenzyme A with  $\alpha$ -ketoisovalerate to yield  $\alpha$ -isopropylmalate; the reaction is catalyzed by  $\alpha$ -isopropylmalate synthetase ( $\alpha$ -isopropylmalate  $\alpha$ -ketoisovalerate lyase (coenzyme A acetylating), EC 4.1.3, IPMS). Thus  $\alpha$ -ketoisovalerate is the intermediate common to both the valine and leucine pathways. The second step involves isomerization of  $\alpha$ -isopropylmalate to  $\beta$ -isopropylmalate, mediated by  $\alpha$ -isopropylmalate isomerase.  $\beta$ -Isopropylmalate dehydrogenase (EC 1.1.1.85) converts  $\beta$ -isopropylmalate to  $\alpha$ -ketoisocaproate by an oxidative decarboxylation process, and the keto acid precursor is aminated to leucine by transaminase.

All the enzymes involved in leucine biosynthesis have since been detected in cell-free extracts of S. typhimurium and N. crassa

(Jungwirth et al., 1963; Gross et al., 1963; Burns et al., 1963) and in S. cerevisiae (Satyanarayana et al., 1968). -IPMS has been highly purified from N. crassa (Webster and Gross, 1965), S. typhimurium (Kohlhaw et al., 1969) and S. cerevisiae (Ulm and Kohlhaw, 1971).

### 1.3. REGULATORY ASPECTS OF THESE PATHWAYS

#### 1.3.1. Control by feedback inhibition

Referring again to fig.1.2.1, it is obvious that there are three points where feedback control could be effected: at the levels of TD, AHAS and IPMS. TD has been shown to be specifically and strongly inhibited by L-isoleucine, AHAS by L-valine and IPMS by L-leucine (Umbarger, 1969). In almost all organisms investigated thus far, these feedback patterns have been observed.

#### 1.3.2. Control of enzyme level in isoleucine-valine synthesis

Regulation of enzyme amount in the isoleucine-valine pathways was shown to be controlled by multivalent repression in S. typhimurium and E. coli (Freundlich et al., 1962); the isoleucine-valine enzymes were repressed only when excess isoleucine, valine and leucine were all present in the growth medium. The enzymes are derepressed when one of these amino acids is absent, even in the presence of an excess of the other two. This pattern of multivalent repression has been demonstrated in bakers' yeast (Magee and Hereford, 1969; Bussey and Umbarger, 1969) and for Serratia marcescens (Kisumi et al., 1971). An interesting exception occurs in Pseudomonas aeruginosa; where only AHAS and IR are multivalently repressed; the formation of the other enzymes in the pathway is not affected by the presence of all three amino acids (Marinus and Loutit, 1969).

In many cases, it appears that it is not the free amino acids

themselves which function as repressors; in such cases studies have shown that they must be activated by their respective tRNA synthetases and charged to their respective aminoacyl-tRNA's (Williams and Freundlich, 1969). Interestingly, leucyl-tRNA is bound specifically and reversibly to an immature form of purified TD from S. typhimurium (Hatfield and Burns, 1970 - to be discussed in more detail later). In eucaryotes, it was shown that isoleucyl-tRNA synthetase is essential for multivalent repression of the isoleucine enzymes in S. cerevisiae (McLaughlin et al., 1969). More recent evidence has accumulated which indicates that the feedback site of the first enzyme of a biosynthetic sequence must be intact for the repression process to occur (Kovach et al., 1969). In this regard, it was recently shown that AHAS in bakers' yeast could not be derepressed to the usual extent in mutants having TD genetically desensitized to isoleucine, or in those with a nonsense mutation in the TD gene which results in only a fragment of TD being synthesized (Bollon and Magee, 1971). Thus the two apparently unrelated regulatory mechanisms of feedback inhibition and repression have in this case a common requirement for one regulatory site of the first enzyme in the pathway.

In E. coli, although all five ilv genes are tightly clustered, Ramakrishnan and Adelberg (1965) demonstrated the existence of two operator genes, one governing TD, DH and TrB (ADE operon), and the second controlling AHAS (ilv B operon). No mutants derepressed for IR (ilv C) have been observed, so there is no evidence for a third operator locus (Umbarger, 1969). While TD, AHAS, DH and TrB are coordinately repressed or derepressed, under appropriate conditions, IR levels follow the same pattern, but are not coordinate with, the other four enzymes. IR appears

to be a substrate-controlled enzyme (Arfin et al., 1969): under conditions which repress TD, AHAS, DH and TrB, IR can be induced by acetolactate and acetoxybutyrate, thus suggesting that substrate induction may be the major means for regulating synthesis of IR, at least in E. coli, S. typhimurium, and probably A. aerogenes.

It is interesting to note here that the first cases demonstrating repression and feedback inhibition of the first enzyme, and induction of intermediate enzymes in the same biosynthetic pathway, were reported by Lacroute (1964) for the uracil pathway in S. cerevisiae, and inferred by Gross (1965) for the leucine pathway in Neurospora.

In S. cerevisiae, multivalent repression controls the levels of the isoleucine-valine enzymes, although the genes specifying them are unlinked (Kakar and Wagner, 1964; Mortimer and Hawthorne, 1969). Whether the IR is subject to substrate induction is both an open question and a difficult one to answer, as yeast is impermeable to the acid intermediates of the pathway (Magee and de Robichon-Szulmajster, 1968a).

### 1.3.3. Control of leucine enzyme levels

In Salmonella, four clustered genes specify the structure of the first three enzymes of the pathway (Margolin, 1963), and they are coordinately repressed by L-leucine (Burns et al., 1966). Leucyl-tRNA is postulated to be involved in the repression process (Calvo et al., 1969).

In Neurospora, the four leu cistrons are unlinked, yet the function of each cistron is regulated in response to specific demands. IPMS is repressed by excess L-leucine, while the two subsequent enzymes seem to be inducible by the product of the first enzyme,  $\alpha$ -isopropylmalate (Gross, 1965).

In bakers' yeast, 10 genetic loci are involved in the control of three of the leucine enzymes (Satyanarayana et al., 1968a). A single locus governs IPMS, which is repressible not by leucine alone, but by leucine plus threonine. The isomerase is specified by 6 loci, and the remaining three govern  $\beta$ -IPM dehydrogenase. These latter two enzymes are repressed by L-leucine alone (Satyanarayana et al., 1968b).

#### 1.4. RECENT DEVELOPMENTS OF INTEREST

Of all the enzymes involved with branched-chain amino acid synthesis, TD is probably the most studied and well-known. As most of the properties of the remaining enzymes will be adequately discussed in the "Results" and "Discussion" to follow, material in this section will be restricted to the TD of S. typhimurium, and to studies on the enzyme from S. cerevisiae, because they will be of more immediate interest to the work in this thesis, from the point of view of biosynthesis and regulation in eucaryotes.

##### 1.4.1. TD of S. typhimurium

This TD has been purified to homogeneity and shown to possess all the properties characteristic of the enzyme in crude extracts: normal threonine saturation curve which is made sigmoidal by L-isoleucine; removal of heterotropic interactions by L-valine and reversal of isoleucine inhibition (Burns and Zarlengo, 1968). It has a molecular weight of 194,000 and consists of four identical subunits of molecular weight 48,500 (Zarlengo et al., 1968). The earlier work of Maeba and Sanwal (1966) which indicated two binding sites for threonine, along with the fact that the native tetramer was shown to bind two molecules of pyridoxal phosphate (PLP), led to the idea that the functional protomer for this enzyme was a dimeric structure composed of two of the polypeptide chains.

Subsequent studies by Hatfield and Burns (1970a) focussed on the intermediate substructure of the enzyme. The stable dimeric intermediate

was isolated by removal of PLP through dialysis, and was found to have a molecular weight of 97,000. Although it was enzymatically inactive, this immature form of the enzyme could be fully activated in the presence of PLP and threonine. PLP alone preincubated with the dimeric intermediate promoted the formation of an inactive holotetramer, which was immediately activated upon addition of threonine. Thus, the maturation of the inactive tetramer into a catalytically-functional enzyme is brought about by a ligand-specific induction. The presence of both isoleucine and valine prevented the maturation process of the inactive tetramer, although each separately could promote maturation to the active form. These authors reasoned that the inactive holotetramer has at least two distinct stereospecific sites, one for either threonine or valine, and the other for isoleucine; when both sites are occupied by ligands, the process is blocked due possibly to steric hindrance of the conformational change in the protein which confers catalytic activity. Leucine had no effect in the maturation process.

As pointed out before, multivalent repression requires the participation of one or another form of all three of the branched-chain amino acids. Binding studies performed on sucrose density gradients revealed that leucyl-tRNA bound specifically only to the immature holotetramer; both isoleucyl-tRNA and valyl-tRNA were not bound, nor did they promote maturation. Neither free leucine nor leucyl-tRNA had any effect on the maturation process (Hatfield and Burns, 1970).

The results described here suggested a model for the mechanism of multivalent repression as observed in Salmonella. The A cistron of the ilv (ADE) operon specifies the basic TD monomers, which associate via disulfide bonds to form dimers. These apodimers are in equilibrium with

apotetramers which, in the presence of PLP, become holotetramers which lack catalytic activity. These holotetramers are activated by threonine, valine or leucine to form native TD. If threonine and isoleucine or valine and isoleucine are simultaneously present, the immature form is maintained. These authors proposed that the immature holotetramer is in fact the aporepressor of the ilv (ADE) operon, and that when it binds leucyl-tRNA, the complex formed is the holorepressor which acts directly on the DNA at the transcriptional level or at some later point in protein synthesis. The levels of ligands in the cell's pools (isoleucine, valine and leucyl-tRNA) thus regulate the amounts of native TD or immature holotetramer which will be present (i.e. derepression or multivalent repression). This is a very interesting model which is consistent with almost all known facts of isoleucine-valine synthesis. It remains to be determined whether a similar situation exists in other bacteria and in eucaryotes.

#### 1.4.2. TD and its regulatory rôle in *S. cerevisiae*

The properties of TD from bakers' yeast have been extensively studied by Cennamo et al. (1964) and by de Robichon-Szulmajster and Magee (1968). As the details of the interactions of TD with its ligands will be presented at length in later sections, only a brief summary will be given here. The yeast enzyme can bind threonine, isoleucine and valine; variation of pH changes the relative affinities of the enzyme towards these ligands. Threonine and isoleucine display heterotropic interactions. Valine exerts no homotropic effects: it increases the apparent affinity for threonine, reverses isoleucine inhibition and eliminates

cooperative effects of threonine. Isoleucine, at low concentrations, also acts as a positive effector. The enzyme has been shown to have a molecular weight of close to 190,000 (Brunner et al., 1969). The enzyme has been recently purified to homogeneity (Ahmed and Magee, 1971).

Although the yeast TD could not be desensitized by chemical means (de Robichon-Szulmajster and Magee, 1968), Betz et al. (1971) have succeeded in isolating mutants resistant to growth inhibition by thiaisleucine; these excrete isoleucine and have TD's 10- and 100- fold less sensitive than wild type enzyme to L-isoleucine as inhibitor. The enzymes' catalytic properties remained unchanged. The pattern of isoleucine activation and inhibition of these mutant TD's yielded interesting information: at low isoleucine levels, the enzymes were activated as are wild type enzyme. However, at high isoleucine concentrations, no inhibition of activity occurred with the mutant enzymes, whereas the inhibition curve for the wild type TD dropped sharply and 100% inhibition could be achieved. These results suggested two sorts of sites for binding isoleucine: one which causes stimulation at low threonine concentrations, and another which inhibits.

A model for TD was elaborated which includes two sites for substrate; two activator sites able to bind valine (preferentially) and isoleucine; and four inhibitor sites able to bind both isoleucine and valine. Isoleucine binds with greater affinity to the T form of the enzyme, while valine binds either to the T or R forms, and reverses isoleucine inhibition by favouring the R form.

It was very recently demonstrated that the TD in S. cerevisiae is involved in the regulation at least of AHAS (Bollon and Magee, 1971). Using a TIL-resistant mutant whose TD is genetically desensitized to

isoleucine, these authors showed that AHAS could be derepressed about 6-fold when cells were transferred from repressing to minimal medium, as compared with the wild type which shows only 2-fold increase in AHAS under the same conditions. A second mutant, a leucine auxotroph with a nonsense mutation in the TD structural gene, could not be derepressed for AHAS activity when grown under minimal conditions or during growth on limiting leucine. These results indicate that intact TD plays a role in the derepression of at least one enzyme of the pathway, through isoleucine limitation. Several models were envisaged to explain this effect, including the involvement of leucyl-tRNA binding to TD. These findings prognosticate that the detailed study of regulation of repression of biosynthetic pathways in eucaryotes will be a most interesting and stimulating area of research.

### 1.5. SCHIZOSACCHAROMYCES POMBE

The physiology and genetics of the fission yeast, Schizosaccharomyces pombe, have been the subjects of two recent and extensive reviews (Mitchison, 1970; Leupold, 1970). Only a short description of this yeast will be given here in order to acquaint the reader with some fundamental properties of this strain.

S. pombe is a simple eucaryotic yeast whose cells are round-ended cylinders, and like other yeasts, possess a tough outer cell wall. It reproduces vegetatively by binary fission, in contrast to the budding process characteristic of S. cerevisiae and other yeasts; a septum or cross-wall forms without any constriction of the original cell wall, and when completed, it divides into two individual walls, and the roughly equal-sized daughter cells then separate. The fission yeast is a haploid organism under normal vegetative growth conditions. Mating response is determined by three alleles: two are heterothallic, that is, cells of opposite mating types  $h^-$  and  $h^+$  can copulate to form a diploid zygote which immediately reduces to form four haploid ascospores which germinate into haploid vegetative cells. The third ( $h^{90}$ ) confers homothallism, i.e. cells of this mating type can copulate to form a zygote.

S. pombe is easily grown and maintained on both defined and complex media, and it can be readily analyzed genetically with all the standard genetic techniques.

In S. cerevisiae, sixteen chromosomes, each with one or more centromere-linked genes, and six fragments which may be new chromosomes or parts of already identified chromosomes, have been identified. Close to 100 genes have been mapped, and as in most eucaryotes, the genes for any

given pathway are, for the most part, unlinked (Mortimer and Hawthorne, 1969). S. pombe is somewhat less defined genetically: six chromosomes have been identified and 78 genes have been demonstrated (Flores da Cunha, 1970; Leupold, 1970).

## 1.6. INTRODUCTION TO THE PROBLEM

### 1.6.1. Background and purpose

Yeasts more than any other microorganisms have been intimately associated with the welfare of man; they have been exploited by man in the making of alcoholic beverages and bread since very early times. Thus, it was only natural for man to want to learn as much as possible about these organisms which contributed so much to his well-being and economic interests. Yeasts were therefore, one of the principal tools used in the elucidation of the biochemical and metabolic processes characteristic of living cells. Although in this respect the same can be said of bacteria, yeast are more closely similar to animal cells by virtue of being eucaryotes, i.e. the DNA carrying the genes is complexed with nucleoproteins and histones, and is organized into discrete chromosomes which are separated by a nuclear membrane from the rest of the cell's machinery. In addition, yeast carry on respiration utilizing distinct mitochondria such as are found only in higher forms of life. Thus, in a yeast, a researcher has an organism which combines the handling ease of bacteria (with respect to growth and genetic manipulation), and the basic structure and differentiation of higher cells.

Of all the yeasts, S. cerevisiae is the most well-known both genetically and biochemically. Only one other eucaryotic microorganism is almost as well characterized, Neurospora crassa; this microorganism has different growth (mycelial), and grows much more slowly, and is thus not as convenient to work with.

The fission yeast S. pombe was chosen for this study for a number of reasons. Its ease of handling and method of cell division have

already been discussed. It has been extensively used for study of the biochemistry of the cell cycle: cultures can be readily synchronized, and the synthesis of a number of catabolic and biosynthetic enzymes have been correlated with the pattern of DNA synthesis during the cell cycle (Mitchison, 1970). S. pombe has been quite well studied genetically, for the standard genetical techniques (mutagenesis, random spore analysis, meiotic and mitotic mapping, etc.) are readily amenable to the growth and physiology of this organism. However, despite the identification of at least 55 genes involved in the biosynthesis of purines, pyrimidines, amino acids and other growth factors (Leupold, 1970), next to nothing is known of the enzymology and regulation of the synthesis of these metabolites. Studies on the biosynthesis of uracil and aspartate transcarbamylase (Megnet, 1958), and the enzymology of the first enzyme of the purine pathway (phosphoribosylpyrophosphate amidotransferase) (Nagy, 1970) have been reported.

The control of the pathways in the aspartic acid family of amino acids has proved to be an immensely important and rewarding model for cell regulation. The regulatory aspects of this system have been well documented in E. coli (see Cohen, 1965), and less well so in S. cerevisiae (de Robichon-Szulmajster, 1967 and 1971). The organization of the genetic material in eucaryotes (unlinked genes) suggests that the regulatory mechanisms must be more sophisticated than those of bacteria, where many genes with related functions (i.e. for a particular pathway) are often arranged into units of function or operons.

The pathways leading to the synthesis of isoleucine and valine are of special interest because four of the steps are common to both pathways and are catalyzed by a common set of enzymes (Umbarger and Davis,

1962). Earlier work had established the regulatory mechanisms in E. coli (Umbarger, 1961), and subsequent studies with other procaryotes (e.g. Salmonella, Bacillus subtilis and Pseudomonas aeruginosa) indicated that, although the same pathways were involved, interesting differences in the regulatory mechanisms occurred. To date, this author has found no reports in the literature of the regulatory aspects of isoleucine-valine biosynthesis in N. Crassa, although the enzymes of the pathways have been demonstrated (Wagner et al., 1964).

Very little work on amino acid biosynthesis and its regulation in S. pombe had been reported. As part of a long-term study of the aspartic acid family, it was decided to investigate the biosynthesis of isoleucine and valine in this organism. At the time when this project was begun, the first results of the gene-enzyme relationships and regulation of the isoleucine-valine pathways in bakers' yeast were appearing (de Robichon-Szulmajster, 1967). The appearance of these results in S. cerevisiae in no way diminished the importance of extending these studies to S. pombe. Although one might not expect to find great differences between two yeast species, interesting differences at the biochemical level are becoming apparent. For example, Heslot et al. (1970) reported that in S. pombe, only nuclear petite mutants (which show Mendelian segregation) are obtained, whereas in S. cerevisiae, one also can obtain cytoplasmic respiratory-deficient mutants; this tends to indicate that the mitochondrial biochemistry of S. pombe will be different from that of bakers' yeast. Striking differences exist in the properties of PRPP-amidotransferase, the first enzyme in purine biosynthesis, in these two yeasts. The S. pombe enzyme exhibits cooperative kinetics with respect to both substrates, glutamine and phospho-

ribosyl pyrophosphate (Nagy, 1970), whereas the kinetics of bakers' yeast enzyme are Michaelian for both substrates (Satyanarayana and Kaplan, 1971). The S. pombe enzyme was strongly inhibited by inosinic and guanylic acids, and was quite insensitive to adenylic acid (AMP); in bakers' yeast, the enzyme was sensitive to AMP (which effector made the kinetics sigmoidal), and almost unaffected by the other two. It was critical, then, to initiate studies in S. pombe to establish some of the basic patterns of amino acid biosynthesis and regulation, and to compare and corroborate the results with those obtained with bakers' yeast.

Studies with E. coli on the synthesis and regulation of the z, y and i genes for the dissimilation of lactose inspired the operon theory as proposed by Jacob and Monod (1961). Many subsequent studies in bacteria indicated that the genes specifying the steps in most well-known pathways were closely associated and controlled in operon-like fashion. However, the demonstration of operons in eucaryotes is extremely rare, as the genes for a pathway are unlinked and reside on several chromosomes. One example is the ura-2 locus in S. cerevisiae, which, on the basis of biochemical and genetic data, appears to be a polycistronic operon coding for carbamyl phosphate synthetase, aspartate transcarbamylase and possibly a regulatory polypeptide, all of which are involved in the early steps of pyrimidine biosynthesis (Kaplan et al., 1970; Lue and Kaplan, 1971). It is only through studies of regulatory mechanisms of various biosynthetic pathways in many eucaryotes that we may come to some fundamental scheme of regulation which is unique to these forms of life.

Studies such as these are fundamental to an understanding of metabolic regulation in higher forms of life, where the patterns are obscured and complicated by differentiation of cells into specific

tissues, by the intervention of histones, and by the complex interplay of nervous control and hormone action.

1.6.2. Statement of the problem

This research project had as its aims:

1. to elucidate the pathways for the biosynthesis of isoleucine and valine in S. pombe, by demonstrating the ability of cell-free extracts to catalyze the individual steps of the pathways;
2. to partially purify TD and characterize the enzyme with respect to its complex kinetic interactions with isoleucine, valine and leucine, and to compare these properties with those of TD from S. cerevisiae;
3. to propose a tentative model for TD to correlate all the facts obtained with current concepts of "allosterie";
4. to characterize AHAS and determine its kinetic relationships with respect to substrate and branched-chain amino acids, and to compare these results with the system from bakers' yeast;
5. to determine the basic kinetic properties of IR, DH and TrB;
6. to elucidate the regulatory patterns governing the synthesis and function of the enzymes involved in isoleucine-valine biosynthesis, i.e. regulation through feedback inhibition at the level of TD and AHAS, and regulation through repression of the ilva enzymes by the end-product amino acids;

7. to examine the first enzyme of the leucine biosynthetic pathway, and determine whether it is subject to feedback inhibition by leucine;
8. to isolate and characterize isoleucine - and isoleucine - valine - requiring mutants, and mutants resistant to analogs of isoleucine and valine, in order to probe more of the regulatory properties of TD and AHAS, and of the repression pattern governing the synthesis of the ilva enzymes in S. pombe.

## 2. MATERIALS

### 2.1. MEDIA AND VITAMINS

Yeast extract, Bacto-peptone, nutrient broth and Bacto-agar were products of Difco Laboratories.

Thiamine HCl, pyridoxine HCl, i-inositol, D-biotin, nicotinic acid, folic acid and para-aminobenzoic acid were obtained from Nutritional Biochemicals Corp. (N.B.Co.), and D-pantothenic acid, Ca salt, came from J. T. Baker Chem. Co.

### 2.2. AMINO ACIDS

All amino acids used were supplied by N.B.Co., except L-leucine (methionine-free), L-valine and L-isoleucine, which were products of Calbiochem.

### 2.3. BUFFER REAGENTS

Sodium barbiturate (sodium barbital) came from Fisher Scientific, and Sigma Chemical Co. supplied monopotassium phosphate ( $\text{KH}_2\text{PO}_4$ ), grade III; dipotassium phosphate ( $\text{K}_2\text{HPO}_4 \cdot 3\text{H}_2\text{O}$ ), grade V; Trizma base (tris (hydroxymethyl) aminomethane); Trizma HCl (tris HCl).

#### 2.4. SUBSTRATES

The sodium salts of pyruvic acid,  $\alpha$ -ketoglutaric acid,  $\alpha$ -ketoisovaleric acid and  $\alpha$ -keto- $\beta$ -methylvaleric acid were obtained from Sigma. Ethyl  $\alpha$ -acetoxy- $\alpha$ -acetolactate, ethyl  $\alpha$ -acetoxy- $\alpha$ -acetohydroxybutyrate, sodium  $\alpha, \beta$ -dihydroxyisovalerate and sodium  $\alpha, \beta$ -dihydroxy- $\beta$ -methylvalerate were purchased from Reef Laboratory.

#### 2.5. COENZYMES

Sigma supplied: thiamine pyrophosphate chloride (cocarboxylase, TPP); flavin adenine dinucleotide, di-sodium salt (FAD); pyridoxal-5'-phosphate (PLP); nicotinamide adenine dinucleotide phosphate, reduced form (NADPH), type II, tetrasodium salt; nicotinamide adenine dinucleotide, reduced form (NADH), disodium salt, type III. Acetyl coenzyme A, trilithium salt, 85% pure, was obtained from P-L Biochemicals.

#### 2.6. STANDARDS

$\alpha$ -Ketobutyric acid, Na salt, and reduced glutathione were from Sigma. Acetoin (3-hydroxy-2-butanone) was obtained from Eastman Organic Chemicals. Bovine serum albumin, crystalline, was purchased from N.B. Co.

## 2.7. ENZYMES

Alcohol dehydrogenase (2 x crystallized, lyophilized) came from Calbiochem.

## 2.8. ANALOGS

From N.B.Co.: N-acetyl-DL-valine, dichloroacetyl-DL-threonine, L-threonine methyl ester, DL-allo-threonine, glycyl-L-isoleucine, chloroacetyl-DL-isoleucine, D-valine, chloroacetyl-DL-valine, L-serine.

From Sigma: glycyl-L-valine, L-valine methyl ester HCl, D-threonine, L-isoleucine methyl ester HCl, D-isoleucine (allo-free), L- $\alpha$ -amino-n-butyric acid.

From Calbiochem: N-methyl-DL-isoleucine.

From Reef Laboratory: 4-thiaisoleucine  $\cdot$  HCl  $\cdot$  H<sub>2</sub>O, and 4-azaisoleucine di HCl.

The following compounds were gifts from several researchers; their generous donations are gratefully acknowledged. L-O-ethyl threonine was supplied by Dr. B.G. Christensen of Merck, Sharp and Dohme Research Laboratories. Dr. M. Rabinovitz of the National Institutes of Health, Bethesda, Md., made available DL-threo  $\alpha$ -amino- $\beta$ -chlorobutyric acid HCl. The fluoro-analogs of isoleucine and valine were generously supplied by Dr. H. S. Anker of the University of Chicago: 4,4,4-DL-trifluorovaline, hexafluoro-DL-valine, 4',4',4'-trifluoro-DL-isoleucine and 5,5,5-trifluoro-DL-isoleucine.

## 2.9. CHROMATOGRAPHIC MATERIALS

Cellex D (DEAE cellulose) and Bio-Gel HTP (hydroxylapatite) were purchased from Bio-Rad Laboratories. Sephadex G-25 (coarse) and Sephadex G-50 were obtained from Pharmacia Inc.

## 2.10. CHEMICALS

All inorganic and organic chemicals used in this work were reagent grade, and were supplied by Fisher Scientific. Ammonium sulfate (enzyme grade, 2 x crystallized) was obtained from N.B.Co.

## 2.11. MISCELLANEOUS

Cleland's reagent (dithiothreitol, DTT) was from Calbiochem. Ellman's reagent (5,5'-dithiobis-(2-nitrobenzoic acid), DTNB) came from Sigma. Phenol reagent (Folin-Ciocalteu) and toluene (spectranalyzed) were supplied by Fisher Scientific.  $\alpha$ -Naphthol was purchased from Eastman Organic Chemicals; this preparation was whitish and required no recrystallization, and gave a light amber solution when first dissolved in 2.5N NaOH. Creatine  $\cdot$  H<sub>2</sub>O (practical grade) was a product of Sigma.

### 3. METHODS

#### 3.1. ORGANISMS

##### 3.1.1. Fission yeast

###### 3.1.1.1. Strains

The wild type strains of the fission yeast, Schizosaccharomyces pombe, used in this study were obtained from two sources. Strain NRC 213002 was obtained through B.F. Johnson from the culture collection of the National Research Council of Canada. Strains 972 ( $h^-$ ) and 975 ( $h^+$ ) were supplied by A. Nasim, A.E.C.L., Chalk River Nuclear Laboratories.

###### 3.1.1.2. Media

Complete medium (YPG) for the growth of precultures consisted of yeast extract (1%), Bacto-peptone (0.5%) and D-glucose (2%). For plates and slants used in maintaining stock cultures, this medium was supplemented with 3% agar.

Minimal or defined medium (HM) was that of Halvorson (1958), as modified by Satyanarayana et al. (1968). The vitamin solution was that of Lucas et al. (1964) modified to contain 1 mg / l D-pantothenate (Ca salt) and 10 mg / l i-inositol, as prescribed for S. pombe by McVeigh and Bracken (1955). Other supplements were employed as indicated in the results. In experiments where repression was investigated (section 4.6), complete medium was HM supplemented with 1% yeast extract and 0.5% Bactopeptone.

### 3.1.1.3. Growth conditions

Cells of S. pombe were precultured in YPG to  $2-3 \times 10^8$  cells per ml, washed once with sterile distilled water and resuspended in water to the same cell density. Cell counts were made in an American Optical Bright-Line Hemocytometer, using a  $1/50$  dilution of the original preculture.

Cells to be used for the preparation of extracts were grown in batch cultures; 5 mls of preculture were innoculated aseptically into 500 mls of HM in triple-baffled 2-litre Erlenmeyer flasks (Bellco Glass Inc., Vineland, N.J.). The cells were incubated at 28-30 C on a New Brunswick gyratory shaker to mid-exponential phase. Growth was measured turbidimetrically in a Klett-Summerson colorimeter using a no. 66 (red) filter.

Cells used for growth curves were grown under similar conditions in 25 or 50 mls of HM in 300-ml sidearm flasks.

### 3.1.1.4. Preparation of extracts

The yeast cells were harvested on glass-fiber filter disks (11.0 cm, 934 AH; Reeve Angel, Clifton, N.J.) by suction filtration (Bussey and Umbarger, 1969). The cells were washed twice on the filter with distilled water, and then washed off the filter with a gentle stream of water into 250-ml Nalgene centrifuge bottles. A cell pellet was obtained after centrifuging 5 min at 10,000 rpm in a Sorvall RC-2B centrifuge. For the preparation of threonine deaminase, the pellet could be frozen and stored at -20 C for several weeks until

needed. In the case of all other enzymes studied, and for repression experiments, fresh cells were used.

The cells were broken in a Braun ISK mechanical cell homogenizer, as described by Kaplan et al. (1969). Details for the preparation of the various enzymes will be described in appropriate sections of the results.

### 3.1.2. Bakers' yeast

Strain D 237-10b of Saccharomyces cerevisiae, from F. Sherman's collection, was kindly supplied by C. Inera, N.R.C., Ottawa, Canada. It is a haploid wild type strain with no genetic markers except that it will not grow on maltose.

This strain was grown on the Halvorson minimal medium as described for S. pombe. The same methods were employed for harvesting the cells and for the preparation of extracts for enzyme assays.

### 3.1.3. Aerobacter aerogenes

#### 3.1.3.1. Strain

The strain of A. aerogenes used here was a standard hospital isolate supplied by Dr. R.V. Iyer of the University of Ottawa Medical School.

#### 3.1.3.2. Media

Precultures were grown in 0.8% Difco nutrient broth at 30 C.

The stock culture was maintained on slants prepared from nutrient broth supplemented with 3% agar.

Cells for the preparation of extracts were grown in the minimal medium of Davis and Mingioli (1950) modified by the omission of citrate (Halpern and Umbarger, 1959).

### 3.1.3.3. Growth of cells and harvest

A 10-ml inoculum of an overnight preculture of A. aerogenes in nutrient broth was added to 1 l of the minimal medium in 2.8 l Fernbach flask. The cells were grown at 30 C on a gyratory shaker for 15 hrs; the culture had reached a Klett reading of 320 (red 660 filter). They were harvested by centrifugation and washed once with distilled water. The cell paste so obtained was used immediately.

### 3.1.3.4. Preparation of acetolactate decarboxylase

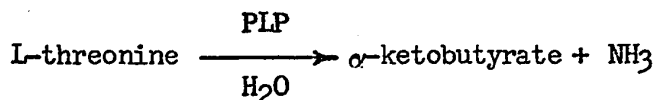
The wet cell paste was suspended in 10 mls 0.1 M sodium phosphate buffer, pH 6.5, containing 1 mM DTT and 1 mM Mg SO<sub>4</sub>. This slurry was then passed twice through a Wabash French pressure cell, and the extract obtained was centrifuged at 4 C for 20 min at 31,000 x g.

The crude extract was subjected to heat treatment at 60 C for 3 min; the heat denatured protein was removed by centrifugation and discarded. Such a heated preparation is still active in acetolactate decarboxylation but inactive in the decarboxylation of pyruvate (Juni, 1952a).

### 3.2. ENZYME ASSAYS

All enzyme assays except those for isomeroreductase and pyruvate decarboxylase were carried out at 30 C, the temperature at which the cells were grown.

#### 3.2.1. Threonine deaminase (TD)



Threonine deaminase activity was measured using the  $\alpha$ -keto acid determination procedure of Friedemann and Haugen (1943) as modified by Datta (1966). The reaction mixture contained (in 1.0 ml): 100  $\mu$ moles potassium phosphate, pH 8.0; 50  $\mu$ moles L-threonine, pH 8.0; 0.01  $\mu$ mole pyridoxal phosphate (PLP) and enzyme preparation. The reaction was started by the addition of enzyme to reaction mixture and stopped by the addition of 0.2 ml of a v/v mixture of 30% trichloroacetic acid (TCA) and 0.2% 2, 4-dinitrophenyl hydrazine (DNPH) in 2 N HCl. After 10 min further incubation at 30 C, 1 ml of 2.5 N NaOH was added. The absorbance of each solution was read, after a further 10 min, at 540 m $\mu$  in a Zeiss PMQ-II spectrophotometer using cuvettes with a light path of 0.5 cm. A reaction mixture which received the TCA-DNPH solution immediately prior to enzyme addition, or a reaction mixture without threonine, served as blank. The keto acid formed was quantitated from a standard curve prepared with authentic  $\alpha$ -ketobutyrate (fig. 3.2.1). Enzyme activity was expressed as  $\mu$ moles ketobutyrate formed per ml enzyme per minute; specific activity is activity per mg protein. Fig. 3.2.2 shows the

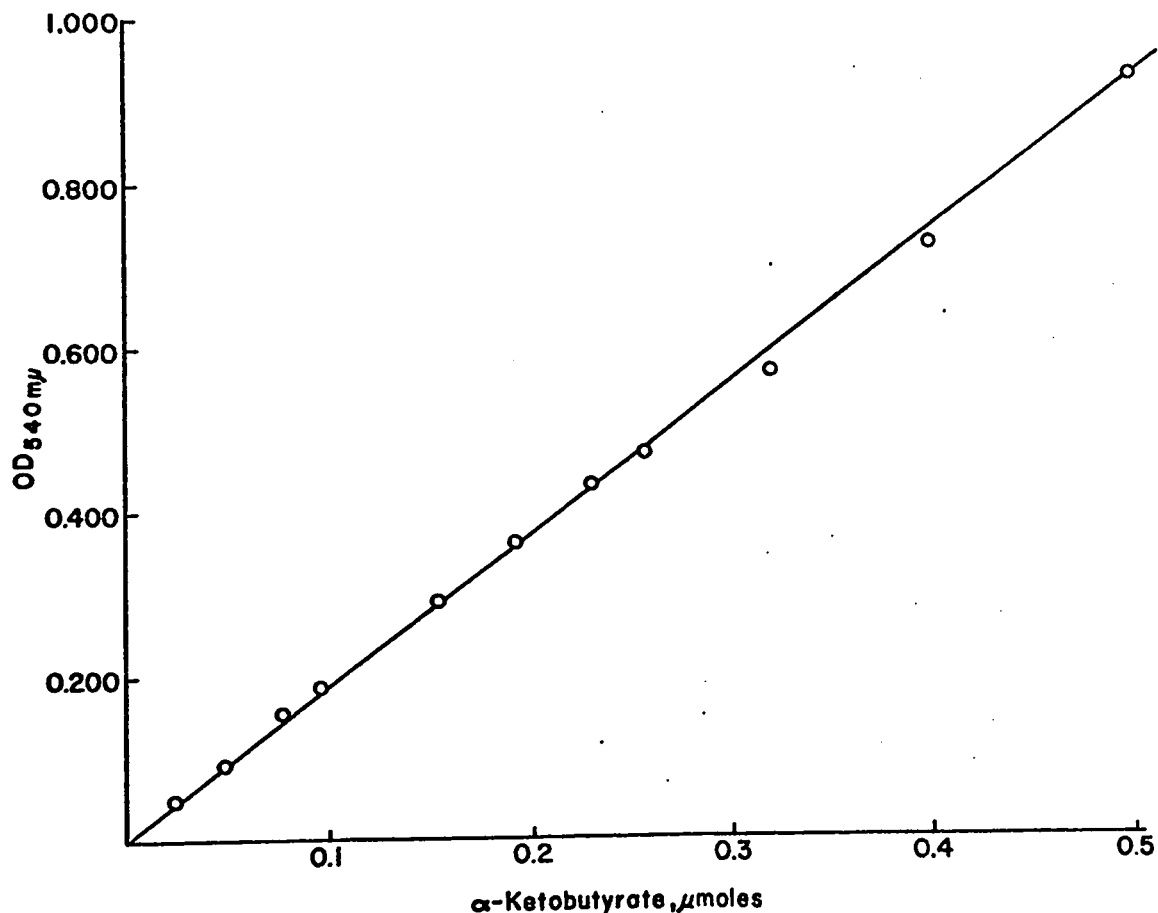


Fig. 3.2.1. Standard curve for  $\alpha$ -ketobutyrate determination. A series of standard reaction mixtures were prepared, containing the indicated concentrations of  $\alpha$ -ketobutyrate. Each sample received 0.2 ml TCA-DNPH solution, and after 10 min at 30 C, 1 ml 2.5 N NaOH. The absorbance of the solutions was read at 540 m $\mu$ , in cuvettes with a 0.5 cm light path.

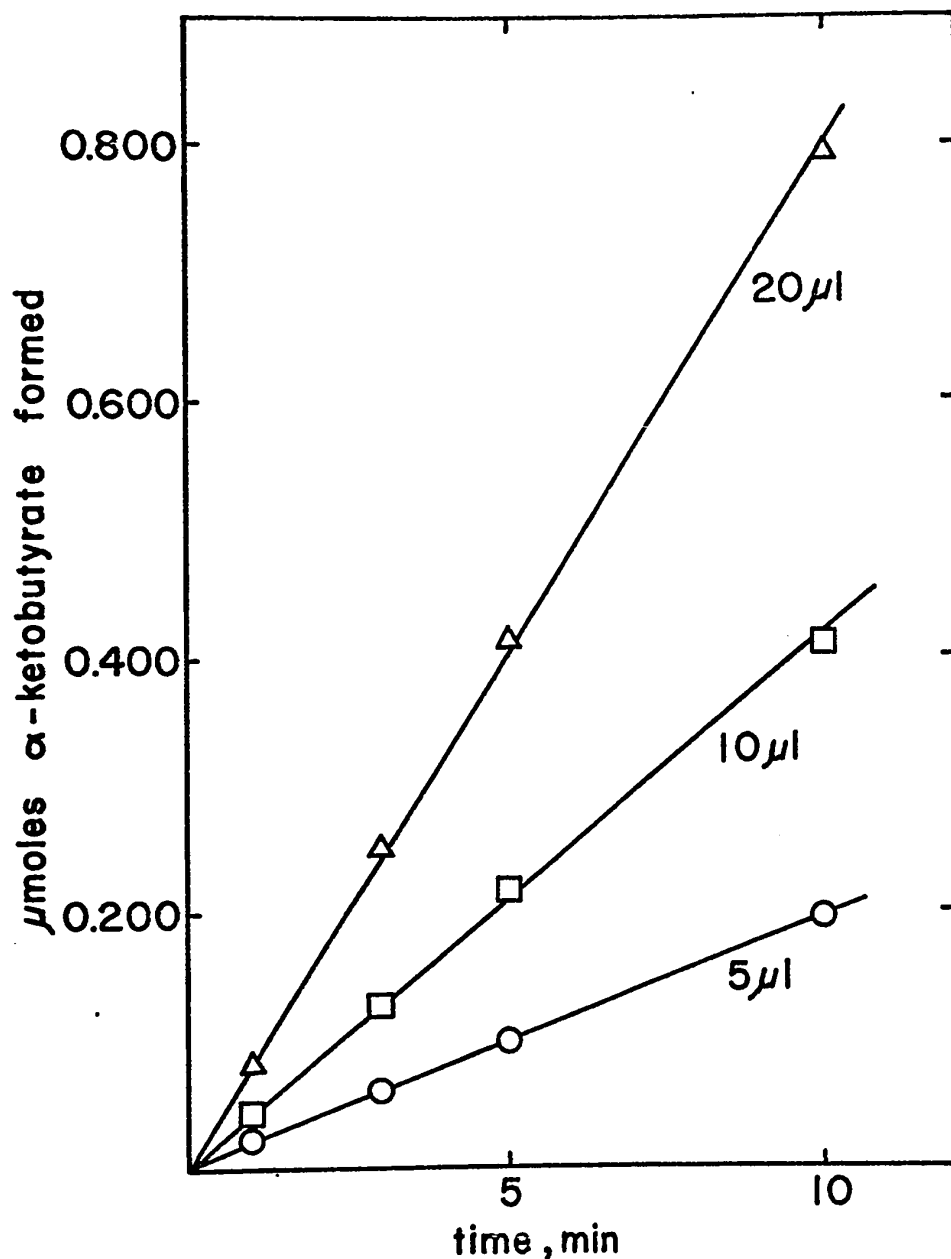
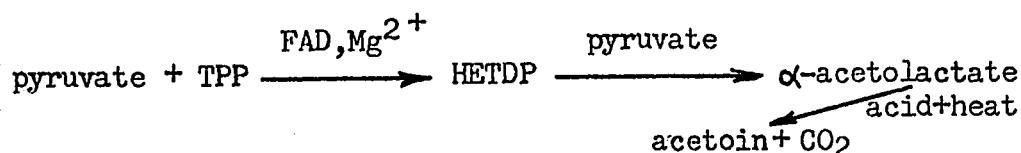


Fig. 3.2.2. Linearity of TD activity with incubation time of the enzyme in the standard reaction mixture. Enzyme was extracted in Veronal buffer, pH 8.0, containing 2 mM DTT and 0.5 mM EDTA. 5, 10 and 20  $\mu$ ls of extract were assayed at 30 C for the indicated times. Linearity of enzyme activity with enzyme concentration is shown by the slopes of the lines: 0.020, 0.042 and 0.081  $\mu$ moles KB/min for 5, 10 and 20  $\mu$ ls enzyme, respectively.

conditions under which TD activity in crude extracts of S. pombe was proportional to time and to enzyme concentration.

### 3.2.2. Acetohydroxy acid synthetase (AHAS)



The assay procedure for AHAS was modified from the differential method of Radhakrishnan and Snell (1960). The reaction mixture contained in a final volume of 1.0 ml: 500  $\mu$ moles potassium phosphate, pH 6.5; 500  $\mu$ moles sodium pyruvate (pH 6.5 with NaOH); 100  $\mu$ g flavin adenine dinucleotide (FAD); 5  $\mu$ moles Mg SO<sub>4</sub>; 100  $\mu$ g thiamine pyrophosphate (TPP) and the appropriate amount of enzyme. After incubation for 15-30 min, the reaction was stopped by the successive addition of 0.1 ml 1 M ZnSO<sub>4</sub> and 0.1 ml 2 N NaOH, and the volume in each tube then brought up to 2.0 mls with water. The white precipitate of Zn(OH)<sub>2</sub> and protein was removed by centrifugation at top speed in a desk-top centrifuge (International, Model HN), and the supernatant fluid was used for determination of acetoin and acetolactate (AL).

Acetoin was determined by the Westerfeld test (Westerfeld, 1945). Two 0.2 ml-aliquots were removed from each deproteinized reaction mixture. One aliquot was made up to 5.0 mls with water ("b" tubes). To the other was added 0.1 ml 5% TCA and the mixture was heated at 60 C for 10 min to chemically decarboxylate any acetolactate present to acetoin. The mixture was then neutralized with 0.4 ml 0.75 M NaHCO<sub>3</sub> and made up to 5.0 mls with water ("a" tubes). To both tubes were then added 1 ml 0.5% creatine and 1 ml 5%  $\alpha$ -naphthol dissolved in 2.5 N NaOH. The

colour was allowed to develop at room temperature for 1 hour, when the tubes were read at 540 m $\mu$  in the Zeiss PMQ-II spectrophotometer, in cuvettes with 1-cm light path. The absorbance of the colour read in "a" tubes represents that contributed by free acetoin in the reaction mixture plus that of acetoin derived from the decarboxylation of AL in acid. Tubes in which only base is present ("b" tubes) give a measure of free acetoin. Thus the difference between the readings for "a" tubes and "b" tubes is a measure of the amount of AL formed during the course of the reaction. Since the Westerfeld test is specific for acetoin, the differential assay affords a sensitive method to measure AHAS activity. Since only pyruvate is used as a substrate in the reaction, only aceto-lactate and acetoin are formed. Quantitation of AL was made from a standard curve constructed with authentic acetoin (fig. 3.2.3). Activity is expressed as  $\mu$ moles AL formed per ml enzyme per min. Specific activity is activity per mg protein.

Under these conditions, AHAS activity in crude extracts was proportional to time up to 45 min, and to enzyme concentrations up to 2 mg protein (50  $\mu$ ls) (fig. 3.2.4).

In later work, the reproducibility of the assay was improved by stopping the reactions with 0.1 ml 2.5N NaOH and bringing the volume to 2.0 mls with water. "B" aliquots were treated as above; "a" aliquots were acidified with 0.1 ml 6 N H<sub>2</sub> SO<sub>4</sub> and heated for 10 min at 60 C. The mixture was neutralized with 1.2 ml 0.75 M NaHCO<sub>3</sub> and brought to 5.0 mls with water. After addition of creatine and  $\alpha$  - naphthol, the tubes were heated at 60 C for 5 min. The colour develops completely in this time, instead of over a period of 1 hr as described above (Magee and de Robichon-Szulmajster, 1968a).

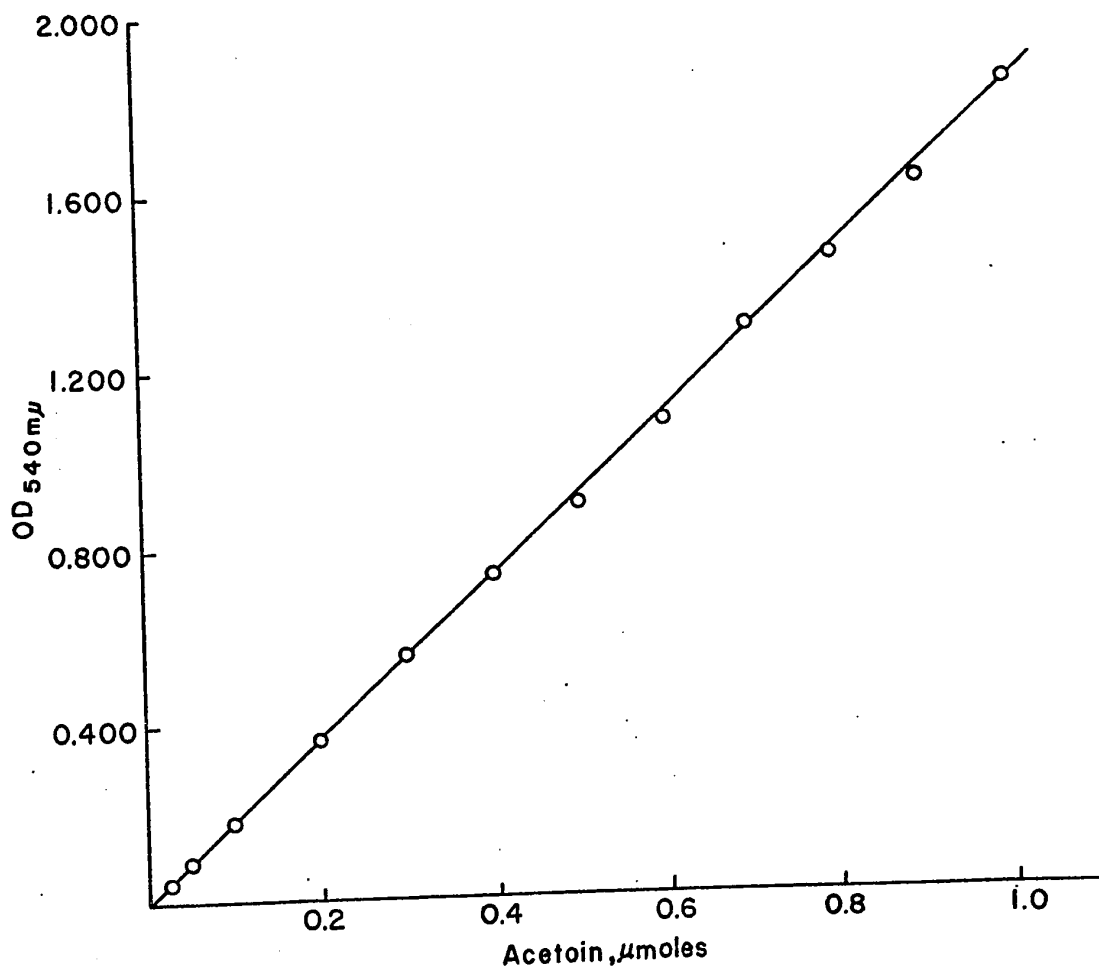


Fig. 3.2.3. Standard curve for quantitative determination of acetoin. To 5 mls of solutions containing the indicated concentrations of acetoin were added, successively, 1 ml 0.5% creatine and 1 ml 5%  $\alpha$ -naphthol in 2.5 N NaOH. The absorbance was measured at 540 mμ after the colour had developed for 1 hr at room temperature.

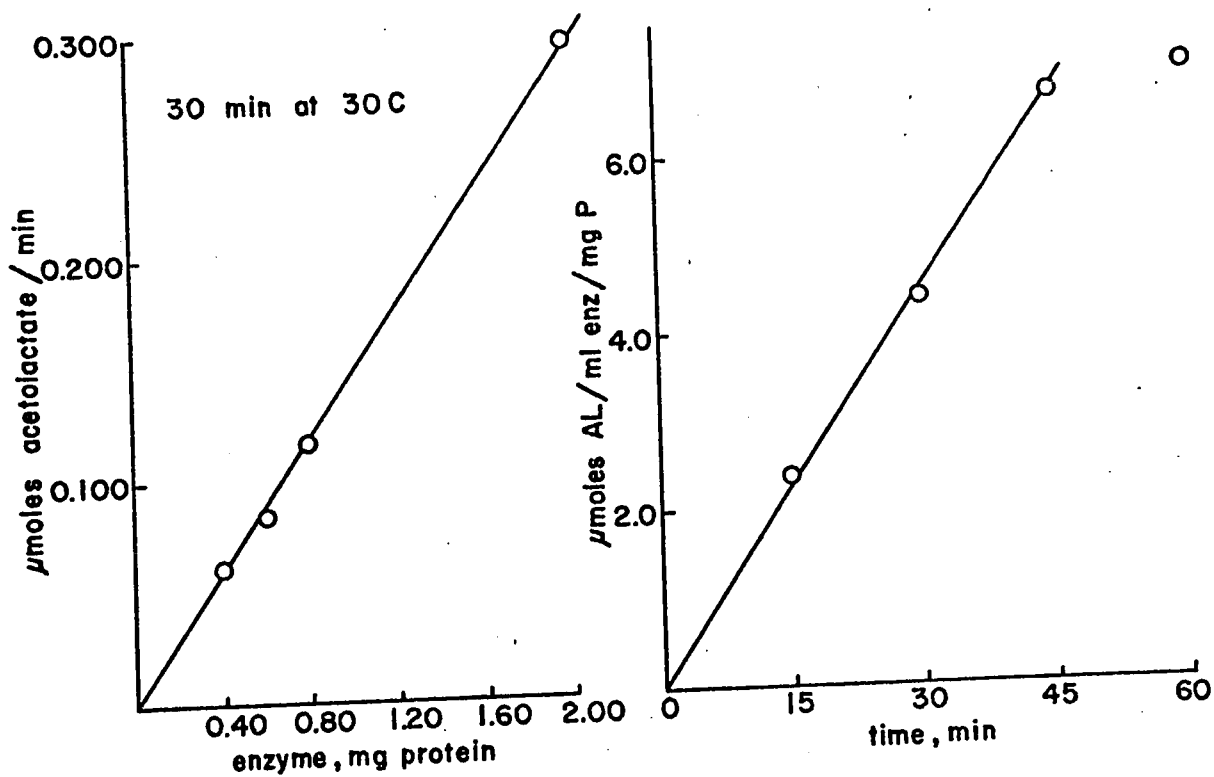
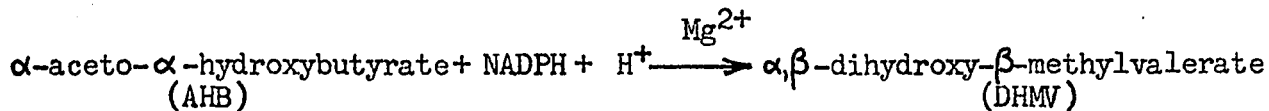
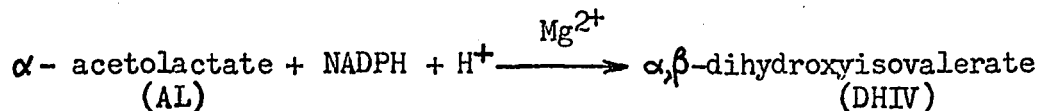


Fig. 3.2.4. Proportionality of AHAS activity with enzyme concentration and time. Cells were grown in HM with 4% glycerol as carbon and energy source; enzyme was extracted into 0.1 M potassium phosphate buffer containing 25% glycerol. Left: 10, 15, 20 and 50  $\mu$ ls of extract were assayed in the standard reaction mixture (section 3.2.2) for 30 min, and AL formed was determined as acetoin in the Westerfeld test. Right: 25  $\mu$ ls extract were incubated in the standard assay mix, at 30 C for 15, 30, 45 and 60 min; AL formed was determined as before.

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### 3.2.3. Isomeroreductase (IR)



The spectrophotometric assay described by Armstrong and Wagner (1961) was used. The decrease in absorbancy at 340 m $\mu$  was followed in a reaction mixture which contained, in a final volume of 1.0 ml: 100  $\mu$ moles potassium phosphate buffer, pH 7.6; 5  $\mu$ moles MgCl<sub>2</sub>; 10  $\mu$ moles AL or AHB; 0.2 mg NADPH (approx.  $2 \times 10^{-4}$ M) and enzyme. A cuvette containing all the above components was placed in the Zeiss PMQ-II spectrophotometer and the slit width was manually adjusted to give a reading of 0.500 OD unit at 340 m $\mu$ . Readings were taken at 30-sec intervals over a period of 2-5 min. A reaction mixture without AL or AHB was always run to correct for endogenous NADPH oxidase activity in the enzyme preparation. Enzyme activity was usually expressed as  $\Delta OD_{340}$  per min per ml enzyme; taking the molar extinction coefficient of NADPH at 340 m $\mu$  as 6.22 cm<sup>2</sup> per  $\mu$ mole, activity can be expressed as  $\mu$ moles NADPH oxidized per min per ml enzyme. Specific activity is activity per mg protein.

IR assays were also carried out in a Gilford Model 2400 recording spectrophotometer. The reaction was followed for 5 min, and the  $\Delta OD$  per min at 340 m $\mu$  was calculated from the chart recordings.

Fig.3.2.5 shows that IR activity in crude extracts was linear with enzyme concentration up to 2.2 mg protein (80  $\mu$ ls crude extract) for 5 min.

The substrates, AHB and AL, were obtained as their ethyl- $\alpha$ -acetoxy

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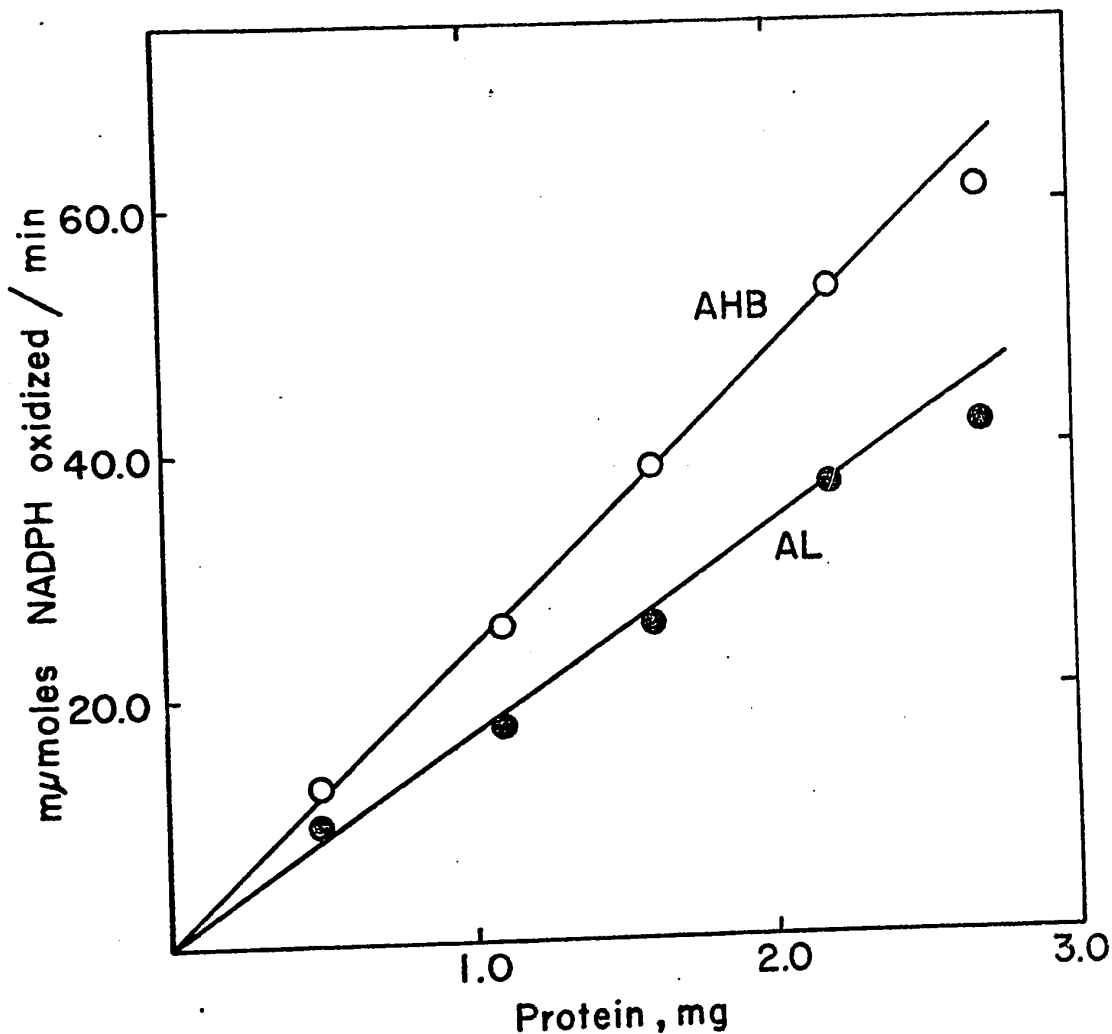
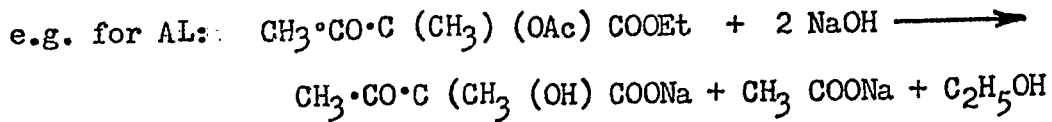


Fig. 3.2.5. Relationship of IR activity and enzyme concentration. 20, 40, 60, 80 and 100  $\mu$ ls of an extract prepared in 0.1 M potassium phosphate buffer, pH 7.5 (27.0 mg protein / ml), were assayed for IR activity, using the coupled assay described in section 3.2.3.

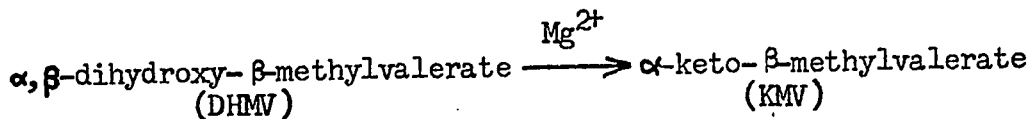
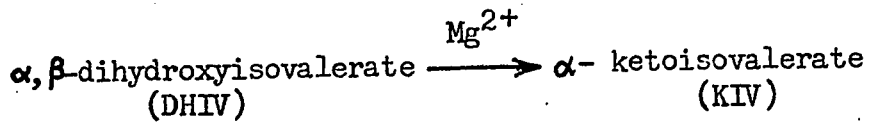
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derivatives. AHB and AL were prepared by saponification of these ethyl esters in the presence of the theoretical amount of NaOH (2 moles base per mole ester):



Saponification was carried out with constant stirring in the cold until only one phase could be seen. The solution was adjusted to about pH 7, and stored frozen.

#### 3.2.4. Dihydroxyacid dehydrase (DH)



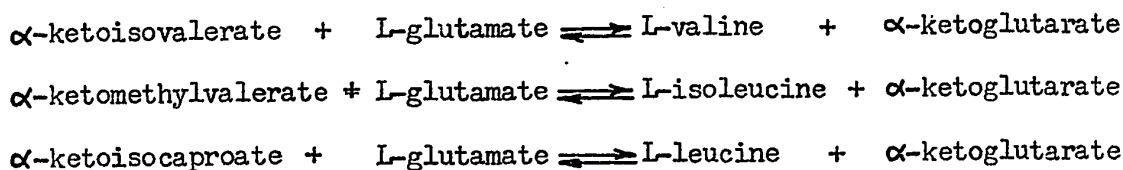
DH activity was estimated by quantitating the amount of  $\alpha$ -keto acid formed from the appropriate substrate by the method of Friedemann and Haugen (1943). The reaction mixture used was modified from that used by Satyanarayana and Radhakrishnan (1964), and contained: 200  $\mu$ moles potassium phosphate, pH 7.6; 10  $\mu$ moles DHIV or DHMV; 5  $\mu$ moles Mg Cl<sub>2</sub> and enzyme, all in a final volume of 1.0 ml. Incubation was at 30 C, and the reaction was stopped by adding 0.2 ml of a v/v solution of 30% TCA and 0.2% DNPH in 2 N HCl. The mixtures were incubated an additional 10 min and 1.0 ml 2.5 N NaOH was added to each tube. After a further 10 min, the tubes were centrifuged at top speed in a desk-top centrifuge to remove the fine black precipitate which forms, and the absorbancy of

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each solution was read at 540 m $\mu$  in the Zeiss PMQ-II. The specific activity of the enzyme was expressed as  $\mu$ moles keto acid formed per min per mg protein.

Standard curves were made using pure samples of KIV and KMV (fig. 3.2.6). DH activity in crude extracts was proportional with enzyme concentration up to 80  $\mu$ ls extract (about 2.5 mg protein) under these conditions; activity is linear with time for up to 20 min (with 50  $\mu$ ls crude extract) (fig. 3.2.7).

### 3.2.5. Transaminase B (TrB)



The assay for TrB employed the reverse reactions where the amino acid is deaminated to its respective  $\alpha$ -keto acid, which is then determined quantitatively by the method of Friedemann and Haugen (1943).

The assay mixture contained (in a final volume of 1.0 ml): 100  $\mu$ moles potassium phosphate, pH 8.0; 5  $\mu$  moles  $\alpha$ -ketoglutarate (neutralized with NaOH); 5  $\mu$ moles branched-chain amino acid (adjusted to pH 8 with NaOH); 0.1  $\mu$ mole PLP and 20  $\mu$ ls of G-25-filtered crude extract. The enzyme was preincubated at 30 C in the reaction mixture minus amino acid for 5 min; the reaction was begun by addition of amino acid. After 10 min, 0.2 ml of each reaction mixture was pipetted into 3 mls of DNPH (0.1% in 2 N HCl), according to the method of Coleman and Armstrong (1971).

After an additional 10 min, 6 mls of toluene were added to each

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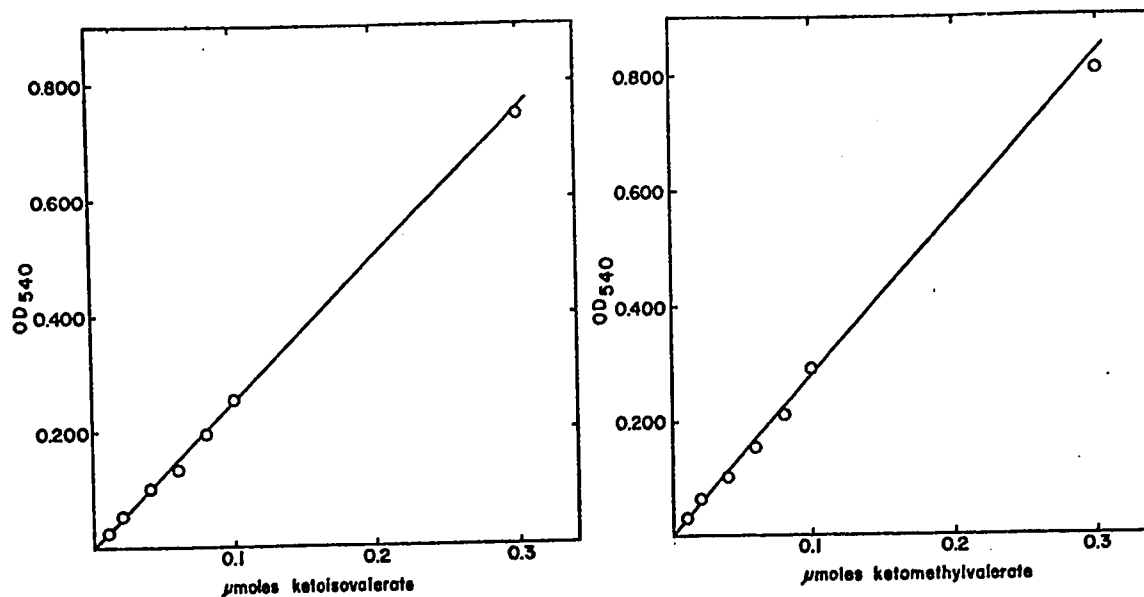


Fig. 3.2.6. Standard curves for DH assay procedure. Ketoisovalerate or ketomethylvalerate were added, in the concentrations given, to standard reaction mixture (section 3.2.4), and the keto acid was determined as its dinitrophenylhydrazone.

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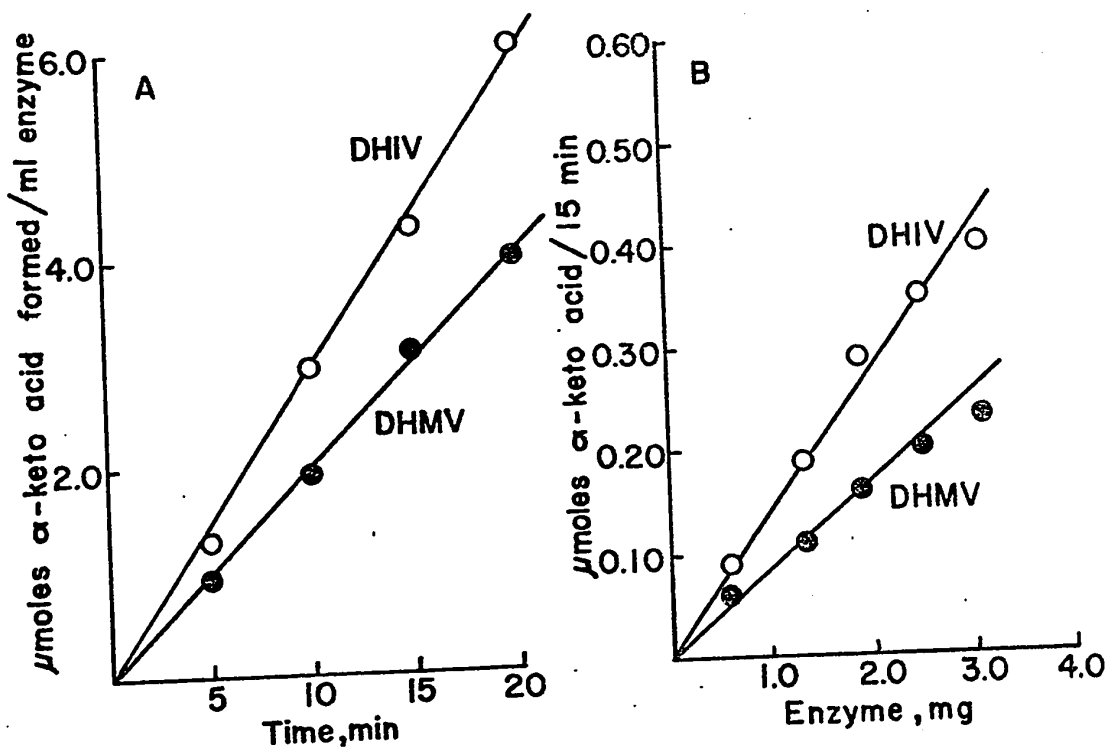
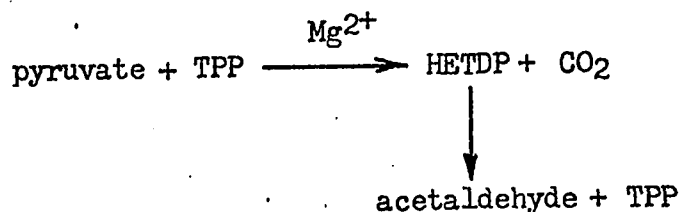


Fig. 3.2.7. Linearity of DH activity with time (A) and enzyme concentration (B). Enzyme was extracted in 0.1 M potassium phosphate buffer, pH 7.5; activity was assayed in the standard reaction mixture containing 200 μmoles / ml Tris- $\text{PO}_4$  buffer and 10 μmoles of either DHIV or DHMV. A: 50 μls extract were assayed for the times indicated. B: 20, 40, 60, 80 and 100 μls enzyme (0.24-1.00 mg protein) were assayed for 15 min. Keto acids formed were determined as described in section 3.2.4.

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tube; the hydrazones of the  $\alpha$ -keto acids were extracted into the toluene layer by vortexing for 10-15 sec on a Vortex Genie shaker. Four mls of the toluene layer were removed to clean tubes and 4 mls 10%  $\text{NaCO}_3$  were added to each tube. The mixtures were vortexed thoroughly for 10-15 sec to extract selectively the hydrazones of KMV (or KIV or KIC) leaving the hydrazone of  $\alpha$ -ketoglutarate behind in the toluene fraction. Three mls of the aqueous carbonate layer were removed to clean tubes and 0.5 ml 20% KOH was added to each. The absorbancy of the coloured solutions was read at 440 m $\mu$  in the Zeiss PMQ-II after 10min. The amount of keto acid formed was calculated from standard curves made with authentic KIV or KMV taken through the extraction steps just outlined (fig. 3.2.8). Specific activity was defined as  $\mu$ moles keto acid formed per min per mg protein, under the conditions described above.

### 3.2.6. Pyruvate decarboxylase (PD) (EC 4.1.1.1)



The assay of PD activity was based on the spectrophotometric method of Holzer et al. (1956), as modified by E. Juni and G.A. Heym (personal communication). The acetaldehyde formed in the reaction is reduced in a coupled reaction involving alcohol dehydrogenase (ADH) and NADH. The assay mixture contained in a final volume of 3.0 mls: 100  $\mu$ moles sodium phosphate, pH 6.5; 36  $\mu$ moles sodium pyruvate; 5  $\mu$ moles EDTA (essential to prevent rapid inactivation of ADH-this concentration does not chelate

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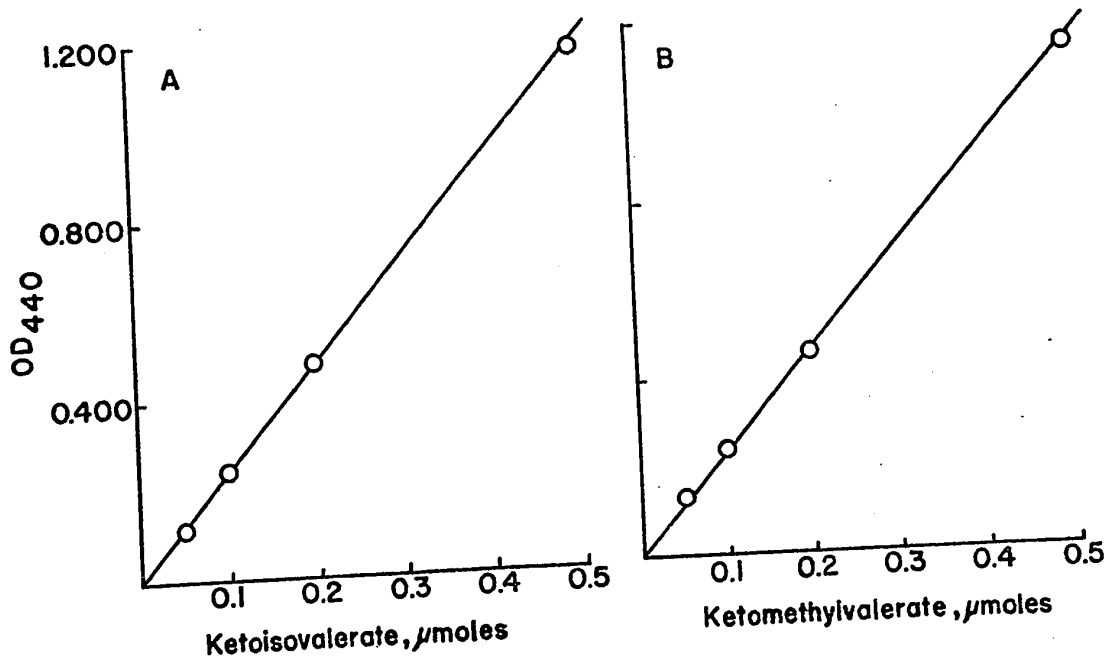


Fig. 3.2.8. Standard curves for ketoisovalerate and ketomethylvalerate determination in the assay for Transaminase B. The indicated concentrations of KIV and KMV were added to the standard reaction mixture, and their amounts estimated quantitatively by the extraction procedures outlined in section 3.2.5.

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the  $Zn^{2+}$  in the ADH); 10  $\mu$ moles  $Mg SO_4$ ; 0.3 mg TPP (approx.  $2 \times 10^{-4}M$ ); 0.05 ml of a 15 mg/ml solution NADH in 0.01 M  $NaPO_4$ , pH 7.5 (final concentration approx.  $3.5 \times 10^{-4}M$ ); 0.05 ml ADH (1.2 mg lyophilized enzyme / ml 0.1 M  $NaPO_4$ , pH 6.5, containing 5 mM EDTA); 0.1 ml diluted PD saturated with TPP and  $Mg^{2+}$ .

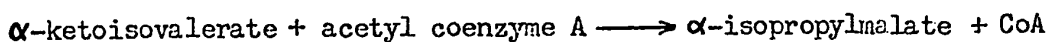
Immediately prior to assay, PD was diluted with 0.1 M sodium phosphate buffer, pH 6.5, containing 10  $\mu$ moles / ml  $Mg SO_4$  and 0.1 mg / ml TPP, and incubated at 30 C for 15 min to saturate the enzyme with TPP and  $Mg^{2+}$ . It was found that a  $1/50$  dilution of PD in crude extracts gave linear reaction rates for at least 5 min when 0.1 ml of such a dilution of PD was added to the reaction mixture.

The rate of NADH oxidation was followed at 366 m $\mu$  in a Gilford Model 2400 recording spectrophotometer, or in a Zeiss PMQ-II; in the latter case, the slit width was set manually at the beginning of the reaction to give an OD reading of 0.500 units, and readings were taken every 30 sec for 5 min. A blank from which pyruvate was omitted was always run to correct for endogenous NADH oxidase activity.

PD activity was defined as  $\Delta OD_{366}$  per ml enzyme per min; this value may be divided by 3.3 ( $E_{NADH}^{25 C} = 3.3 \times 10^6 \text{ cm}^2/\text{mole}$ ) to express activity as  $\mu$ moles NADH oxidized per ml enzyme per min. Specific activity is units of activity per mg protein, under the conditions employed. The reaction rate was linear with time up to 5 min, and with enzyme concentration up to 0.1 ml diluted PD preparation (see fig. 4.2.11).

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### 3.2.7. $\alpha$ -Isopropylmalate synthetase (IPMS)



The DTNB assay for IPMS, as described by Kohlhaw and Leary (1970) was used in this study. The procedure is based on the determination of free-SH groups (of coenzyme A) using Ellman's reagent (5, 5' - dithio (bis) -2-nitrobenzoate, DTNB). The reaction mixture contained the following, in a final volume of 0.25 ml: 50  $\mu$ moles Tris-HCl buffer, pH 8.0; 2  $\mu$ moles KCl; 0.2  $\mu$ mole acetyl CoA; 0.5  $\mu$ mole  $\alpha$ -ketoisovalerate (KIV), adjusted with NaOH to pH 8; and G-25-filtered crude extract. A blank without KIV was always run to correct for endogenous CoA generation. The reaction was started by addition of enzyme, and the mixture was incubated at 30 C for up to 10 min, when the reaction was stopped by addition of 0.75 ml ethanol. To each tube was then added 0.5 ml DTNB solution (1 mM in 20 mM tris buffer, pH 8.0; freshly-prepared), and all tubes were then centrifuged at top speed in a desk-top centrifuge for 5 min to remove precipitated proteins. The yellow colour of the solutions was read at 412 m $\mu$  in the Zeiss PMQ-II, using cuvettes with a 1.0-cm light path and 1.0 ml capacity.

The method was calibrated with a standard curve, using reduced glutathione as a source of SH groups. From fig. 3.2.9, it can be seen that colour formation by 0.01-0.10  $\mu$ mole glutathione is linear with optical density from 0-1.000 units. The slope of the line is 9.55 OD units /  $\mu$ mole glutathione.

The effects of time and protein concentration on the formation of  $\alpha$ -isopropylmalate by a G-25-filtered crude extract from S. pombe are shown in fig. 3.2.10. Enzyme activity is linear with time for up to 10

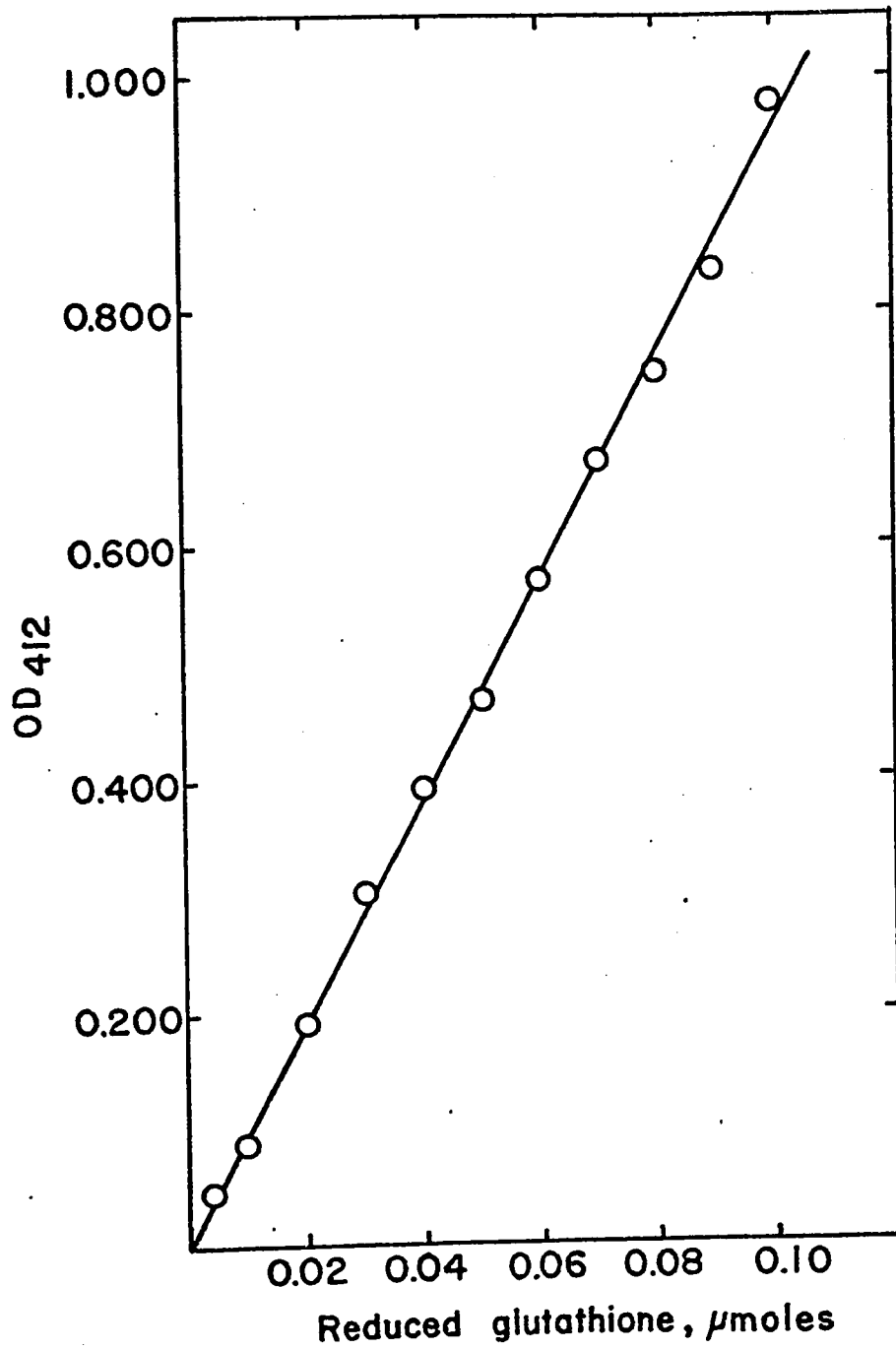


Fig. 3.2.9. Standard curve for quantitation of -SH groups by the DTNB method. The indicated amounts of reduced glutathione (neutralized with KOH) were added to standard reaction mixtures, and were determined chemically in the DTNB assay described in section 3.2.7.

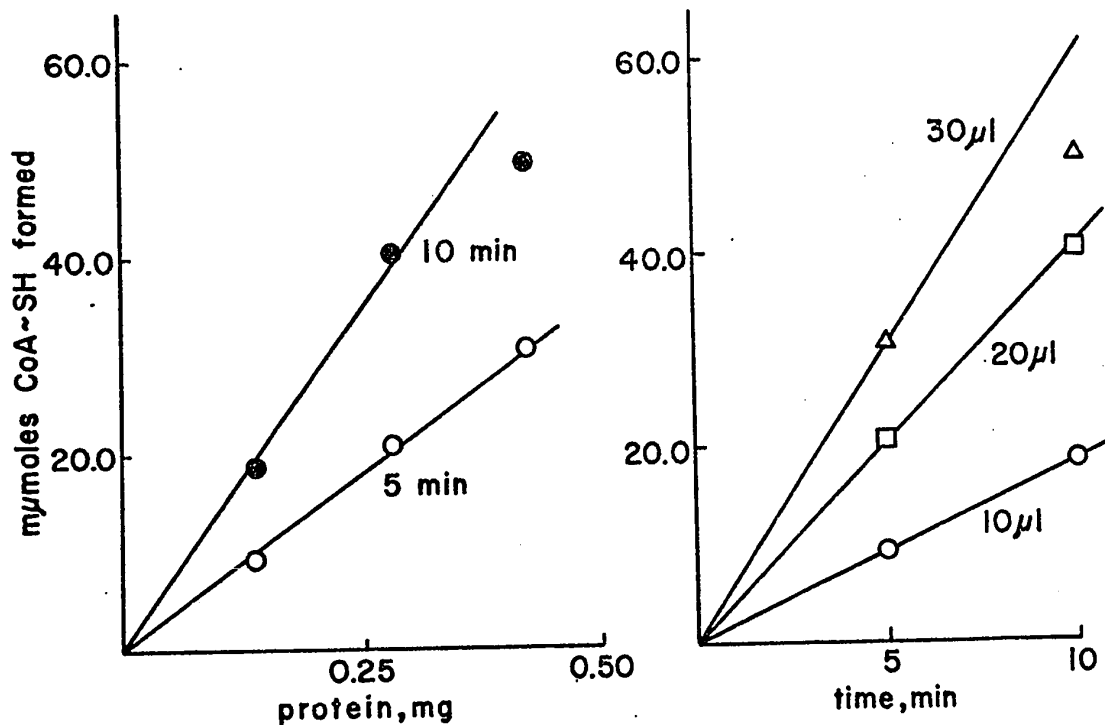
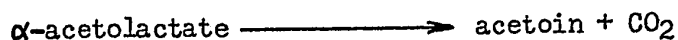


Fig. 3.2.10. Proportionality between IPMS activity and enzyme concentration (left), and time (right). The enzyme was extracted in 0.1 M Tris-HCl buffer, pH 8.0, containing 20% glycerol, 1 mM DTT and 0.1 mM L-leucine. One ml of the extract was filtered through Sephadex G-25, and the eluate used for assay. The reaction mixture described in section 3.2.7 was used. 10, 20 and 30 μls extract (0.12-0.42 mg protein) were assayed for 5 and 10 min at 30 C.

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min incubation with 10 and 20  $\mu$ ls of extract; it is linear with enzyme concentration to 0.42 mg protein (30  $\mu$ ls extract) for 5 min incubation periods, and to about 0.30 mg protein (20  $\mu$ ls extract) for 10 min incubations.

3.2.8. Acetolactate decarboxylase (AD) (EC 4.1.1.5)



AD activity was assayed in a reaction mixture containing (in 1.0 ml): 500  $\mu$ moles potassium phosphate, pH 6.5; 10  $\mu$ moles  $\alpha$ -acetolactate; 5  $\mu$ moles Mg SO<sub>4</sub>; 100  $\mu$ g thiamine pyrophosphate, and the appropriate amount of crude extract from S. pombe cells. The reaction was stopped by addition of 0.1 ml 2.5 N NaOH, and the mixture was made up to 2.0 mls with water. Acetoin was determined in a 0.2 ml aliquot of diluted reaction mixture as previously described (section 3.2.2).

Activity may be expressed as  $\mu$ moles acetoin formed per ml enzyme per min, using the standard curve prepared with acetoin (fig. 3.2.3). Specific activity is units of activity per mg protein.

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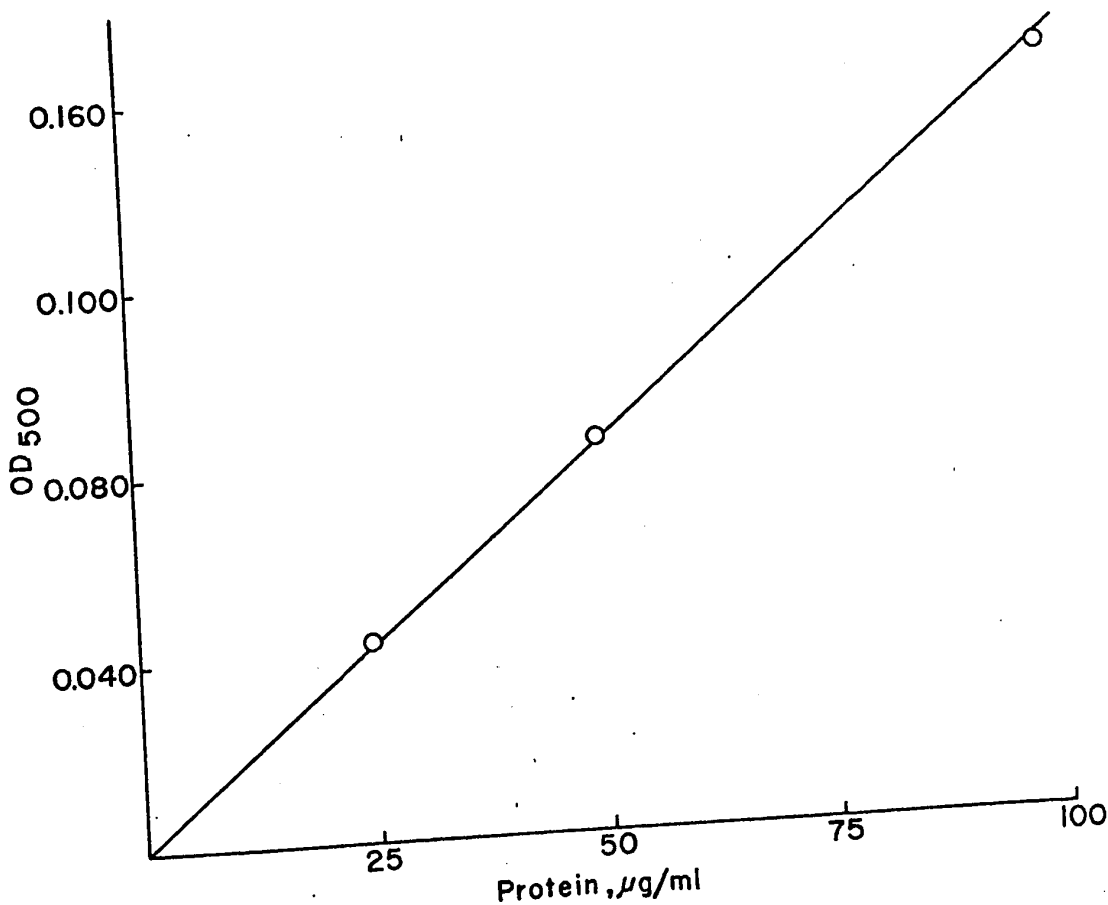


Fig. 3.3.1. Standard curve relating colour formation to protein concentration in the Lowry method. Solutions containing 25, 50 and 100  $\mu\text{gs} / \text{ml}$  bovine serum albumin were prepared, and the protein content was determined quantitatively with the Folin-Ciocalteu phenol reagent, as described in section 3.3.1. Each point is the average of at least sixteen separate determinations.

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#### 4. RESULTS

##### 4.1. THREONINE DEAMINASE

###### 4.1.1. Partial purification

###### a) Preparation of crude extract

Routinely, TD from cells of S. pombe was extracted with Veronal-HCl (barbiturate) buffer (0.03 M with respect to sodium barbiturate and potassium acetate, pH 8.0). The buffer was supplemented with  $8 \times 10^{-4}$ M L-isoleucine,  $5 \times 10^{-4}$ M EDTA and  $2 \times 10^{-3}$ M Cleland's reagent (dithiothreitol, DTT). L-Isoleucine is well known to stabilize TD activity (Changeux, 1961), and the chelating agent and sulfhydryl reagent were found to protect enzyme activity.

Three grams of fresh or freshly-thawed cell paste were suspended in 5 mls of extraction buffer and combined with 8 g glass beads (0.45-0.50 mm) in a glass homogenization bottle. The cells were broken in the Braun MSK mechanical cell homogenizer; the mixture was subjected to three 35-sec homogenizations with cooling by liquid CO<sub>2</sub>. The mixture was transferred to 50-ml Nalgene centrifuge tubes and centrifuged at 31,000 x g for 20 min in a Sorvall RC-2B at 0-5 C, to remove cell debris and unbroken cells. The clear amber-colored supernatant after smashing and removing debris by centrifugation was the crude extract (CE), and this preparation routinely contained about 23-26 mg protein / ml.

In some later experiments, 0.1M potassium phosphate buffer was employed. Pyridoxal phosphate (PLP) at  $5 \times 10^{-5}$ M was found to be an efficient stabilizer for TD activity, and was used routinely in cases where

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use of isoleucine was inadvisable. TD in crude extracts stored at -20 C remained active for several weeks.

b) Protamine sulfate treatment

A slight purification was obtained through precipitation of nucleic acids and some protein with protamine sulfate (PS). The PS treatment routinely gave supernatants which were much clearer, and which contained all the TD activity of the CE.

Solid PS to give a final concentration of 0.2% (w/v) was dissolved in a small volume of the buffer used to make the CE; this volume was 1/10 the volume of the CE. The PS solution was added dropwise to the CE which was continuously stirred at 4 C. Stirring was continued for 15 min after all the PS was added; the resultant mixture was thick and creamy-white. The solution was centrifuged in the cold (15 min at 16,000 rpm) and the clear supernatant was reserved for subsequent ammonium sulfate fractionation.

c) Ammonium sulfate fractionation

Salt fractionation was carried out according to the following procedure. The supernatant from the PS step was brought to 38% saturation by addition of solid ammonium sulfate; the amount of salt to be added was taken from the monogram of Dixon (1953). The  $(\text{NH}_4)_2 \text{SO}_4$  was added slowly (over a period of about 10-15 min) to the PS supernatant, which was stirred constantly in the cold. The solution was stirred constantly for 15 min after addition of salt, and then centrifuged. The supernatant fraction was then brought to 44% saturation with solid  $(\text{NH}_4)_2 \text{SO}_4$ , and the resultant precipitate was collected by centrifugation and dissolved

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in a small volume of 0.05 M potassium phosphate buffer, pH 8.0, containing DTT, isoleucine and EDTA (buffer-III). During all these steps, the pH of the enzyme preparations remained between 7.0 and 8.0. This concentrated solution of TD was dialyzed in the cold in a Sartorius colloid membrane filter assembly against three changes of buffer. The same result was achieved by filtering the ammonium sulfate fraction through a small column of Sephadex G-25 (coarse) equilibrated with the buffer in which the enzyme was dissolved; this technique, however, results in a two-to three-fold dilution of the enzyme without any loss of specific activity. Such a desalted preparation of TD could be stored at -20 C for at least a month without change in specific activity or regulatory properties.

In later experiments, only DTT and PLP were included in the buffers, along with 20% glycerol. Ammonium sulfate preparations made in this buffer and filtered through G-25 were equally stable when stored frozen. In addition, such a preparation is stable to at least 2 cycles of freezing and thawing, and no loss of activity or regulatory properties was observed when it was stored at 4 C for at least two weeks.

d) Chromatography on ion-exchange cellulose

Cellex D (diethyl aminoethyl (DEAE) cellulose, Bio-Rad Laboratories) was suspended with gentle stirring in 0.005 M potassium phosphate buffer, pH 8.0, and then allowed to settle. The supernatant containing "fines" was decanted and the cellulose resuspended and washed twice in more of the same buffer. The Cellex was then suspended in a volume of the same buffer to give approximately a 50% (v / v) slurry which was used to pour the column. All chromatographic procedures were performed at 4 C.

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A 1.5 x 30 cm column of DEAE cellulose was washed and equilibrated with 0.005 M potassium phosphate buffer, pH 8.0, containing isoleucine, DTT and EDTA. It was then charged with 2 mls of a dialyzed ammonium sulfate preparation of TD. Sixty mls of buffer were passed through the column at a flow rate of 12 mls / hr; 5-ml fractions were collected. A gradient of potassium chloride from 0-0.3 M was begun, and the bulk of the enzyme was eluted in fractions 29-33, representing a volume of 25 mls, containing 2.3 mg protein. The overall purification after these steps was about 87-fold with a yield of 25% (table 4.1.1.). The active fractions were pooled and  $(\text{NH}_4)_2 \text{SO}_4$  added to 55% saturation; the protein was so dilute in the pooled fractions, however, that the recovery of TD activity was poor.

No concentrated effort was made to purify this enzyme to homogeneity, as our interest lay mainly in the physiological and regulatory aspects of the entire pathway. Because the S. pombe enzyme is quite stable and is well-protected by PLP, it should be relatively easy to isolate it in pure form by employing one or two more column steps. Preliminary evidence has been obtained that hydroxylapatite (as Biogel HTP, Bio-Rad Laboratories) will adsorb TD from a dialyzed ammonium sulfate preparation. Phosphate buffer, 0.01 M, pH 8.0, containing DTT, EDTA and isoleucine was used. After the unadsorbed protein had been washed out in the void volume, the enzyme was eluted using a gradient of KCl from 0.05 M to 1.0 M; a purification of 3-4 fold over activity in the  $(\text{NH}_4)_2 \text{SO}_4$  fraction was achieved. Hydroxylapatite is the only substance to which the TD from bakers' yeast would adsorb (P.T. Magee, personal communication); good purification and yield were obtained.

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Table 4.1.1. Partial purification of TD from S. pombe

Fraction	Volume mls	Total protein mgs.	Total Activity umoles KB/min	Specific activity	Purif.	% recovery
Crude extract	37	814	115.4	0.142		100
PSN	34	595	105.1	0.177	1.2	91.1
(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> 38-44%	2	37.2	40.5	1.09	7.8	35.1
Dialyzed AS Fraction	2	32.8	35.4	1.08	7.8	30.7
DEAE cellulose	25	2.3	28.5	12.4	87.3	24.7

This adsorbent, however, has a very slow flow rate and tends to pack when pressure via a peristaltic pump is used to speed up the flow. Calcium phosphate gel as modified by Kosicki (1968)<sup>1</sup> to give Ca PO<sub>4</sub>-sucrose-cellulose would be worth trying, as it has a much higher flow rate.

As previously stated, our interest lay mainly in the physiology and regulation of the isoleucine-valine-leucine pathways. In many of the experiments dealing with the regulatory properties of the enzyme, crude extracts, in some cases filtered through Sephadex G-25, were used. No significant differences have been noted between the properties of the semi-purified TD and those of the enzyme in crude extracts.

1. This reference was inadvertently omitted in the preparation of the references; it is cited at the end of the bibliography.

4.1.2. Cofactor requirement of *S. pombe* threonine deaminase

Threonine deaminase from many microorganisms requires pyridoxal phosphate (PLP) for optimal activity (Wood, 1969). TD in crude extracts of bakers' yeast shows no requirement for PLP, but partially purified preparations do show a 50% increase in activity upon addition of 10 uM PLP (de Robichon-Szulmajster and Magee, 1968).

Fission yeast TD shows a stimulation by PLP even in crude extracts (Table 4.1.2); this stimulatory effect becomes more pronounced in more purified preparations, no doubt because endogenous PLP is being removed

Table 4.1.2. Effect of PLP on *S. pombe* TD reaction

Enzyme source	PLP, 20 uM	Specific activity	% activation
crude extract	-	0.125	154
	+	0.193	
(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> fraction (38-44%)	-	0.386	195
	+	0.751	
(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> fraction dialyzed 16 hrs	-	0.037	327
	+	0.121	

in the purification procedure. PLP was, therefore, included in the reaction mixture routinely in order to measure optimal activity of TD.

No attempts were made to measure the PLP-content of *S. pombe* TD as none of the preparations were pure enough to justify the effort.

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#### 4.1.3. Effect of pH on TD activity and isoleucine inhibition

Preliminary experiments revealed that L-isoleucine, as in other systems, is an effective inhibitor of the S. pombe TD, causing 50% inhibition at about  $10^{-4}M$ .

Fig. 4.1.1 shows the effect of pH on the activity of partially purified TD. Optimal activity of the S. pombe enzyme occurs at about pH 9.0, in contrast to pH 8.0 for S. cerevisiae TD (de Robichon-Szulmajster and Magee, 1968). As most bacterial TD's have pH optima in the range pH 9.0-9.5, the S. pombe enzyme more closely resembles the bacterial enzymes.

Inhibition by isoleucine is markedly affected by pH; the enzyme is most sensitive at pH 7.4, and inhibition decreases as the pH increases above this value. Because of our interests in the regulatory aspects of this enzyme, TD activity was routinely assayed at pH 8.0, where activity is strong and there is marked inhibition by isoleucine (69% at  $10^{-4}M$ ).

The results of a more detailed study of the effects of pH on isoleucine inhibition are presented in fig. 4.1.2. The inhibition curves are sigmoidal, an indication that subunit interactions are involved in the inhibition process at all 3 pH's tested. At pH 7.4, low concentrations of isoleucine ( $10^{-5} - 2 \times 10^{-5}M$ ) may cause a slight activation of TD, but the effect is negligible at pH 8.0 and 9.0. The enzyme can be almost totally inhibited at all pH's, but higher concentrations of isoleucine are required at higher pH's. The concentrations of isoleucine required to produce 50% inhibition (a rough approximation of  $K_i$ ) are presented in table 4.1.3.

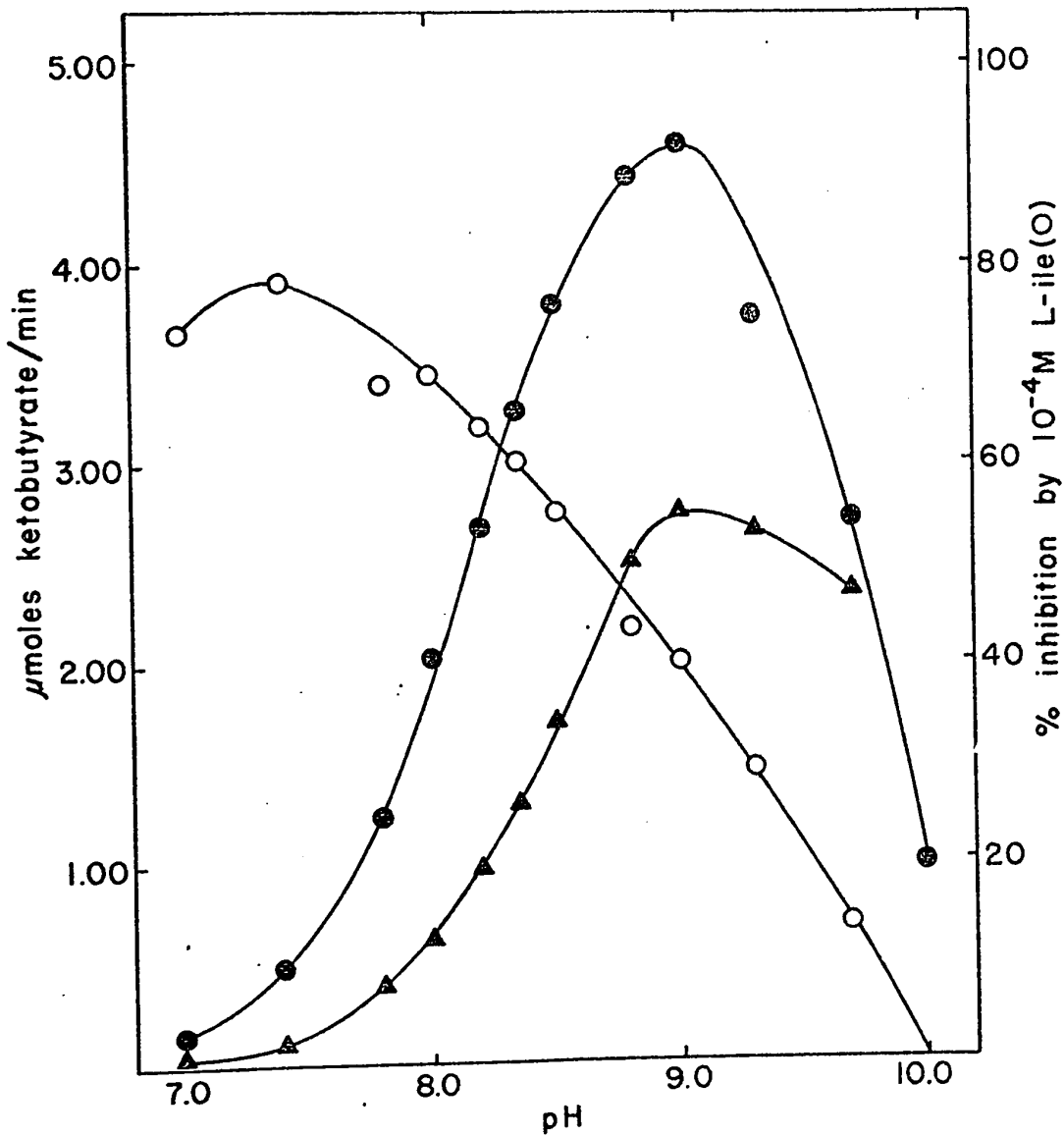


Fig. 4.1.1. pH dependence of threonine deaminase catalytic and regulatory properties. Enzyme (35-50%  $(\text{NH}_4)_2 \text{SO}_4$  fraction) was added to standard assay mixtures containing 100  $\mu\text{moles}$  Tris- $\text{PO}_4$  buffer at the pH values indicated. Feedback sensitivity was measured in the presence of 0.1 mM L-isoleucine. ●—●, enzyme activity with 50 mM L-threonine; ▲—▲, activity in presence of threonine + 0.1 mM L-isoleucine; ○—○, % isoleucine inhibition.

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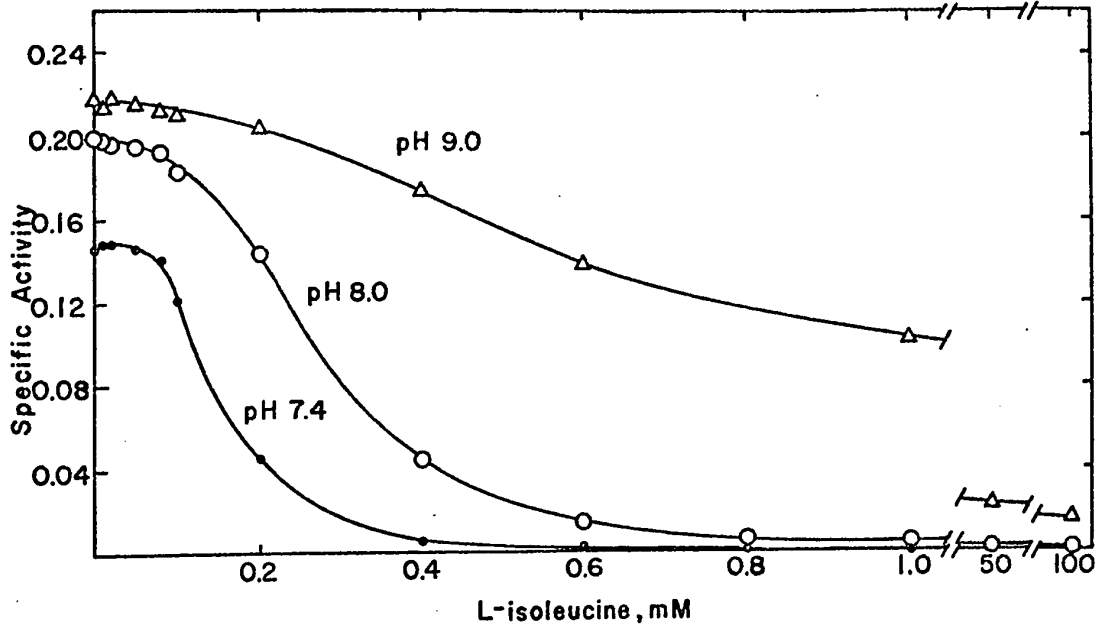


Fig. 4.1.2. Effect of increasing L-isoleucine concentration on TD activity as a function of pH. Crude extract (0.23 mg protein) assayed for 10 min in the standard assay mixture made with Tris- $\text{PO}_4$  buffers at the pH values indicated.

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Table 4.1.3. Effect of pH on apparent  $K_i$  for isoleucine

pH	apparent $K_i$
7.4	$1.5 \times 10^{-4}M$
8.0	$2.7 \times 10^{-4}M$
9.0	$1.1 \times 10^{-3}M$

It is evident that the apparent  $K_i$  increases as the pH increases, indicating that the affinity of the enzyme for isoleucine decreases with increasing pH.

4.1.4. Effect of purification on the feedback properties of TD

The effect of varying concentrations of isoleucine on TD in a crude extract and in a partially purified preparation are illustrated in fig. 4.1.3. The partially purified enzyme appears to be more sensitive to isoleucine, probably because many of the effectors present in the crude extract have been removed. Both activity curves are sigmoidal, suggestive of interactions between subunits of the enzyme. To check whether the homotropic interactions with the enzymes might have been modified by the purification steps employed, the data were replotted according to the empirical Hill equation (Atkinson et al., 1965), and the results are presented in fig. 4.1.4. The slope of the line so obtained, the Hill coefficient ( $n$ ), is an empirical estimate of the degree of interaction between subunits ( $n$  is a function of the number of interacting substrate-binding sites per enzyme molecule and of the strength of the interaction). This value is often used as a measure of the degree of cooperativity between ligands interacting with the enzyme. In the classical case where there

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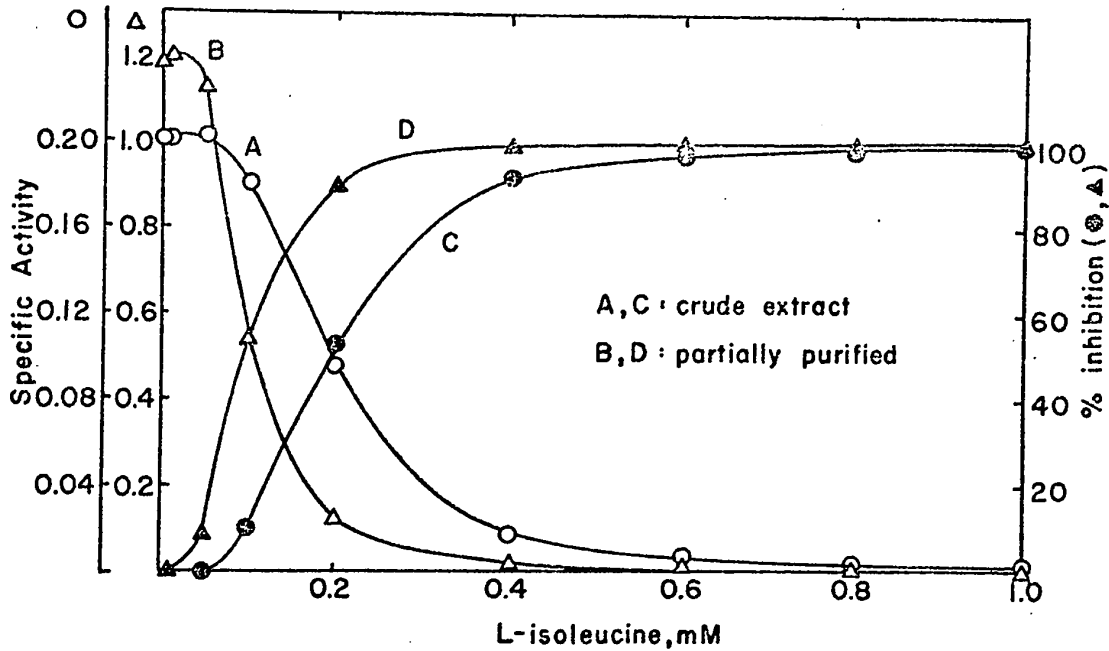


Fig. 4.1.3. Isoleucine sensitivity of TB in a crude extract and in a partially purified preparation. Enzyme activity was assayed as described in "Methods"; incubation time, 10 min. Curves A and C: specific activity and % inhibition in crude extract (0.22 mg protein); curves B and D: specific activity and % inhibition of 38-44%  $(\text{NH}_4)_2 \text{SO}_4$  preparation (0.08 mg protein).

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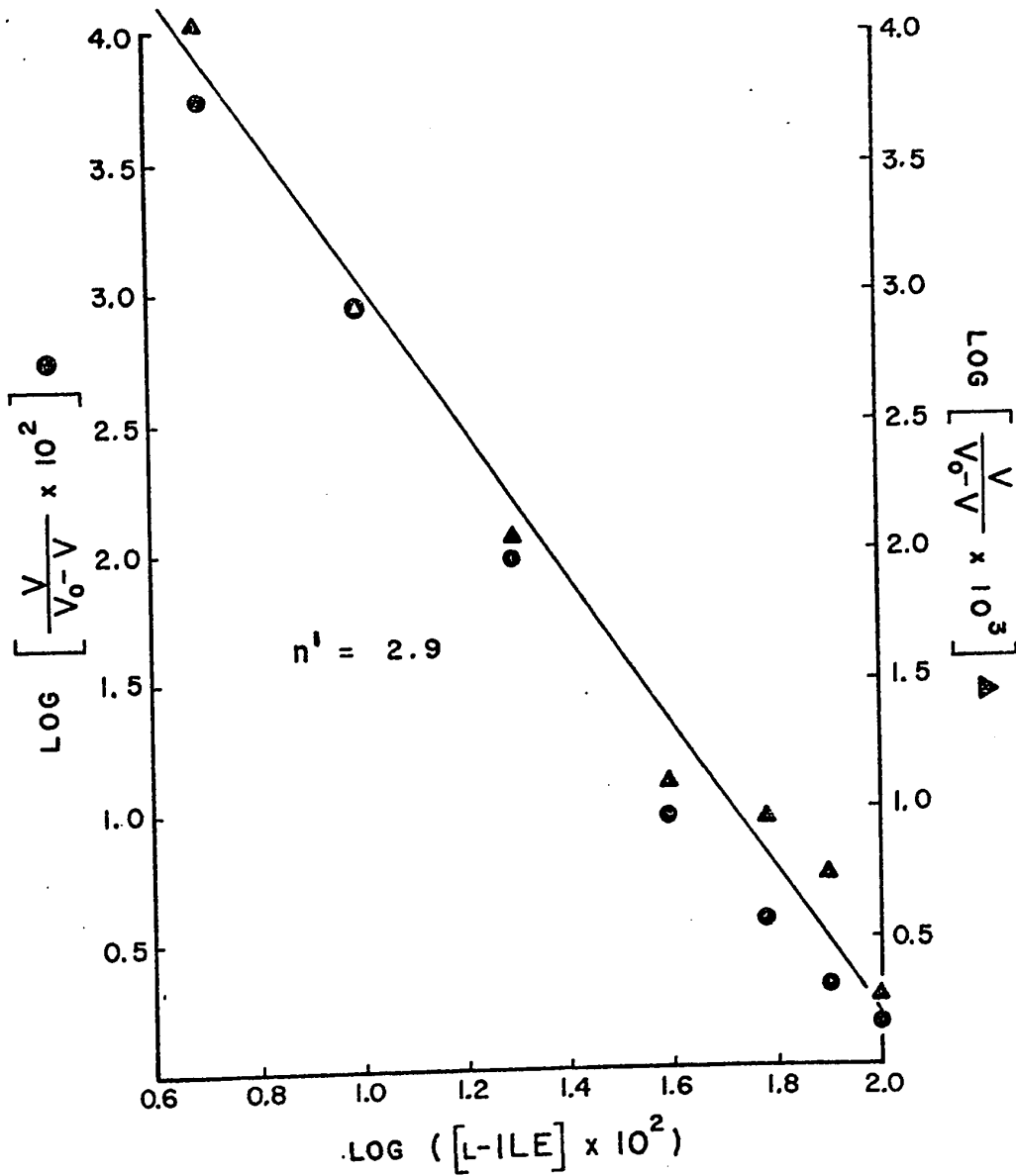


Fig. 4.1.4. Influence of L-isoleucine on TD activity as shown by the empirical Hill plot. The data were taken from fig. 4.1.3.

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are no subunit interactions, a value of  $n = 1$  is obtained from such a plot. The Hill value here ( $n^H = 2.9$ ) indicates that at least two binding sites (possibly more) for L-isoleucine exist per molecule of enzyme. The fact that a common line can be drawn to fit the data from both enzyme preparations is evidence that the regulatory properties of the enzyme have not been affected by the purification steps.

#### 4.1.5. Multiple effects of isoleucine

In S. cerevisiae, isoleucine is reported to have several effects on TD activity (de Robichon-Szulmajster and Magee, 1968). Concentrations lower than 20  $\mu\text{M}$  have no effect at any pH, while in the range 50-200  $\mu\text{M}$ , isoleucine stimulates TD activity, this stimulation being greater at higher pH (stimulation up to 125%). Concentrations of isoleucine greater than 200  $\mu\text{M}$  cause inhibition of enzyme activity.

The effects of isoleucine at low concentrations were investigated using a partially purified TD preparation from S. pombe. The results obtained are illustrated in fig. 4.1.5. It can be seen that, at pH 8.0, L-isoleucine has a very slight, if any, stimulatory effect on TD activity in the range 0.01-0.10 mM, when threonine is saturating. All concentrations of isoleucine greater than 0.10 mM cause inhibition of enzyme activity. The apparent  $K_i$  was estimated at 0.4 mM, in close agreement with the value in table 4.1.3.

In low threonine (20 mM), isoleucine is a much more effective inhibitor: the estimated  $K_i$  in this case is 0.07 mM (ca. 0.4 mM at saturating threonine); no stimulatory effect is evident, even though the curve is sigmoidal.

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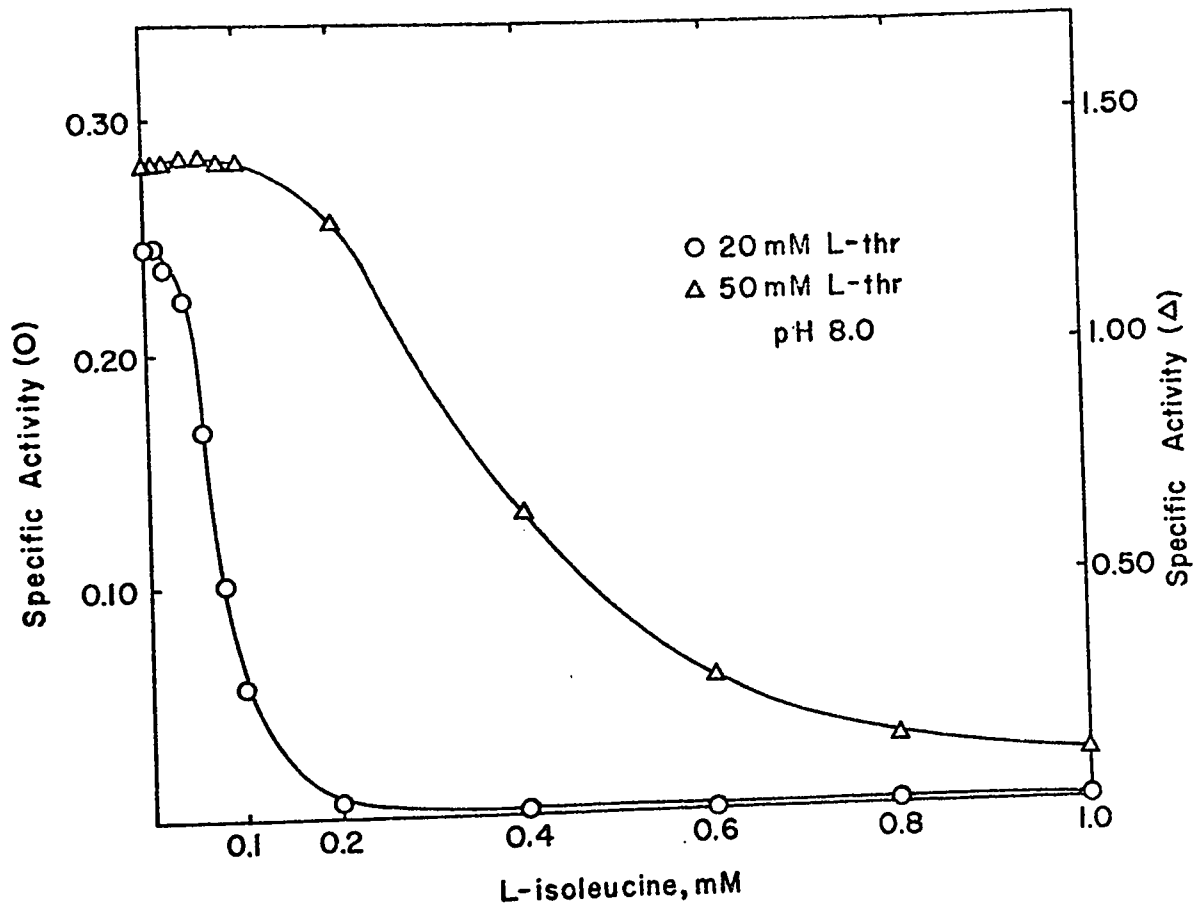


Fig. 4.1.5. Influence of L-isoleucine at low concentrations on TD activity as a function of substrate. TD was purified 10-fold by  $(\text{NH}_4)_2\text{SO}_4$  fractionation (38-44%) and Sephadex G-25 filtration; the enzyme was stabilized with PIP in the buffers instead of isoleucine. Activity was assayed (30  $\mu\text{g}$  protein for 10 min) in standard reaction mixture supplemented with L-isoleucine at the indicated concentrations; threonine concentrations were 50 mM ( $\Delta-\Delta$ ), and 20 mM ( $\text{O}-\text{O}$ ).

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#### 4.1.6. Effects of analogs of isoleucine on TD activity

The ability of analogs of isoleucine to inhibit TD activity was tested to investigate the specificity of isoleucine inhibition. Some of these analogs were tested to find one which could inhibit both TD activity and the growth of wild-type S. pombe, in the hope that resistant mutants could then be isolated; such mutants might have TD with altered feedback sites (see 4.1.18).

TD in an  $(\text{NH}_4)_2 \text{SO}_4$  fraction passed through Sephadex G-25 was used as a source of enzyme in these experiments. With substrate present at 50 mM, each analog was tested at a concentration of 1.0 mM with respect to the L-isomer. The inhibition caused by each analog was compared with the inhibition by 1.0 mM L-isoleucine, and the results are presented in table 4.1.4. The D-isomer of isoleucine was only 2.5% as effective as the natural L-isomer. Trifluoroisoleucine (fluorine substituted for hydrogen at carbon 5) caused only 18.3% inhibition, indicating that the increased electron density (and charge density) about carbon 5 greatly decreases the inhibitory effectiveness of isoleucine. TFI with fluorine at carbon 4 gave similar results.

A free amino group on isoleucine is essential for inhibition, as N-methyl-isoleucine caused only about 18% inhibition of TD activity. The relative positions of the amino group and the carboxyl group of isoleucine are critical for the binding site to recognize isoleucine: glycyl-L-isoleucine was only 6% as effective as L-isoleucine.

$\alpha$ -Carboxyl-substituted analogs (chloroacetyl-DL-isoleucine and isoleucine methyl ester) caused less than 6% inhibition of TD activity, evidence that free  $\alpha$ -carboxyl groups are required for inhibitory activity at the isoleucine site.

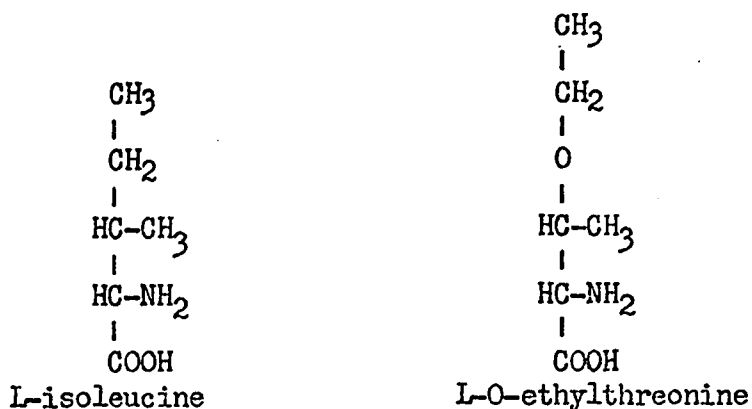
Table 4.1.4. Inhibition of TD activity by analogs of isoleucine

Compound tested 1.0 mM re L-form	% inhibition
L-isoleucine	97.0
D-isoleucine	2.4
5', 5', 5'-trifluoro-DL-isoleucine (TFI)	18.3
N-methyl-DL-isoleucine (NMI)	17.8
glycyl-L-isoleucine (GI)	5.9
chloroacetyl-DL-isoleucine (CAI)	4.1
L-isoleucine methyl ester (IME)	5.9
DL-thiaisleucine (TIL)	13.6
L-O-ethylthreonine (OET)	15.5

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Thiaisoleucine (2-amino-3-methylthiobutyric acid), an analog in which a sulfur is introduced in place of the methylene group at the 4-position of isoleucine (McCord et al., 1965), permitted Betz et al. (1971) to isolate mutants of bakers' yeast which had TD's with altered sensitivity to isoleucine. 5mM TIL increased the generation time of wild-type S. cerevisiae from 5.2 hrs to 14.9 hrs, while 50 mM TIL allowed no growth; no mention is made of the effect of TIL on TD activity (Betz et al., 1971). In S. pombe, TIL at 1.0 mM caused only 14% inhibition of TD activity. In E. coli, TIL inhibits growth completely at 5 mM, and was shown to compete with isoleucine for binding to TD, TrB and isoleucyl tRNA synthetase (Szentirmai and Umbarger, 1968).

L-O-ethylthreonine (OET) is reported to be an antagonist of L-isoleucine (Shigeura et al., 1969), in the sense that it suppressed incorporation of <sup>14</sup>C-isoleucine into isoleucyl-tRNA, and isoleucine competitively reversed this effect of OET. It is also incorporated into Sarcoma cell protein. In E. coli, isoleucine was the most effective at reversing growth inhibition by OET; no effect on TD activity is reported. In S. pombe, OET caused only slight inhibition of growth, and only 16% inhibition of TD activity. This compound has a longer "backbone" than



isoleucine because of the oxygen interposed between carbons 3 and 4; the molecule may thus be too long to "fit" properly at the isoleucine binding site.

#### 4.1.7. Regulatory role of valine

The isoleucine inhibition of TD can be relieved by valine in bacteria (Umbarger, 1969) and in yeast (Cennamo et al., 1964; de Robichon-Szulmajster and Magee, 1968). That such is the case for the S. pombe enzyme is demonstrated in fig. 4.1.6A. A sigmoidal curve was obtained, and valine, at a concentration of 10 mM, was found to relieve isoleucine inhibition of activity up to about 90%. The concentration of valine required to effect 50% reversal of 98% inhibition by isoleucine is about 3 mM; for bakers' yeast, this value is 4 mM (de Robichon-Szulmajster and Magee, 1968). The slope of the line obtained when these data were converted to the Hill plot was  $n = 1.8$ , indicating homotropic interactions of valine, and at least two sites on the enzyme molecule for L-valine (fig. 4.1.6B).

Fig. 4.1.7 illustrates the effect of pH on the ability of valine to reverse isoleucine inhibition in the presence of two substrate levels. Under all these conditions, 10 mM valine could reverse isoleucine inhibition 90-100%; at the low substrate concentration (5 mM), valine was effective only at concentrations greater than 3 mM.

Table 4.1.5 summarizes these results by giving the molar ratio valine / isoleucine for 50% reversal of isoleucine inhibition at various pH and threonine concentrations.

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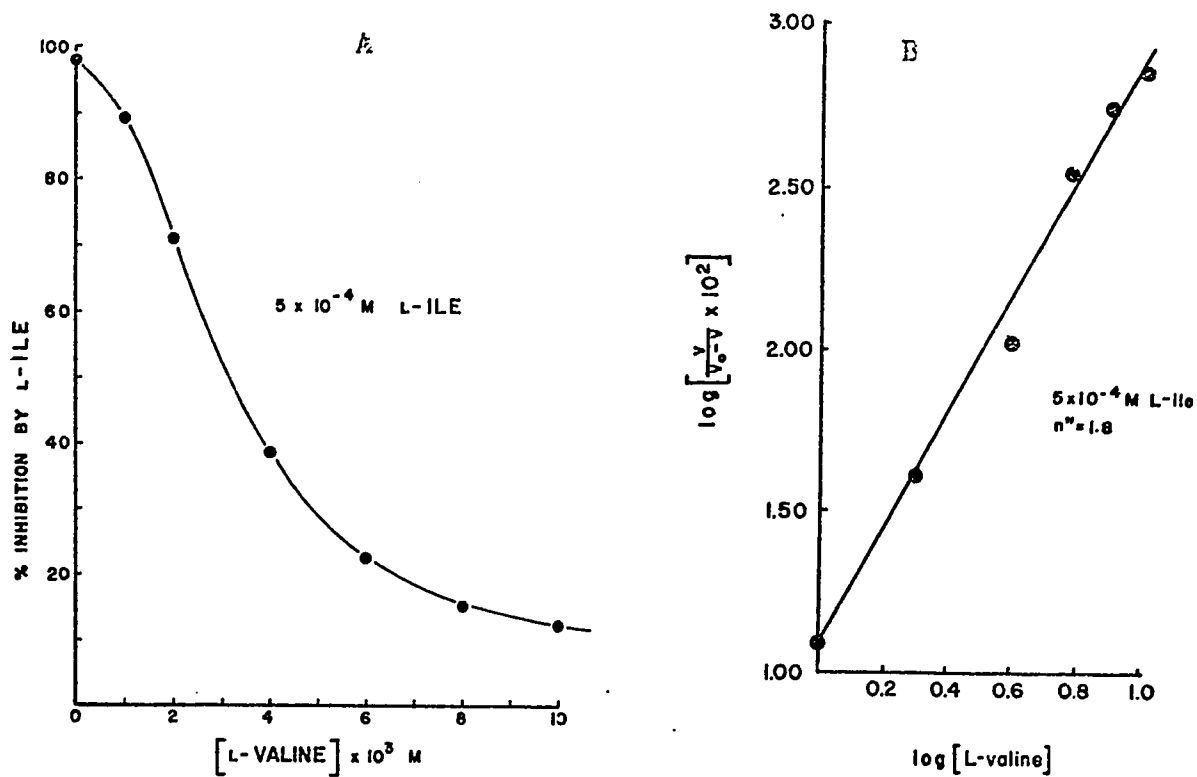


Fig. 4.1.6. Reversal of L-isoleucine inhibition of TD activity by L-valine. Crude extract (10  $\mu$ l) was assayed in standard reaction mixture supplemented with 0.5 mM L-isoleucine and L-valine at the indicated concentrations; incubation time was 10 min. A: % inhibition by isoleucine at each valine concentration. B: Hill plot derived from data in A.

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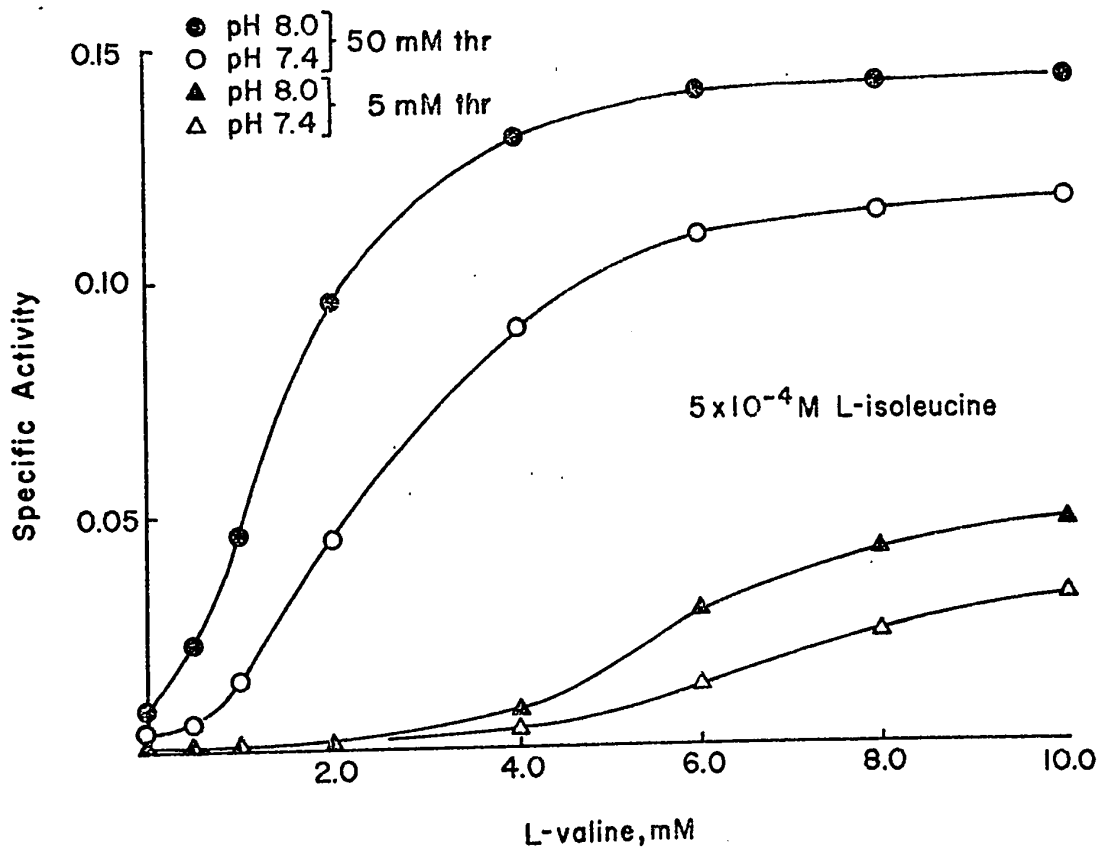


Fig. 4.1.7. Influence of pH on reversal of L-isoleucine inhibition by L-valine. Crude extract (0.34 mg protein) assayed at pH 7.4 and 8.0 for 10 min. Standard assay mixtures containing 50  $\mu$ moles / ml L-threonine (○, ●), or 5  $\mu$ moles / ml L-threonine (△, ▲), 0.5 mM L-isoleucine and L-valine, as indicated.

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Table 4.1.5. Molar ratio  $\frac{\text{valine}}{\text{isoleucine}}$  for 50% reversal of inhibition by  $5 \times 10^{-4}M$  isoleucine

threonine, mM	molar ratio valine/isoleucine	
	pH 7.4	pH 8.0
5	13.0	11.5
50	5.2	3.0

4.1.8. Role of valine as a positive effector

Valine has often been reported to activate the biosynthetic TD (Freundlich and Umbarger, 1963). In the S. pombe system, valine was found to exert positive heterotropic effects only at very low concentrations of threonine and at low pH values (fig. 4.1.8, p.84-85). At pH 7.0 and 7.4, the substrate curves with threonine only are sigmoidal, indicating cooperative homotropic effects of threonine on the activity of the enzyme. Valine at 10 mM normalizes these curves to rectangular hyperbolas. TD functions optimally at threonine concentrations greater than 35 mM at pH 7.0, and 20 mM at pH 7.4. Reciprocal plots from these data (insets) show apparent competitive antagonism between threonine and valine. At pH 8.0, valine stimulates TD activity at threonine concentrations in the range 0.5-10 mM; at pH 9.0, there is very little activation in the range 0.5-4.0 mM threonine, and valine inhibits activity at higher threonine concentrations. Cooperative effects of threonine on enzyme activity are slight at pH 8.0, and at pH 9.0 are no longer evident. In these and other experiments, valine (at concentrations as high as 50 mM) caused no more than 4-10% inhibition of TD activity. Table 4.1.6

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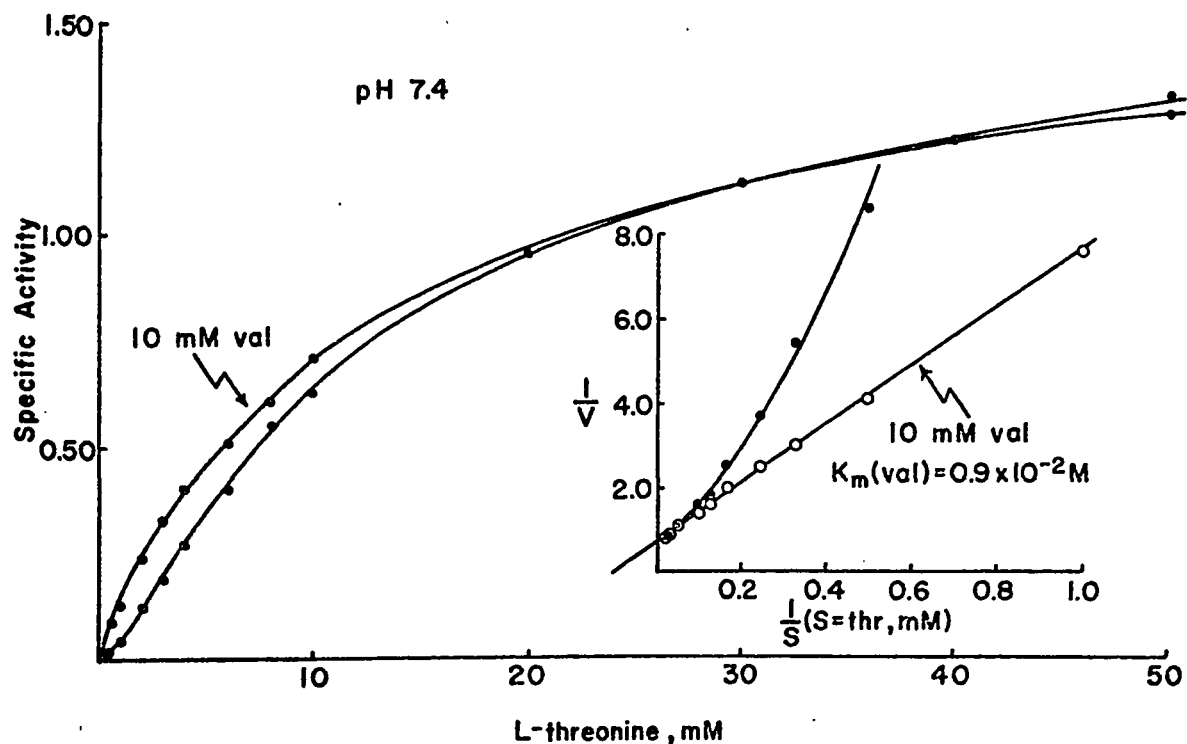
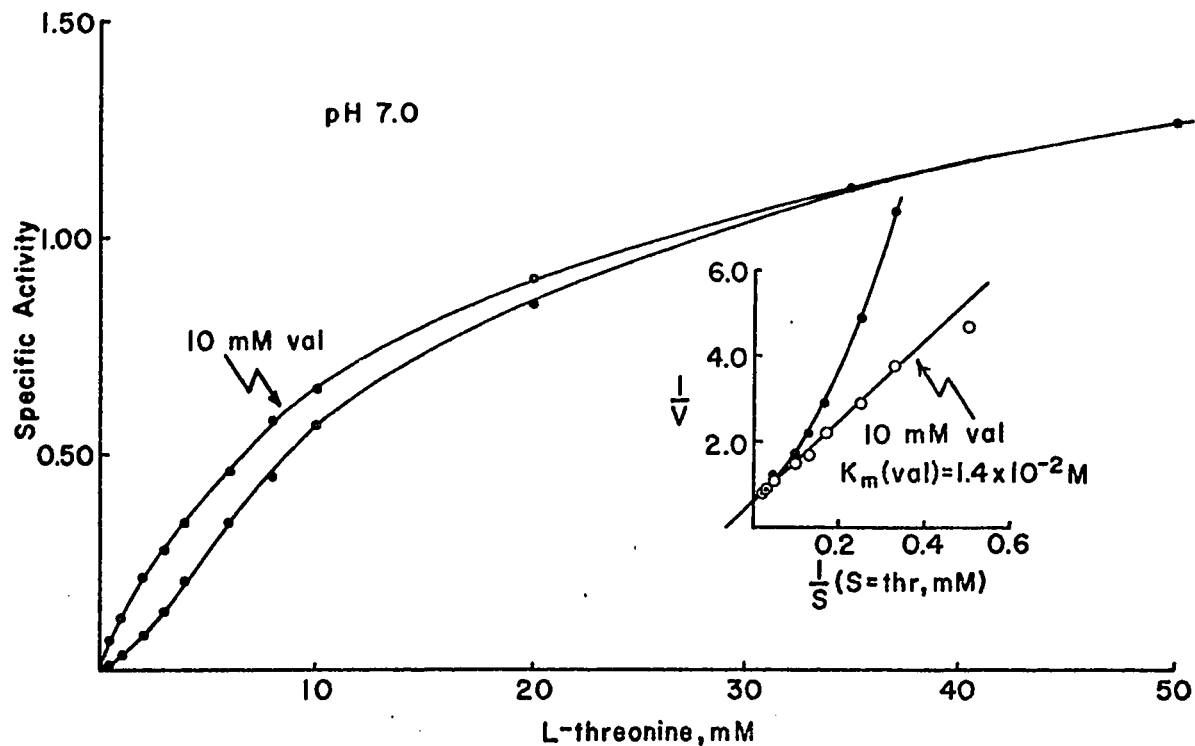


Fig. 4.1.8. The effect of pH on the activation of TD by L-valine. 30  $\mu\text{g}$  of enzyme (10  $\mu\text{l}$  of  $(\text{NH}_4)_2 \text{SO}_4$  fraction filtered through Sephadex G-25) were assayed at the four pH values indicated in increasing concentrations of L-threonine + 10 mM L-valine. Insets show Lineweaver-Burk transformations of the same data. Figure 4.1.8. continued next page (85).

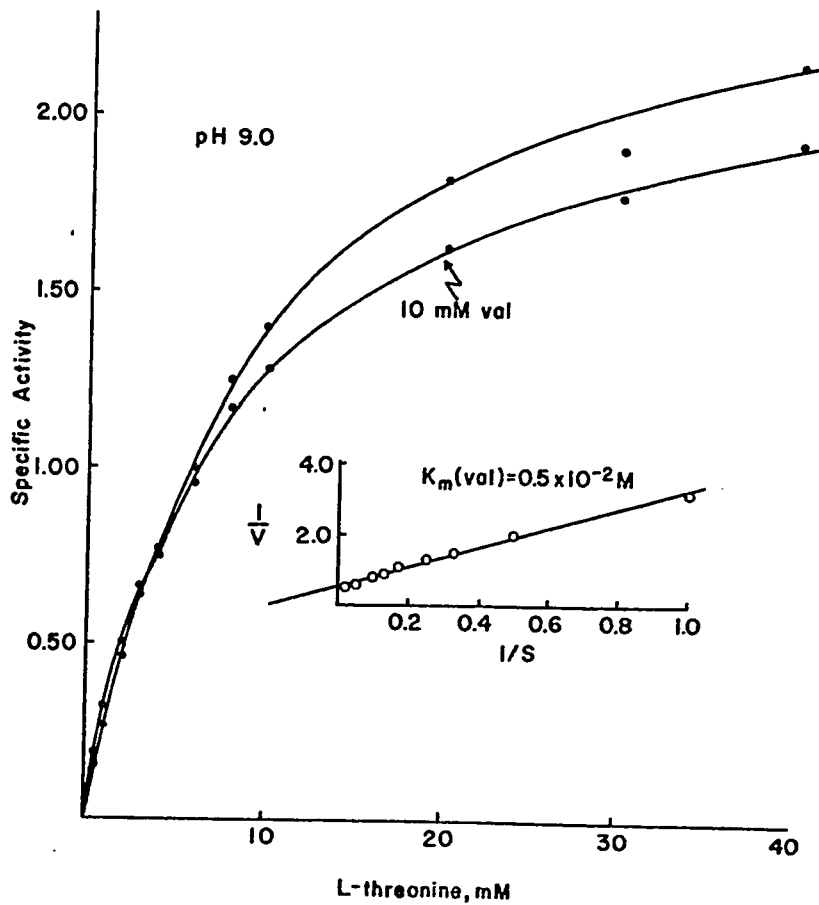
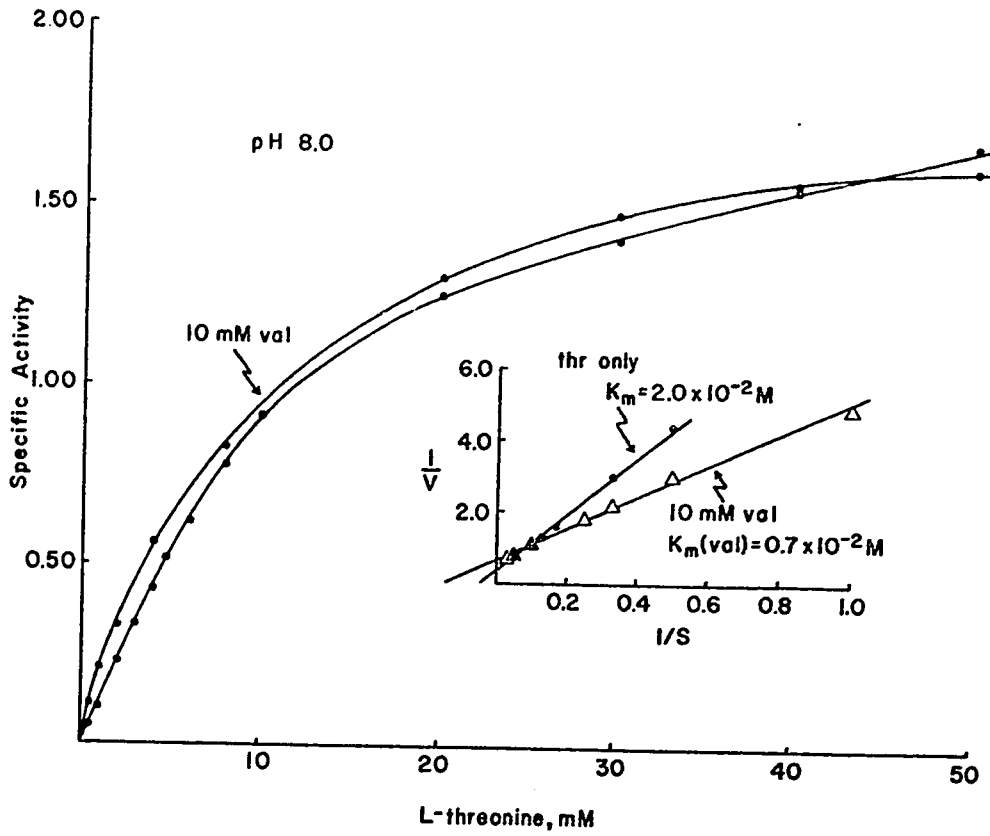


Fig. 4.1.8.

shows the activating effect of 10 mM valine on TD activity in the presence of 0.5 mM threonine, as a function of pH:

Table 4.1.6. Effect of pH on valine activation of TD activity.

pH	% activation
7.0	515
7.4	387
8.0	244
9.0	122

Hill coefficients were derived from the data in fig. 4.1.8, and the plots are presented in fig. 4.1.9. The n values of TD for threonine and threonine + valine at the four pH's tested are given in table 4.1.6a; these data amplify the results just described.

Valine appears to exert its effect as a positive or activating ligand by decreasing the apparent  $K_m$  of TD for threonine from 14 mM at pH 7.0 to 5 mM at pH 9.0, thereby increasing the affinity of TD for its substrate.

Table 4.1.6a. Hill coefficients of TD for threonine and threonine+ valine

Ligand	n value at			
	pH 7.0	pH 7.4	pH 8.0	pH 9.0
L-threonine	1.5	1.5	1.3	1.1
L-threonine + 10 mM L-valine	1.0	1.1	1.0	0.9

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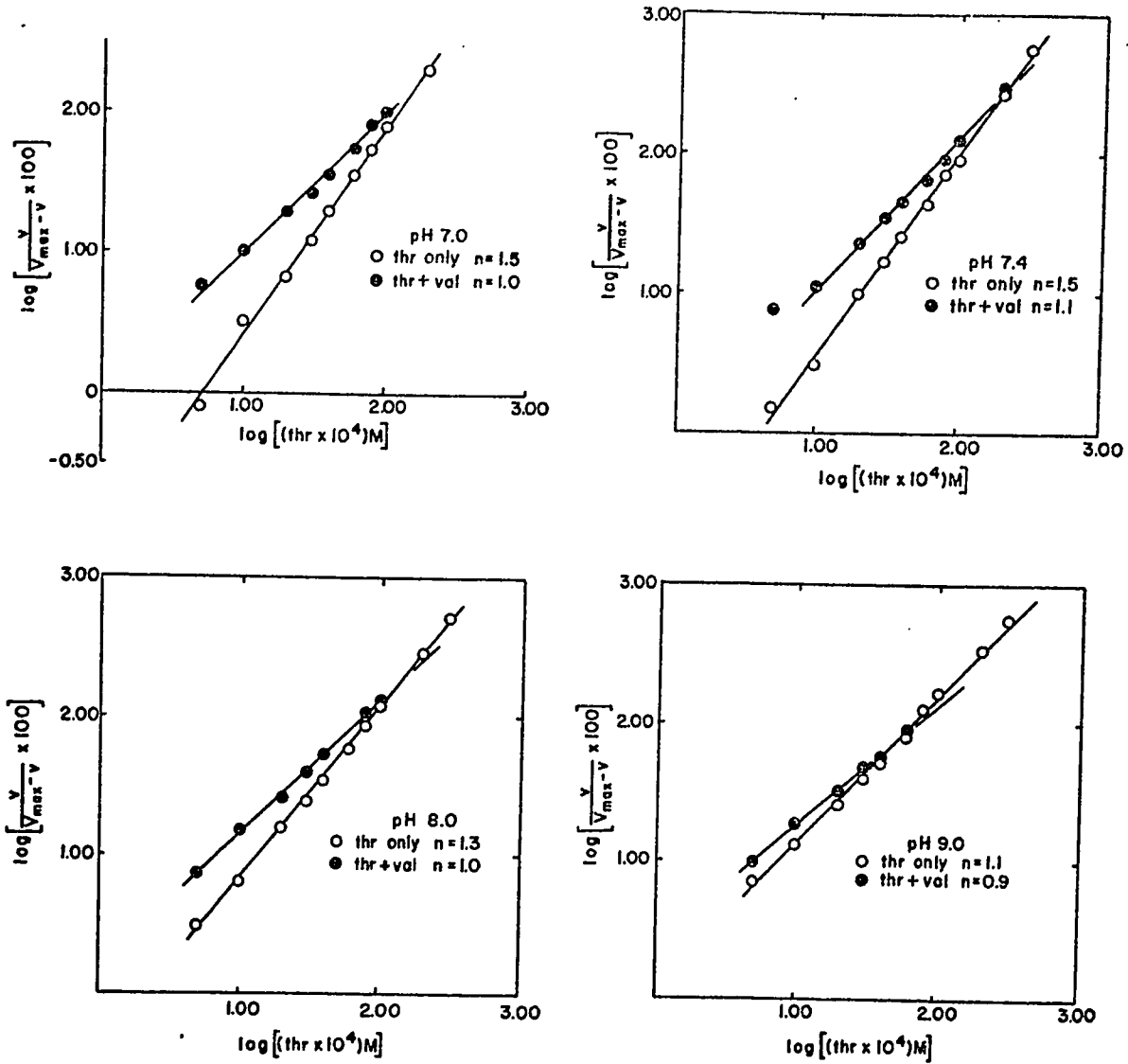


Fig. 4.1.9. Effect of pH on the activation of TD by L-valine, as shown by the Hill plots. Data are taken from fig. 4.1.8.

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Valine exerts similar activating effects in the presence of inhibitory concentrations of isoleucine; these effects will be described in a later section.

#### 4.1.9. Inhibitory effects of valine

Valine alone can inhibit the activity of TD in R. spheroides up to about 75% (Datta, 1966). The B. subtilis enzyme is also sensitive to valine (Hatfield and Umbarger, 1970), but the yeast enzyme does not appear to be (de Robichon-Szulmajster and Magee, 1968). The TD from S. pombe can be inhibited, to a very small degree, in the crude extract, by valine at concentrations greater than 10 mM (fig. 4.1.10). The inhibition curve is sigmoidal, further evidence for more than one binding site for valine.

Valine was tested, using a partially purified enzyme preparation, at low threonine concentration, with the hope that the inhibitory effect of valine might be more pronounced in this condition. In the concentration range 1 - 4 mM, valine stimulated enzyme activity at both lower pH values (fig. 4.1.11); higher valine caused 10 - 15% inhibition. At pH 9.0, no stimulation occurred; a smooth inhibition curve was obtained, presumably because at this pH the enzyme exists in its most active form.

A feature to be noted in fig. 4.1.8 is the crossover points of the threonine and threonine + valine curves, at which threonine concentration valine begins to be inhibitory. This concentration of threonine is 30 mM at pH 7.4, and decreases to 4 mM at pH 9.0. Since the concentration of valine used in these experiments (10 mM) was high enough to cause inhibition by valine itself, further experiments were performed at pH 7.4 with 2 mM valine, and at pH 9.0 with 1 mM valine. Fig. 4.1.12 shows that,

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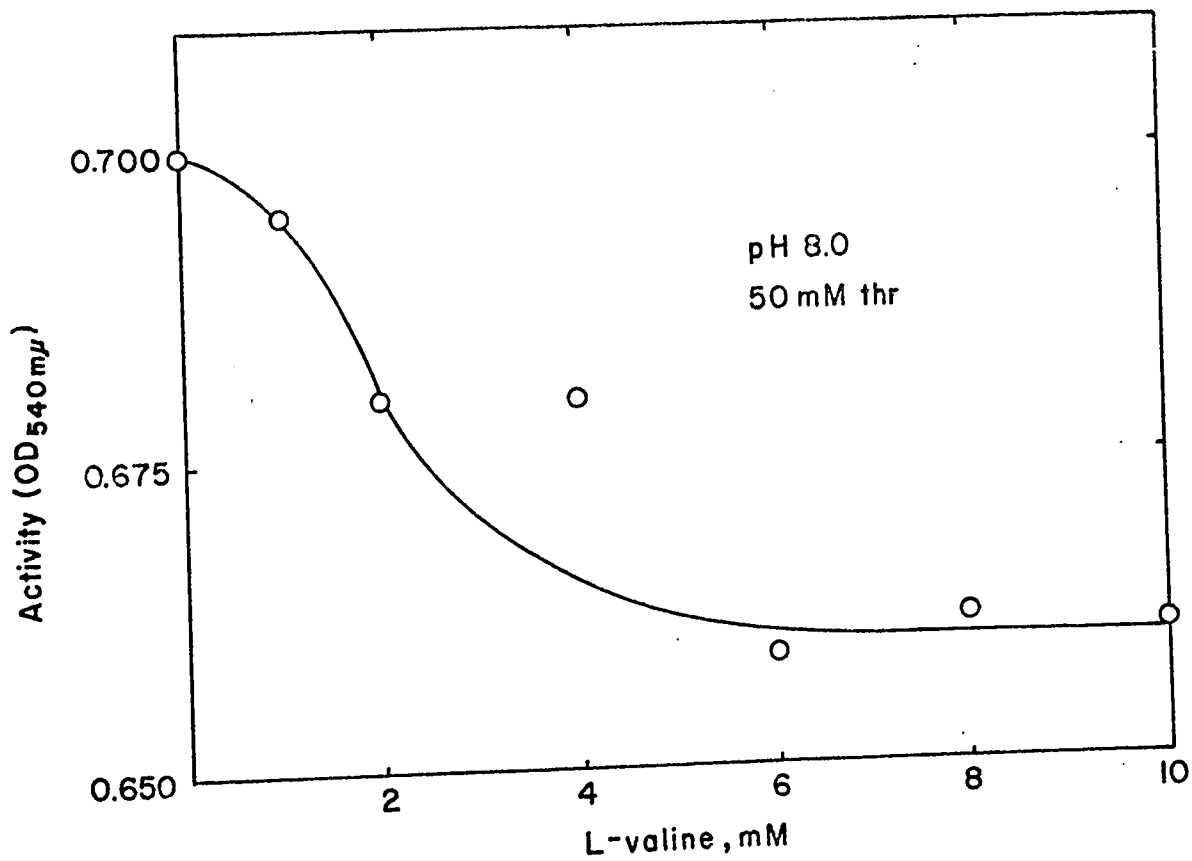


Fig. 4.1.10. Inhibition of TD activity by L-valine. Crude extract (10  $\mu$ l) assayed for 10 min in standard assay mixture supplemented with valine at the concentrations indicated.

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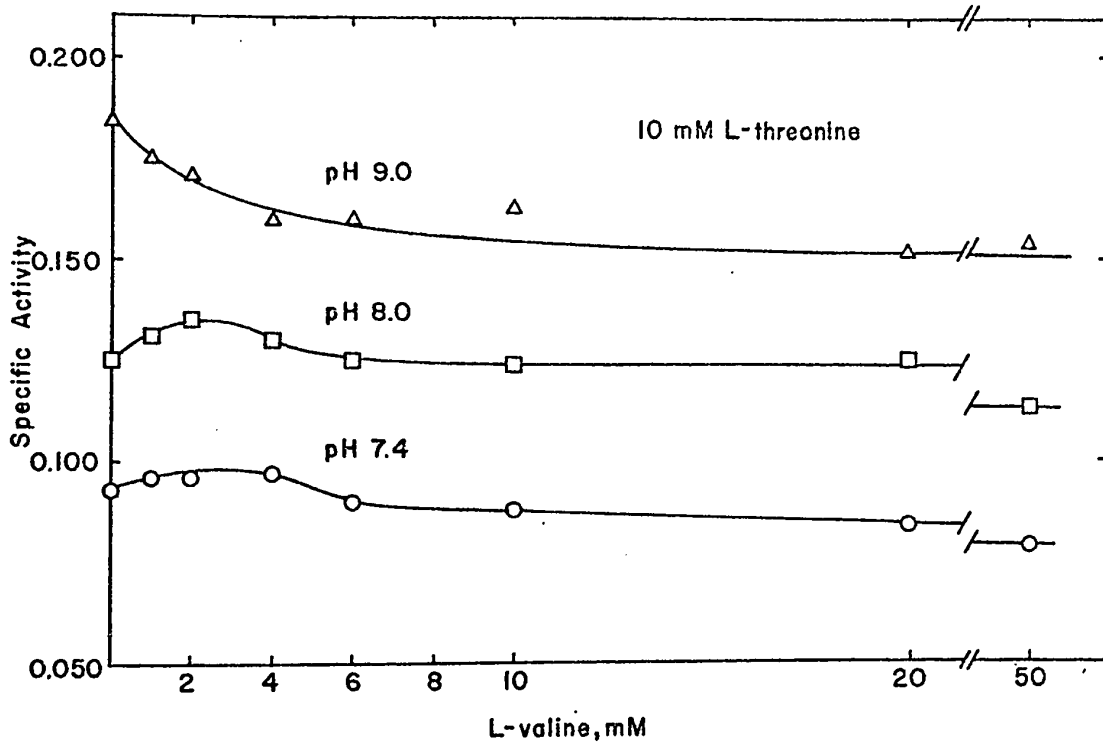


Fig. 4.1.11. Influence of pH on valine inhibition of TD activity at low substrate concentration. An  $(\text{NH}_4)_2\text{SO}_4$  fraction passed through Sephadex G-25 was assayed (18  $\mu\text{g}$  protein for 10 min) in the standard assay mix containing 10  $\mu\text{moles / ml}$  L-threonine, at the three pH values indicated. L-Valine was included at the concentrations given.

Fig. 4.1.12. Activating effect of low L-valine concentrations at the pH optimum for isoleucine inhibition and at the pH optimum for catalytic activity. Same enzyme preparation as in fig. 4.1.11; 25  $\mu$ g protein for 10 min. Standard reaction mixture with varying concentrations of L-threonine; L-valine included at 2 mM at pH 7.4 (upper figure), and at 1 mM at pH 9.0 (lower figure).

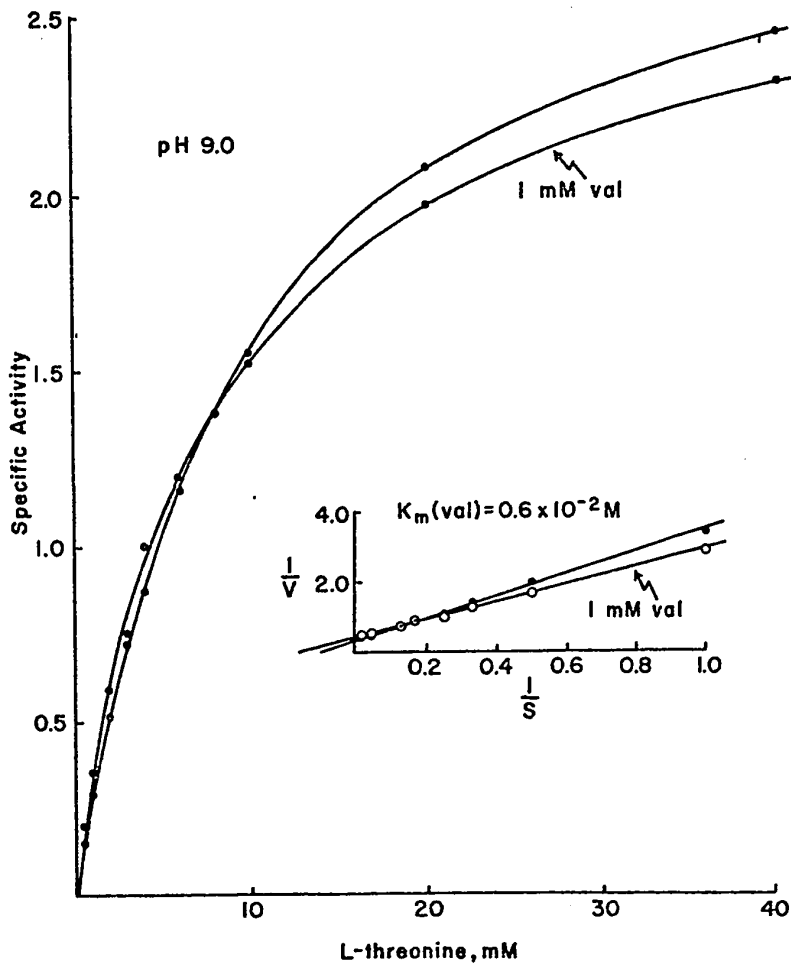
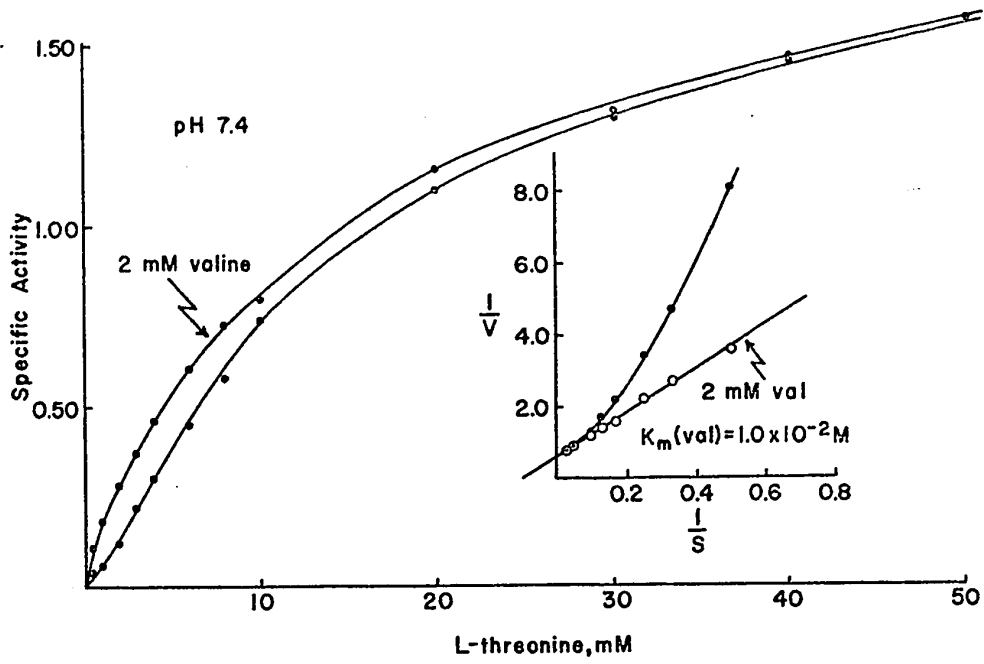


Fig. 4.1.12.

at pH 7.4, in the presence of a stimulatory concentration of valine (2 mM), the two curves meet at saturating (.50 mM) threonine. From the reciprocal plot (inset), the antagonism between valine and threonine is clearly competitive. At pH 9.0, with 1 mM valine, the crossover point now occurred at 8 mM threonine, and valine was inhibitory at higher threonine concentrations. Similar stimulatory and inhibitory effects of valine were observed for E. coli TD by Freundlich and Umbarger (1963).

In summary, valine activates TD at low threonine concentrations by decreasing the  $K_m$  of the enzyme for threonine. This effect is most pronounced at lower pH, where valine normalizes the sigmoidal threonine saturation curve and reduces the corresponding Hill number to 1. The kinetic relationship between valine and threonine is competitive in nature. At pH values below 9.0, valine at 4 mM or higher causes slight inhibition of TD activity.

#### 4.1.10. Analogs of valine and reversal of isoleucine inhibition

Analogs of valine were tested for their ability to reverse the inhibition of TD activity caused by  $5 \times 10^{-4}M$  isoleucine. An  $(NH_4)_2 SO_4$  fraction of TD gel-filtered on Sephadex G-25 was used in these experiments. The results in table 4.1.7 show that there is considerable specificity in the binding site (s) for valine on TD. Reversal of isoleucine inhibition by the D-isomer of valine was only 12% that observed with the natural L-isomer.

The substitution of fluorine for hydrogen in a compound changes the electron density of the site into which it has been introduced without changing the overall geometrical shape of the molecule (Bergmann, 1961); these analogs generally behave as toxic antimetabolites. Trifluorovaline

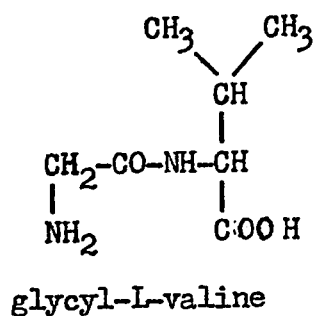
Table 4.1.7. Ability of valine analogs to reverse isoleucine inhibition of TD activity. Threonine at 50 mM.

Compound tested 10 mM	% activity in presence of $5 \times 10^{-4}$ M L-isoleucine
L-valine	91.8
D-valine	10.9
4, 4, 4-trifluoro-DL-valine (TFV)	55.9
4, 4, 4, 5, 5, 5-hexafluoro-DL-valine (HFV)	0
N-acetyl-DL-valine (NAV)	2.7
glycyl-L-valine (GV)	8.0
chloroacetyl-DL-valine (CAV)	7.3
L-valine methyl ester (VME)	17.1
L- $\alpha$ -amino-n-butyric acid (AB)	44.0
DL- <u>threo</u> - $\alpha$ -amino- $\beta$ -chloro-butyrac acid-HCl (ACB)	28.0

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was found to be 61% as effective as valine in reversing isoleucine inhibition, while hexafluorovaline was ineffective in this role.

The negligible effect of N-acetylvaline shows that a free amino group is necessary for valine's role. Glycyl-L-valine, a dipeptide, was only slightly more effective than NAV; this would tend to indicate that the binding site (s) cannot tolerate any shift in the position of the amino



group relative to the  $\alpha$ -carboxyl group on the valine molecule.

$\alpha$ -Aminobutyrate, a natural analog of valine, was 48% as effective as valine, whereas aminochlorobutyrate was only 31% as active in reversing isoleucine inhibition. The loss of a methyl group at the  $\beta$ -carbon of valine (as in ACB) changes the stereospecificity of the valine molecule considerably, indicating that the aliphatic side-chain plays a significant role in the binding of valine, or at least in the alignment of the valine molecule at its binding site.

#### 4.1.11. Effect of leucine on TD activity

TD activity of *S. pombe* is inhibited by L-leucine. (table 4.1.8). Higher concentrations, up to 50 mM leucine, caused little further inhibition of TD activity in the partially purified form.

Table 4.1.8. Effect of L-leucine on TD activity

Enzyme	% inhibition by 10 mM L-leucine
crude extract	40.2
partially purified prep	37.2

The effect of leucine in the presence of isoleucine and isoleucine - valine was tested (fig. 4.1.13). Concentrations of leucine in the range 0.1-1.0 mM are able to reverse inhibition by  $10^{-4}$ M isoleucine up to about 30% (curve B). Concentrations of leucine greater than 1.0 mM (in the presence of  $10^{-4}$ M L-isoleucine) cause increasing inhibition of TD activity; the inhibitory effects of leucine and isoleucine were not found to be additive. Curve C shows that valine at 4 mM reversed 50% of the inhibition by isoleucine, while valine plus concentrations of leucine up to 0.5 mM reversed the isoleucine inhibition a further 15%. Greater than 0.5 mM leucine, the combination (isoleucine + valine + leucine) was inhibitory to enzyme activity.

Valine concentrations up to 20 mM could not reverse inhibition of TD activity by 5 mM leucine.

The data from curve A (same figure) were rearranged for a Hill plot (fig. 4.1.13), and yielded a straight line with slope corresponding to an n value of 0.7 for leucine. This indicates that, unlike both isoleucine and valine, leucine exerts no cooperative effects in its interaction with TD.

These effects of leucine were verified using a partially purified TD preparation (fig. 4.1.14). The saturation curve in the presence of

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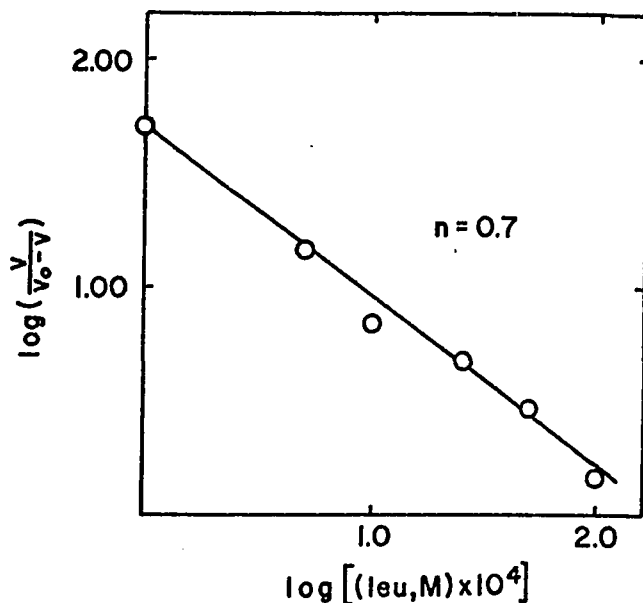
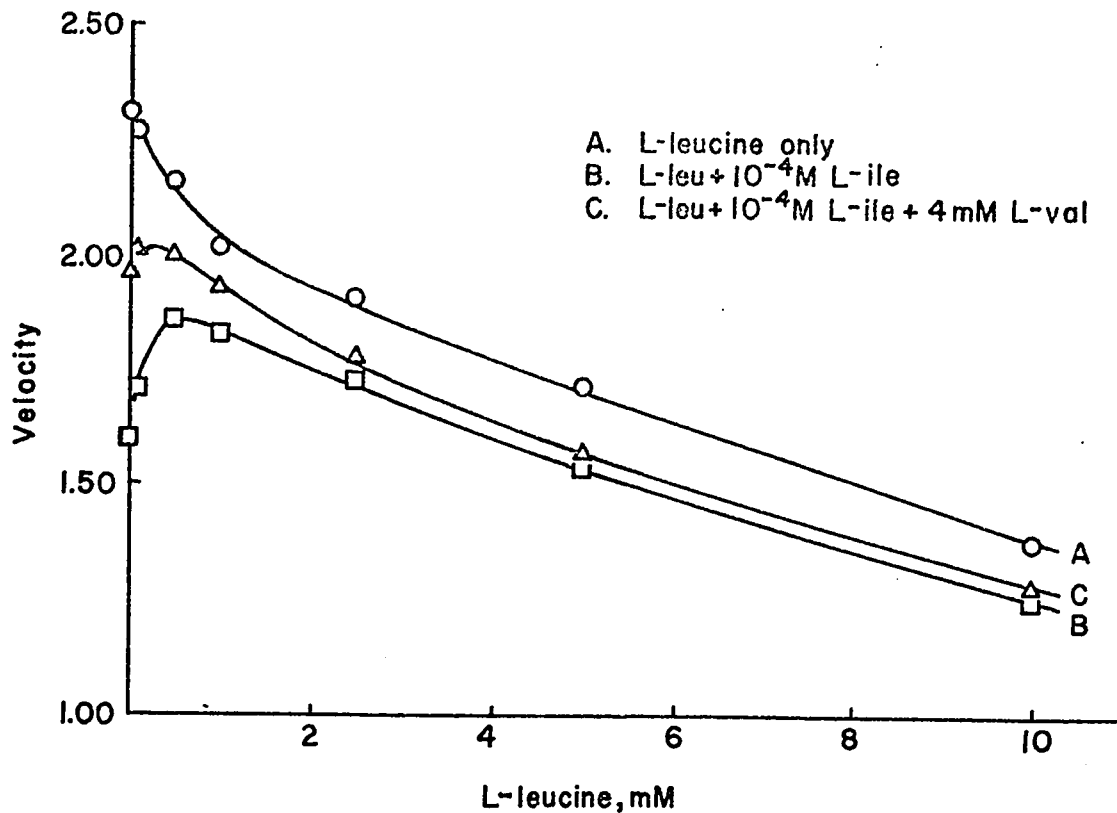


Fig. 4.1.13. Effect of L-leucine on activity and regulatory properties of threonine deaminase. Crude extract served as source of TD in the standard assay mixture (10  $\mu$ l assayed for 10 min). Upper figure: the concentration of L-leucine was varied as indicated. Curve A: L-leucine only; curve B: L-leucine varied with 0.1 mM L-isoleucine; curve C: L-leucine varied in the presence of both 0.1 mM L-isoleucine and 4 mM L-valine. Lower figure: Hill plot for leucine inhibition; data from curve A (upper figure).

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Fig. 4.1.14. Effect of L-leucine on L-iso-leucine inhibition of TD. Enzyme used was Sephadex G-25-filtered  $(\text{NH}_4)_2 \text{SO}_4$  fraction. Standard assay procedure was used: 20  $\mu\text{g}$  protein for 15 min.

Upper figure:

curve A: activity in varying L-threonine concentrations;  
curve B: as in A, but with 10 mM L-leucine added;  
curve C: as in A, with 0.1 mM L-iso-leucine added;  
curve D: as in C, with addition of 0.5 mM L-leucine.

Lower figure: empirical Hill plots with data from upper figure.

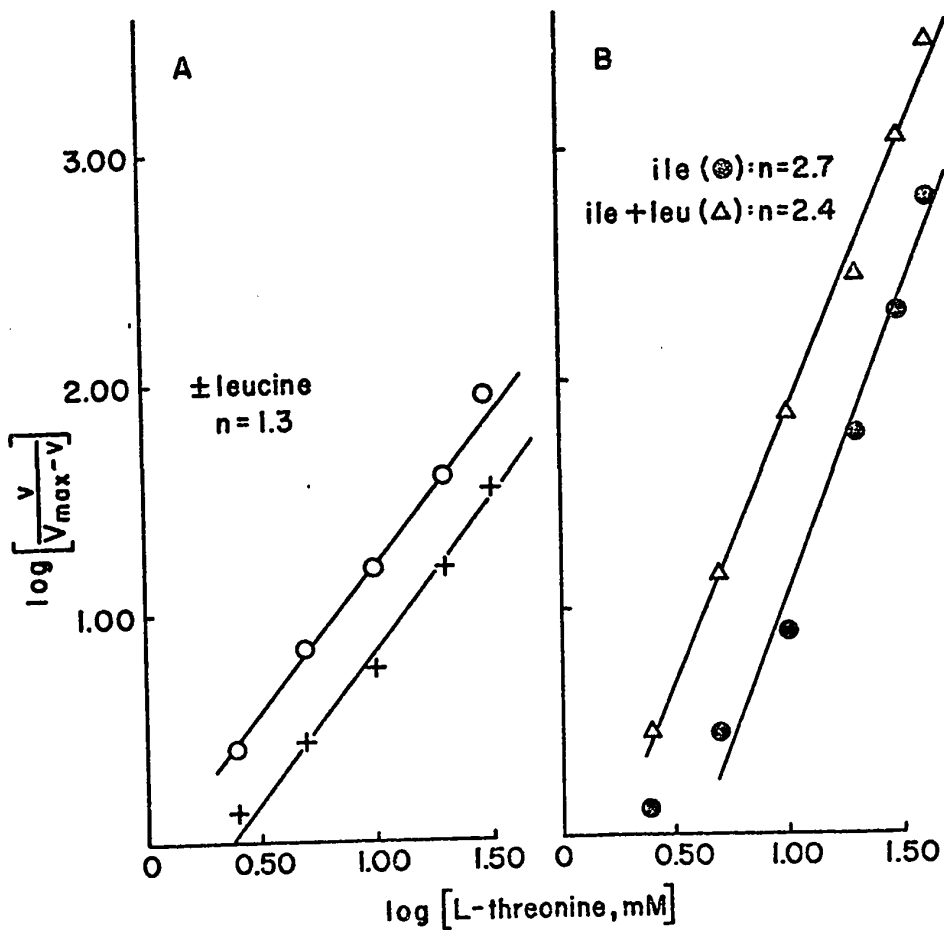
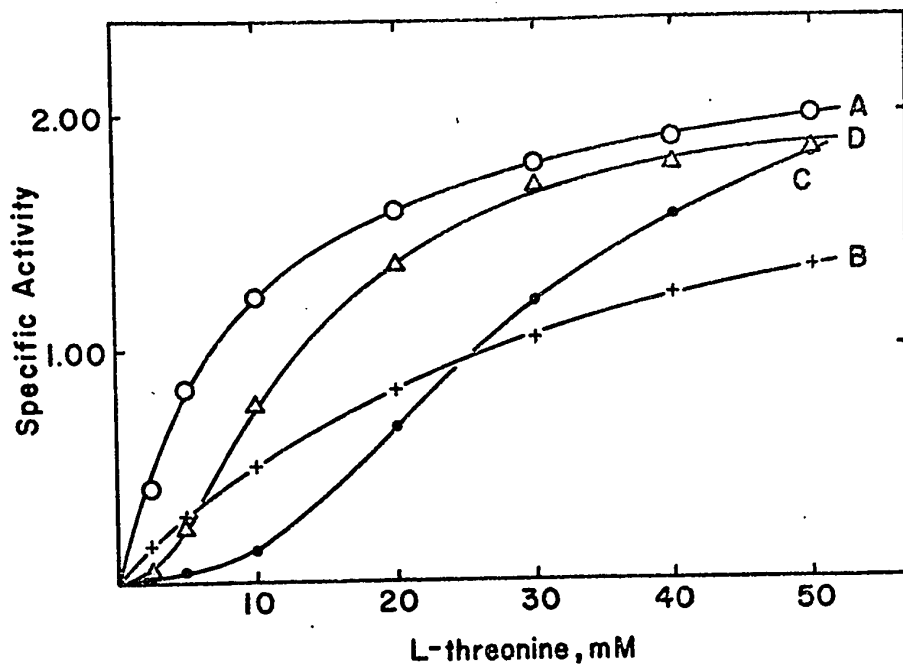


Fig. 4.1.14.

leucine (B) is still hyperbolic. As before,  $10^{-4}M$  isoleucine rendered the curve sigmoidal (C); when leucine at  $5 \times 10^{-4}M$  was also included (D), the curve remained sigmoidal - no normalization or activation occurred as when valine was present. A double reciprocal plot (fig. 4.1.15) showed that leucine inhibition of TD activity is competitive with respect to threonine, as the  $K_m$  is changed.

Hill plots obtained from these data show that leucine alone cannot cause cooperative effects (fig. 4.1.14 A), nor can it eliminate the cooperative effects of isoleucine (fig. 4.1.14 B).

#### 4.1.12. Effects of isoleucine and valine on TD activity as a function of threonine concentration

In a previous section (4.1.8), it was shown that at pH 7.0 and 7.4, the substrate saturation curve of TD in the presence of threonine alone was sigmoidal, i.e. at these pH's, threonine exerts homotropic cooperative effects on enzyme activity. At higher pH's, these effects are diminished, and the substrate curve is a rectangular hyperbola at pH 9.0. At all pH values, valine exerted an activating effect on activity, most pronounced at low threonine concentrations; the sigmoidal substrate curves were normalized ( $n = 1.0$ ) in the presence of valine.

At the routine assay pH of 8.0, plots of TD activity vs threonine concentration gave nearly rectangular hyperbolas. This was true for enzyme activity in crude extracts, in G-25-filtered crude extracts, and in partially-purified preparations. This was also true for activity assayed in potassium phosphate, sodium phosphate and Tris-HCl buffers. The Hill

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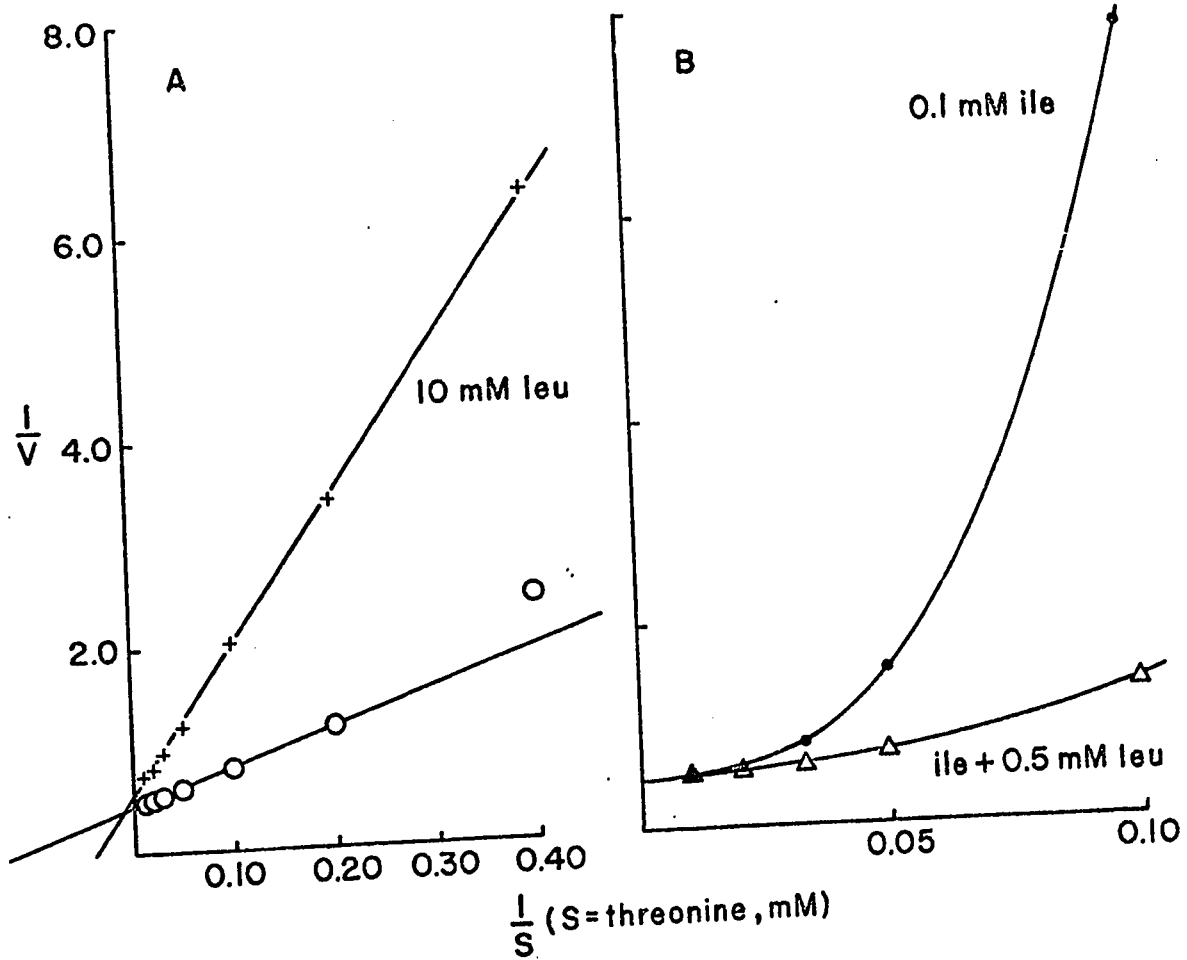


Fig. 4.1.15. Effect of leucine on TD activity and inhibition by L-isoleucine. Data from fig. 4.1.14 rearranged in Lineweaver-Burk plot. A: inhibition by L-leucine; B: antagonism between L-isoleucine and L-leucine.

coefficient of TD for threonine only was found to be  $1.22 \pm 0.07$  (average of 6 separate determinations); it can be concluded that, at pH 8.0, cooperative effects of threonine are extant to some degree.

Fig. 4.1.16 shows the effects of isoleucine and isoleucine + valine on threonine deamination as a function of threonine concentration. In the absence of effectors (curve A), the plot of activity vs threonine concentration is very slightly sigmoidal; the points can be fitted by eye to a hyperbola, but the Hill  $n=1.3$  (fig. 4.1.17) indicates cooperative effects for threonine ( a Hill number of 1.0 is obtained from a true rectangular hyperbola). Similar results were shown in figs. 4.1.8 and 4.1.14, where partially purified TD's were used.

Curves B and C (fig. 4.1.16) show the effect of two concentrations of isoleucine: as isoleucine concentration is increased, the saturation curves become increasingly sigmoidal, as isoleucine increases the cooperative effects of threonine (fig. 4.1.17:  $n=2.4$  with  $5 \times 10^{-5}M$  isoleucine,  $n=2.8$  with  $10^{-4}M$  isoleucine). Moreover, at threonine concentrations over 50 mM, the isoleucine curves tend to the same maximum velocity attained in the absence of isoleucine, indicating that inhibition by isoleucine is apparently competitive (or pseudocompetitive). When these data are transformed to double reciprocal plots, the curves may be extrapolated to the same  $V_{max}$  value (fig. 4.1.18), verifying apparent competitive inhibition by isoleucine. The double reciprocal plot for threonine only shows a small upward swing about the points corresponding to the two lowest threonine concentrations; this, along with the Hill coefficient of 1.3, indicates that there are slight homotropic effects between threonine molecules at low substrate concentrations at pH 8.0. A straight line can be fitted to the points corresponding to the higher threonine

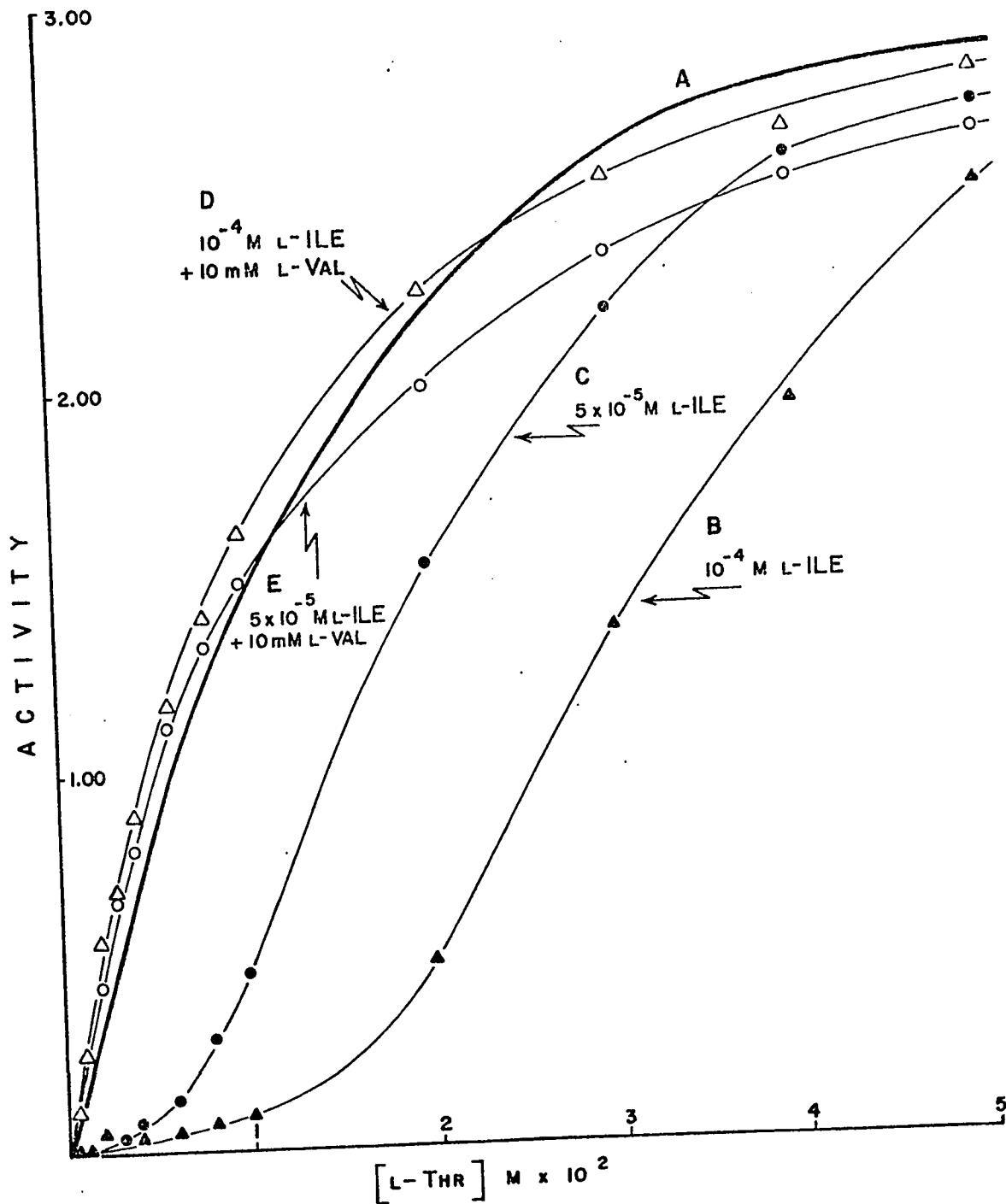


Fig. 4.1.16. Effects of L-isoleucine and L-valine on TD activity as a function of L-threonine concentration. Crude extract was assayed (10  $\mu$ l for 10 min) in standard reaction mixtures containing increasing concentrations of L-threonine and the indicated levels of L-isoleucine and L-valine; pH was 8.0. Activity is expressed as  $\mu$ moles  $\alpha$ -ketobutyrate per ml enzyme per min. Curve A: threonine only; B: threonine + 0.1 mM isoleucine; C: threonine + 0.05 mM isoleucine; D and E: as in B and C, respectively, with 10 mM valine.

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Fig. 4.1.17. Kinetics of TD in the presence of L-isoleucine and L-valine as shown by the Hill plot. Data are from fig. 4.1.16.

Upper figure: L-threonine only

Lower figures: left, 0.1 mM L-isoleucine ± 10 mM L-valine; right, 0.05 mM L-isoleucine ± 10 mM L-valine.

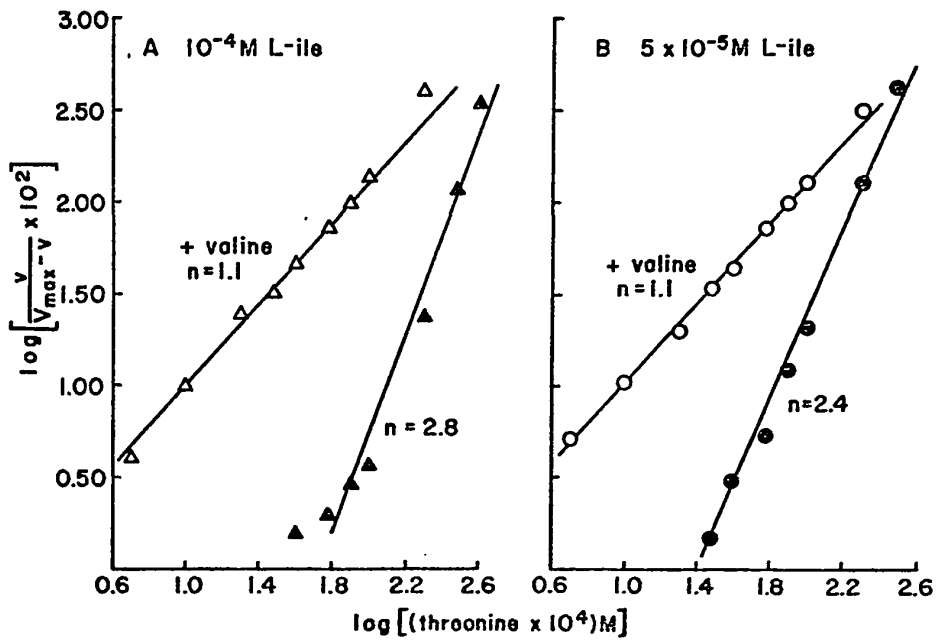
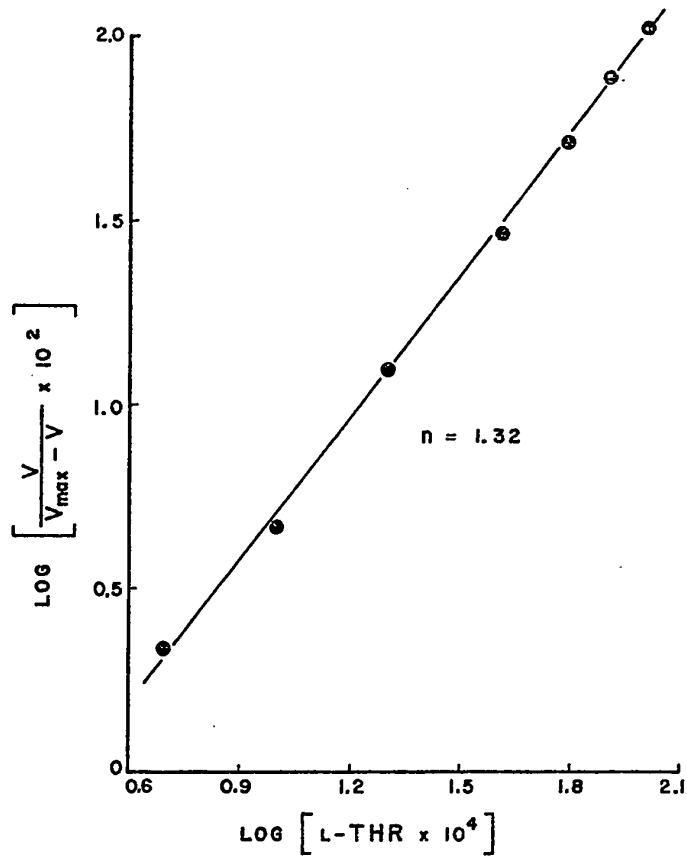


Fig. 4.1.17.

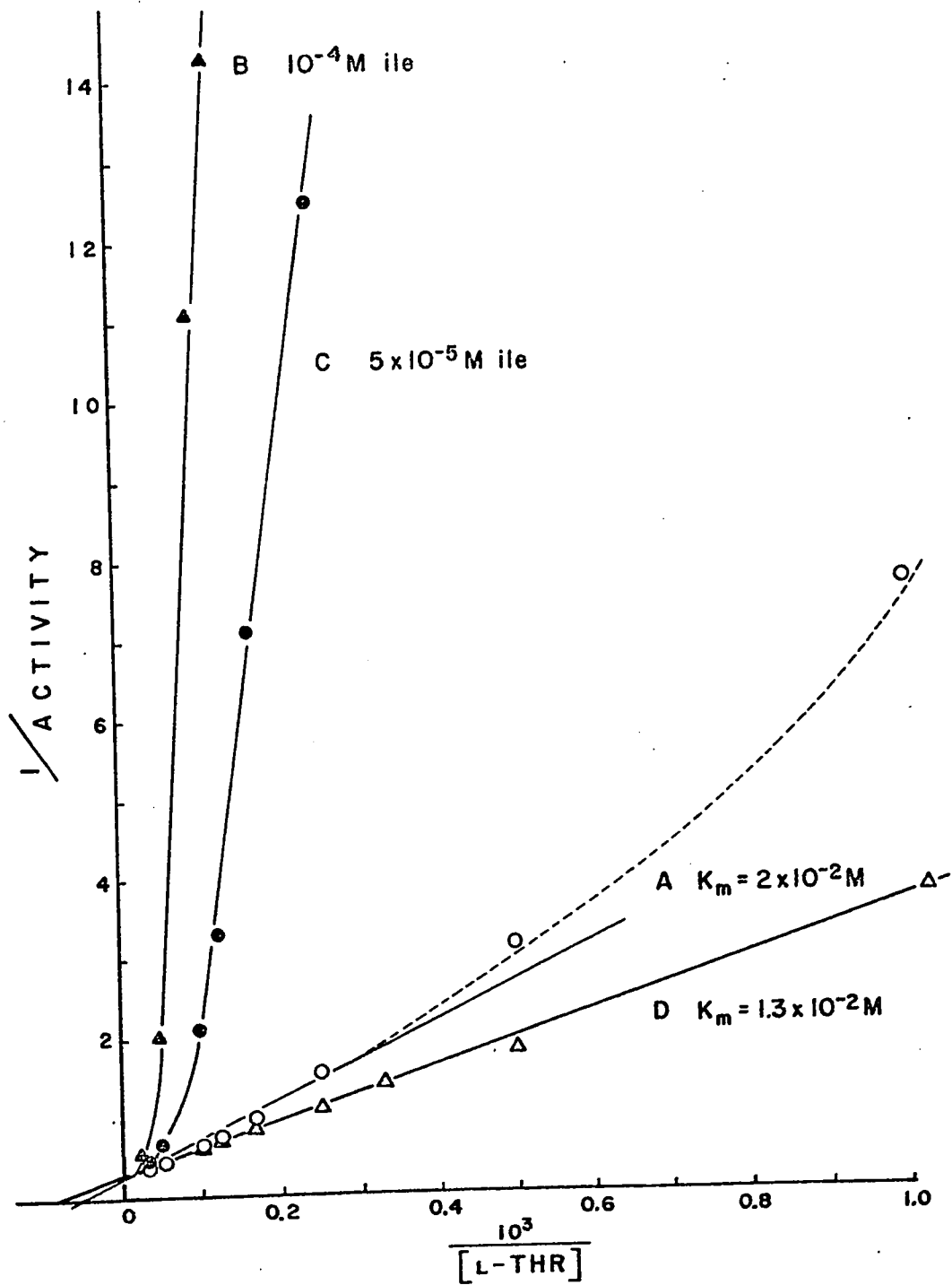


Fig. 4.1.18. Kinetics of TD in the presence of L-isoleucine and L-valine. Data from fig. 4.1.16 rearranged in Lineweaver-Burk plot.

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concentrations, and an apparent  $K_m$  of  $2 \times 10^{-2}M$  can be estimated from this data.

Curves D and E (fig. 4.1.16) show the results of including 10 mM valine in the assay mixtures along with threonine and isoleucine. The inhibitory effects of isoleucine are reversed and the shape of the substrate curves is normalized by valine ( $n=1.1$  in both cases where 10 mM valine is present with the two isoleucine concentrations, fig. 4.1.17). At low threonine concentrations plus isoleucine, valine also activates TD activity about 200-220% in the range 0.5-1.0 mM threonine; this effect is no longer observed when threonine reaches concentrations greater than 10 mM. As shown before, at pH 8.0, the activating effect of valine in the presence of 0.5 mM threonine only was 244% (table 4.1.6); thus valine exerts its activating role at low threonine concentrations even in the presence of isoleucine. The stimulatory effect of valine appears to be more pronounced with  $10^{-4}M$  isoleucine (curve D) than with  $5 \times 10^{-5}M$  isoleucine (curve E).

The  $K_m$  value obtained in the presence of  $10^{-4}M$  isoleucine + valine (curve D, fig. 4.1.18) was  $1.3 \times 10^{-2}M$ , which is lower than the  $K_m$  with threonine only (cf.  $2.0 \times 10^{-2}M$ ). Since the  $V_{max}$  values of all the curves (fig. 4.1.16) tend to the same value, there would seem to be competitive antagonism between valine and isoleucine.

#### 4.1.13. Activity of TD with analogs of L-threonine

The specificity of TD for its natural substrate, L-threonine, was examined by testing the ability of several analogs of threonine to serve as substrates for TD. L-Serine has long been known to be deaminated by TD preparations from E. coli (Wood and Gunsalus, 1949); H. crassa (Yanofsky and Reissig, 1953); bakers' yeast (Boil and Holzer, 1965) and C. tetanomorphum (Whiteley and Tahara, 1966); a more detailed analysis of serine as substrate for S. pombe TD will be presented in the following section.

Threonine analogs at 50 mM (with respect to the L-isomer) were substituted for L-threonine in the usual assay mixture; partially-purified TD (ammonium sulfate fraction filtered through Sephadex G-25) was incubated with each analog for 10 min, and the amount of keto acid formed from the analog in that time was determined as previously described (3.2.1). Controls containing analog to which stopping mixture was added prior to enzyme addition were run to correct for color contributed by certain of the analogs (CAT and TME). Table 4.1.9 presents the results of this study.

The enzyme is specific for the L-isomer of threonine; D-threonine is deaminated only about 1% under identical conditions. The diastereoisomers, D- and L- allothreonine, do not serve as substrates for TD. Thus, of four possible stereoisomers of threonine, TD acts only on L-threonine.

The low efficiency of chloroacetyl threonine (i.e. 2% that of L-threonine) suggests that a free  $\alpha$ -amino group on threonine is essential, possibly for binding at the active site (s).

Table 4.1.9. Interaction of TD with analogs of L-threonine

Compound tested 50 mM	Specific activity	% activity of L-threonine
L-threonine	1.68	
D-threonine	0.02	1.2
DL-allothreonine (AT)	0.07	4.2
dichloroacetyl-DL-threonine (CAT)	0.03	1.8
L-threonine methyl ester (TME)	1.05	62.5*

\* This compound was not tested for saponification products.

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Threonine methyl ester was about 63% as efficient as L-threonine, so the  $\alpha$ -carboxyl group apparently does not play a large part in the reaction.

#### 4.1.14. Activity of TD on L-serine

The ability of TD to deaminate L-serine is illustrated in fig. 4.1.19; the curve is a shallow hyperbola without any noticeable plateau. The inset shows the data rearranged in a double reciprocal plot; the apparent  $K_m$  of TD for serine was estimated to be 0.4 M. Thus, the enzyme has very low affinity for serine (the apparent  $K_m$  for threonine is about 40 times lower than that for serine).

#### 4.1.15. Action of ammonium ion on TD activity

It has been reported by Holzer et al. (1964) that TD from bakers' yeast is activated by ammonia, which is a product of the enzyme reaction. However, the enzyme from S. typhimurium was shown to be competitively inhibited by  $NH_4^+$  (Maeba and Sanwal, 1966). The effect of  $NH_4^+$  on partially purified TD from S. pombe is illustrated in fig. 4.1.20; ammonia non-competitively inhibits the reaction when activity is assayed in phosphate buffer.  $NH_4^+$  stimulated TD activity when Tris-HCl buffers were used in the preparation of the enzyme and in the assay mixtures. However, the enzyme activity assayed under these conditions (i.e. tris buffers and  $NH_4Cl$ ) was always less than activity measured in phosphate buffers. For this reason, phosphate buffer was routinely used for assays.

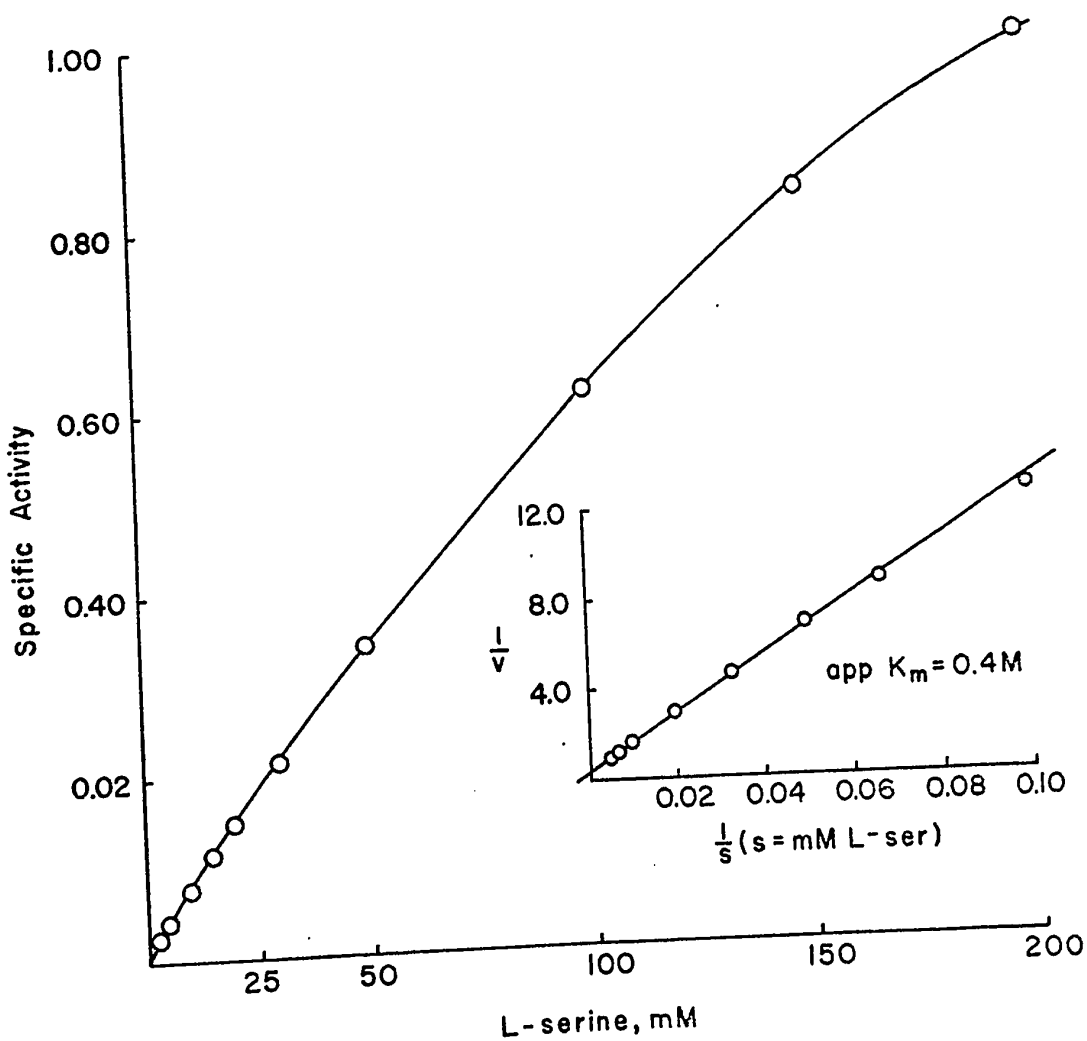


Fig. 4.1.19. Deamination of L-serine by TD from *S. pombe*. The standard reaction mixture, with varying concentrations of L-serine substituted for L-threonine, was used. A Sephadex G-25-filtered  $(\text{NH}_4)_2 \text{SO}_4$  fraction was used in the assay (0.07 mg for 10 min). Pyruvate was determined as for  $\alpha$ -keto-butyrate (section 3.2.1). Inset shows data rearranged in double reciprocal plot for  $K_m$  determination.

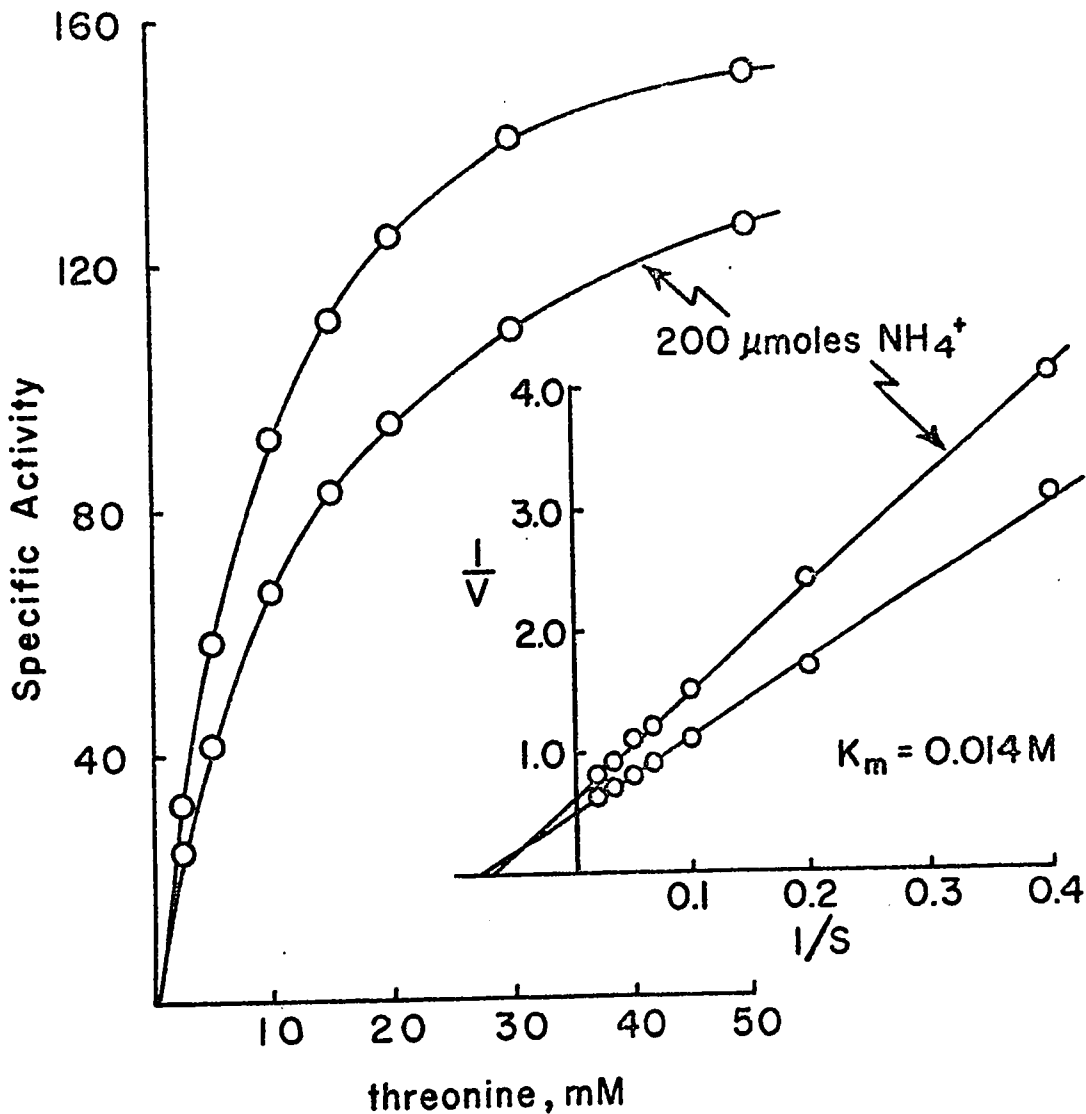


Fig. 4.1.20. Influence of ammonium ion on TD activity. A partially purified preparation ((NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> fraction desalted on Sephadex G-25) was assayed in a reaction mixture containing 200 μmoles / ml NH<sub>4</sub> Cl and increasing concentrations of L-threonine up to 50 mM. Inset shows Lineweaver-Burk transformation of the results obtained.

4.1.16. Effects of temperature on TD activity

A temperature study on a regulatory enzyme provides another dimension in which to study the activity of the enzyme and its inter-relationships with effectors. Consequently, the activity of S. pombe TD was assayed at various temperatures ranging from 0-55 C. Appropriate amounts of enzyme and incubation times were chosen for each temperature in order to be in the linear range of activity. The results are presented in fig. 4.1.21. At 0 C, there is detectable enzymatic activity (open circles); activity rises steadily as the temperature is increased to 37 C, above which temperature activity declines rapidly to almost nil at 55 C. Inhibition by isoleucine is very temperature-dependent (closed circles); it decreases rapidly as the temperature rises to 37 C, above which temperature it remains constant at about 13%. It is not apparent from this experiment whether heat is affecting the enzyme protein itself, or whether it is acting at the level of the enzyme-substrate-inhibitor (ESI) complex. In other words, is heat acting on the protein structure of TD so that it no longer reacts with isoleucine, or is isoleucine affecting the ability of the ES complex to respond to the increase in temperature, making the ES complex more sensitive to isoleucine at lower temperatures and less sensitive at higher temperatures? However, in other experiments to be reported, where TD was heated at 55 C for up to 10 min in an attempt to desensitize the enzyme, activity could still be almost completely inhibited by higher concentrations of isoleucine (ca.  $10^{-3}M$ ), when the activity was subsequently assayed at 30 C. It seems, thus, to be an effect of isoleucine on the ES system, and the enzyme is less sensitive to isoleucine with increasing temperature.

These data were rearranged to give an Arrhenius plot (fig. 4.1.22).

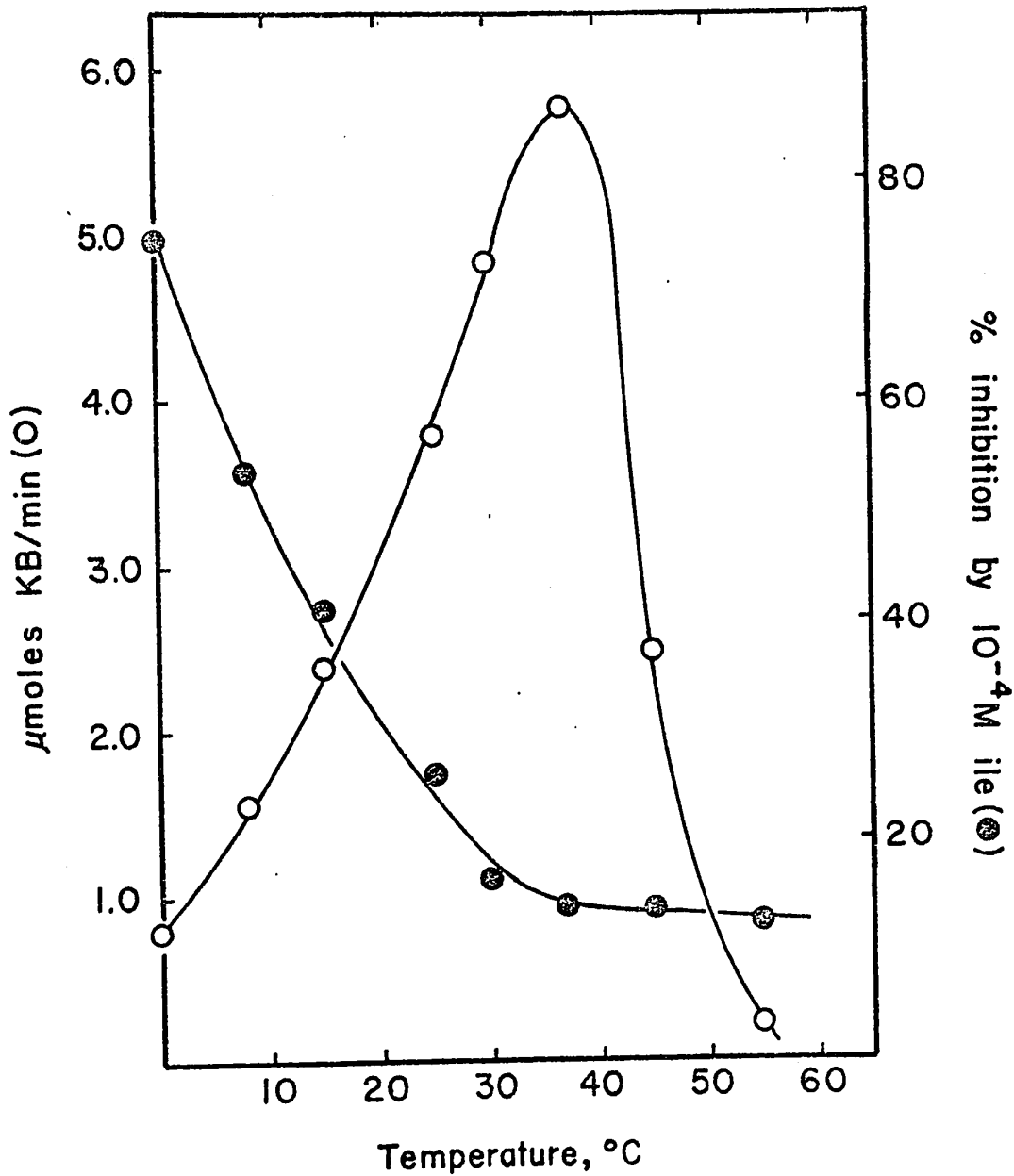


Fig. 4.1.21. Influence of temperature on TD activity and regulatory properties. TD activity in a crude extract was assayed in the standard assay mixture at the temperatures indicated. Feedback sensitivity was measured in the presence of 0.1 mM L-isoleucine.  $\circ$ — $\circ$ , enzyme activity;  $\bullet$ — $\bullet$ , % inhibition by L-isoleucine.

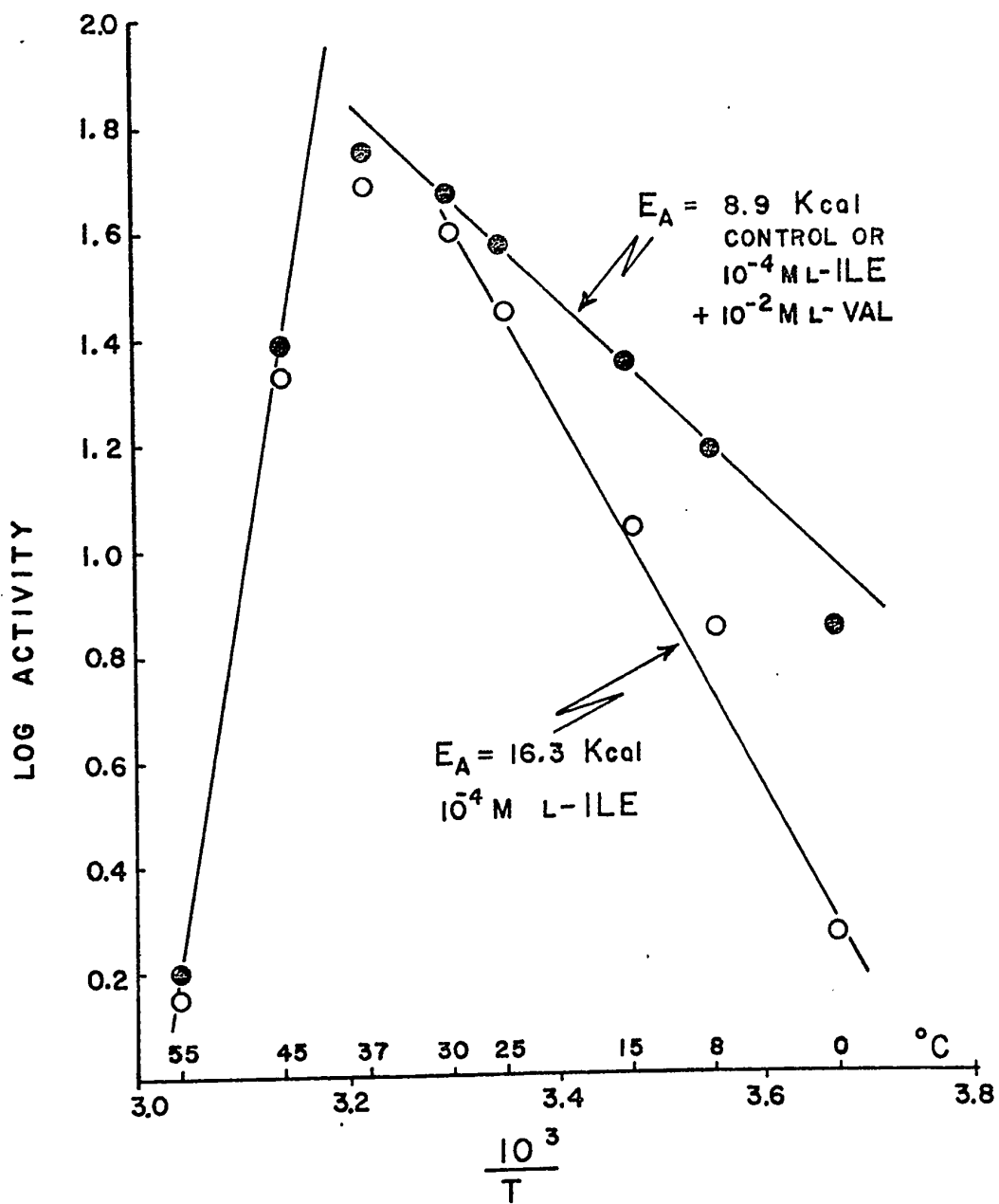


Fig. 4.1.22. Arrhenius plot of activity of TD and effects of L-isoleucine and L-valine. Data are from fig. 4.1.21. L-Valine was added to reaction mixtures at a final concentration of 10 mM.

A good fit to the Arrhenius equation was obtained in the temperature range 0-30 C. The experimental energy of activation ( $E_A$ ) for threonine was computed from the slope of the line to be about 9 Kcal / mole. Isoleucine increased the  $E_A$  of the system to 16 Kcal / mole, whereas when both isoleucine and valine were present, the  $E_A$  was identical with that for threonine only.

A similar observation was made by Kaplan et al. (1967) for the aspartate transcarbamylase of S. cerevisiae; the retroinhibitor UTP increased the  $E_A$  of the ES system from about 6 Kcal / mole to 11 Kcal / mole.

#### 4.1.17. Initial velocity studies and effects of isoleucine and valine

Hatfield and Umbarger (1970) have reported that TD isolated from B. subtilis showed a time-dependent acceleration of the rate of reaction when the enzyme was preincubated in isoleucine and the reaction was initiated by addition of threonine. The time course of the reaction was linear when threonine alone was present. A similar activation effect was described for the bakers' yeast enzyme by de Robichon-Szulmajster and Magee (1968), but it was shown to be an artifact of the coupled assay procedure which they used. It was of interest, then, to investigate the effects of isoleucine and valine on the initial rates of the TD reaction in S. pombe.

In fig. 4.1.23 (upper left), it can be seen that enzyme activity is strictly linear with time in the absence of isoleucine (A). When enzyme is preincubated with isoleucine and the reaction is begun by addition of threonine (C and D; also curve B, middle right), there is an acceleration of the rate of reaction from the initial inhibited rate to a steady rate

Fig. 4.1.23. Influence of L-threonine, L-isoleucine and L-valine on initial velocity of TD. A partially purified TD preparation (38-44%  $(\text{NH}_4)_2 \text{SO}_4$  fraction desalted on Sephadex G-25) was used in all these experiments. For each experiment, 20  $\mu\text{l}$ s of enzyme were preincubated 5 min at 30 C in two mls of standard reaction mixture minus L-threonine; the reaction was begun by addition of 50  $\mu\text{moles}$  L-threonine. At 20-sec intervals, 100  $\mu\text{l}$ s of the reaction mixture were added to 0.2 ml TCA-DNPH solution. Colour was developed with NaOH as usual.

Upper left: A, threonine added to a reaction mixture containing enzyme; B, threonine added to enzyme preincubated 5 min in 0.5 mM isoleucine + 10 mM valine; C and D, threonine added to enzyme preincubated with 0.3 mM and 0.5 mM isoleucine, respectively.

Lower left: A, threonine only; B, threonine + 0.3 mM isoleucine added simultaneously to reaction mixture containing enzyme.

Upper right: A, threonine only; B, enzyme added to reaction mixture containing threonine + 0.3 mM isoleucine.

Middle right: A, threonine added to reaction mixture containing enzyme, and 0.3 mM isoleucine added where indicated; B, threonine added to enzyme preincubated in 0.3 mM isoleucine.

Lower right: A, threonine only; B, 5  $\mu\text{moles}$  threonine added to reaction mixture containing enzyme  $\pm$  10 mM valine; C, 30  $\mu\text{moles}$  threonine added to enzyme preincubated with 0.3 mM isoleucine, and 2 mM valine added at  $t = 3$  min.

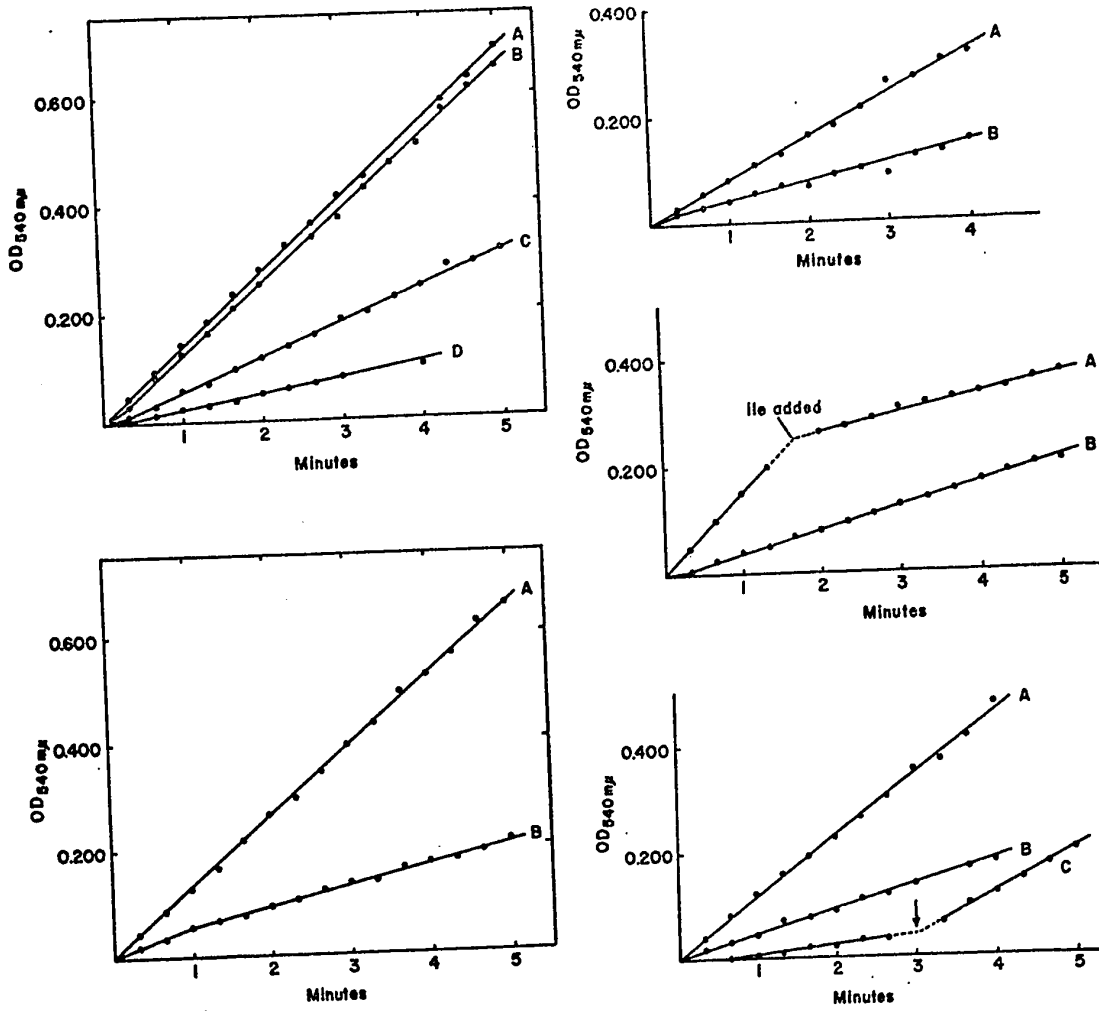


Fig. 4.1.23.

which depends on the level of isoleucine present. With this colorimetric assay, the accelerated rate begins about 20 sec after the addition of threonine. If the enzyme is preincubated with both isoleucine plus valine, a similar curve results (B, upper left), except that the final steady rate achieved is the same as that with threonine alone.

If isoleucine is added to a reaction already in progress (curve A, middle right) there is an immediate break in the curve as the reaction decelerates to an inhibited rate (the two lines extrapolate to the same time point). Curve B (lower left) illustrates the delayed action of isoleucine when it and threonine are added simultaneously to a reaction mixture containing enzyme only; the reaction proceeds at an inhibited rate for about 60 sec when it changes to a second more inhibited state. When enzyme is added to a reaction mixture containing threonine + isoleucine (upper right), the rate decreases from one close to that with threonine alone (A) to an inhibited rate (B) which depends upon the isoleucine concentration.

Curve B (same fig., lower right) shows that if TD is preincubated with valine and the reaction is started with 5 mM threonine, the rate achieved is identical to that of the enzyme in threonine only; no activation effect by substrate in the presence of valine was observed. When 30 mM threonine is added to enzyme preincubated with isoleucine, the acceleration of the reaction rate begins at about 40 sec (curve C, lower right); when valine was added to the system at 3 min, an immediate acceleration occurs and a new steady state rate is obtained.

A similar activating effect of threonine on the rate of TD activity in the presence of isoleucine was observed using crude extracts.

In summary, when threonine is added to an enzyme-isoleucine complex,

a time-dependent lag is observed before the steady-state inhibited rate is attained. A gradual decrease in reaction velocity occurs when threonine and isoleucine are added simultaneously to preincubated enzyme, or when enzyme is added to threonine-isoleucine. The activating effect of valine is exerted slowly, for a lag was observed before the stimulated steady-state velocity was achieved when threonine was added to an enzyme-isoleucine-valine complex. A rapid deceleration of reaction rate occurs when isoleucine is added to enzyme-threonine complex, and a similarly rapid acceleration of rate occurs when valine is added to an enzyme-isoleucine-threonine complex.

#### 4.1.18. Attempts at desensitization of *S. pombe* TD

A characteristic of bacterial regulatory enzymes is the relative ease with which they may be caused to lose their sensitivity to their feedback inhibitors. By incubating the enzyme in the proper concentration of mercuric salts, urea or guanidine HCl, or by heating for several minutes at 50 C, one can obtain enzyme which is no longer feedback-sensitive and which has lost little or none of its catalytic activity (Changeux, 1961; Freundlich et al., 1963; Gerhart and Pardee, 1963). All such efforts to desensitize the TD from bakers' yeast were unsuccessful (Magee and de Robichon-Szulmajster, 1968). Similarly, the ATCase of yeast could not be desensitized (Kaplan et al., 1967), nor could the ATCase of *Halobacterium cutirubrum* (Liebl et al., 1969).

An attempt was made to desensitize the TD of *S. pombe*. Aliquots of a crude extract were preincubated for 5 min at 30 C in increasing concentrations of Hg Cl<sub>2</sub>; the samples were then chilled and assayed for TD activity and sensitivity to isoleucine. The results are presented in

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table 4.1.10. At  $5 \times 10^{-4}M$   $Hg^{2+}$ , enzyme activity was inhibited 64%, while isoleucine caused the same inhibition as it did for untreated enzyme. Mercury thus appears to be inactivating the enzyme, but is not

Table 4.1.10. Effect of mercury on TD activity and its regulatory properties

$Hg^{2+}, M$	Activity $\mu moles\ KB / ml\ enz / min$	% inhibition by $Hg^{2+}$	% inhibition by $5 \times 10^{-4}M$ ile
0	4.36	0	94.3
$0^{-5}$	4.38	0	93.7
$5 \times 10^{-5}$	3.78	13.4	91.5
$10^{-4}$	3.57	18.2	92.8
$5 \times 10^{-4}$	1.57	64.0	89.8

causing any differential effect with respect to isoleucine sensitivity. If mercury is causing inhibition by disrupting SH bonds as it is known to do (forms mercaptides with thiol groups), this suggests that the aggregated state of the enzyme is required for TD activity.

In another experiment, aliquots of crude extract were heated at 55 C for increasing periods of time, chilled in ice and assayed for activity and response to isoleucine and valine. From table 4.1.11, it can be seen that although there was fairly rapid inactivation of TD activity (ca. 99% after 10 min), feedback inhibition by 1 mM isoleucine and its reversal by valine remained essentially constant for each period of heating. Thus the TD from S. pombe appears to be as resistant to desensitization as the enzyme from bakers' yeast.

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Table 4.1.11. Effect of heating on TD activity and its regulatory properties

Min at 55 C	Activity $\mu$ moles KB/ml E/min	% inhibition by $10^{-3}$ M ile	% activity in 10 mM val
0	4.27	99.6	57.5
1	3.50	99.7	48.5
3	1.51	98.9	44.0
5	0.47	100	54.5
10	0.05	85.7	42.9
15	0.02	—	—

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Cennamo et al. (1964) report that bakers' yeast TD is almost completely insensitive to inhibition by  $3.3 \times 10^{-4}$ M isoleucine at pH 8.55, while activity remains strong. They interpret this result to mean that the inhibitor site is distinct from the active site. de Robichon-Szulmajster and Magee (1968) confirmed this finding, but showed also that activity could be 100% inhibited up to pH 9.0 by higher isoleucine concentrations.

Enzyme activity and sensitivity to isoleucine were tested at pH 8.0 and 10.0, using a G-25-filtered ammonium sulfate preparation. Tris-phosphate buffer was used in the reaction mixture, and the threonine and isoleucine solutions were adjusted to the appropriate pH with NaOH prior to addition to reaction mixture. Isoleucine at  $5 \times 10^{-4}$ M was used at pH 8.0 and  $10^{-2}$ M at pH 10.0 (it had been shown before (fig. 4.1.3) that this isoleucine concentration caused 93% inhibition at pH 9.0). The results are presented in table 4.1.12. Half the specific activity

Table 4.1.12. Effect of high pH on isoleucine sensitivity of S. pombe TD

pH	isoleucine	Specific activity	% inhibition
8.0	—	1.83	
	0.5 mM	0.12	93.5
10.0	—	0.89	
	10.0 mM	0.85	4.5

remains at pH 10.0, whereas 10 mM isoleucine causes only 4.5% inhibition. The TD from S. pombe appears to be desensitized to isoleucine at high

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pH, suggesting that the catalytic (threonine) site is distinct from the inhibitor (isoleucine) site. No tests were made to determine if this desensitization is reversible (i.e. by lowering pH).

The possibility exists that the isoleucine and threonine sites overlap (perhaps sharing a common group for binding), and that high pH differentially affects the binding of isoleucine at this site. Although the results of this experiment do not exclude this interpretation, the two site mechanism is the most attractive, and the one which will be used here.

Perhaps the only way to obtain a truly desensitized TD is through genetic means: to select for mutants which are resistant to an analog of isoleucine which inhibits growth of wild type cells. This approach has been used successfully by Betz et al. (1971), who isolated mutant strains of S. cerevisiae resistant to thiaisoleucine; these strains were shown to have TD's with decreased sensitivity to isoleucine as inhibitor.

Numerous attempts to isolate such mutants of S. pombe were made, using fluoro-analogs, thiaisoleucine,  $\alpha$ -aminobutyrate, O-ethyl-threonine, norvaline, norleucine, aminochlorobutyrate, cyclopentaneglycine, aza-isoleucine; all were unsuccessful. S. pombe appears to be naturally resistant to most common amino acid analogs.

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Table 4.1.13. Comparison of TD from bakers' and fission yeasts

Property	<u>S. cerevisiae</u>	<u>S. pombe</u>
1. pH optimum	8.0-8.2	9.0
2. <u>Isoleucine</u> pH for opt. inhibition	7.15	7.4
type of inhibition	pseudocompetitive	pseudocompetitive
apparent $K_i$	0.25 mM at pH 7.15	0.15 mM at pH 7.4
effect of increasing pH	increases app. $K_i$	increases app. $K_i$
stimulation	by 0.05-0.02 mM	none
3. <u>Valine</u> effects	activates, reverses ile inhibition	activates, reverses ile inhibition
inhibition	none	slightly at higher pH
4. <u>Saturation kinetics</u> <u>threonine only</u>	normal at pH 7.15 (n = 1.2) sigmoidal at pH 8.0 (n = 1.9) v. sigmoidal at pH 9.0 (n = 3.0)	sigmoidal, pH 7.0-7.4 (n = 1.5) slightly sigmoidal, pH 8.0 (n = 1.3) normal at pH 9.0 (n = 1.1)
Km	decreases with increasing pH 6.6 x 10 <sup>-2</sup> M 6.7 x 10 <sup>-3</sup> M	decreases with increasing pH 1.4 x 10 <sup>-2</sup> M 0.5 x 10 <sup>-2</sup> M
pH 7.0		
pH 9.0		
effect of isoleucine	partially normalizes curve at pH 8.0	increases sigmoidicity
effect of valine	no effect at pH 7.15 normalizes at higher pH's	normalizes at all pH
5. Effect of NH <sub>4</sub> <sup>+</sup>	activation	non-competitive inhibition

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#### 4.1.19. Discussion of TD data

Before discussing the results in terms of a unifying model, a brief summary of the most important experimental findings will be presented. It is impossible to categorize the results as "pH effects", "isoleucine effects", etc. because there is much overlap; they will be listed in the approximate order in which they will be referred to throughout the discussion.

##### 4.1.19.1 Summary of findings pertinent to the model

1. TD in S. pombe is optimally active at pH 9.0, and optimally inhibited by isoleucine at pH 7.4.
2. All isoleucine inhibition curves are sigmoidal ( $n' = 2.9$ ), and the affinity of TD for isoleucine decreases as the pH increases. TD can be almost completely inhibited by isoleucine at all pH up to 9.0; at pH 10.0, the enzyme is desensitized to isoleucine although still active catalytically.
3. Valine reverses isoleucine inhibition and shows cooperative effects in so doing ( $n'' = 1.8$ ).
4. Valine acts as a positive effector, normalizing threonine saturation curves at pH 7.0-8.0; it increases the affinity of TD for threonine by lowering the  $K_m$ .
5. Threonine saturation curves are sigmoidal at pH 7.0 and 7.4, and become hyperbolic at pH 9.0. Isoleucine renders the substrate curves increasingly sigmoidal, while valine normalizes the saturation kinetics and relieves isoleucine inhibition.
6.  $K_m$  for threonine decreases with increasing pH.

7. Isoleucine is a pseudocompetitive inhibitor with threonine, and the antagonism between isoleucine and valine is competitive in nature
8. Leucine is a non-competitive inhibitor of TD activity, and can partially reverse isoleucine inhibition of TD; antagonism between isoleucine and leucine is competitive in nature
9. TD activity increases with temperature up to 37 C, while inhibition by isoleucine decreases with increasing temperature. Isoleucine increases the  $E_A$  of the enzyme-substrate system
10. If TD is preincubated with isoleucine, addition of threonine causes an acceleration in the rate of reaction after a discrete measurable time interval
11. S. pombe TD cannot be desensitized by heat or mercurials without concomitant loss of catalytic activity.

#### 4.1.19.2. Proposal of a model for S. pombe TD

The results from the relatively crude steady-state kinetic studies reported here do not allow one to choose between the two general models for allosteric regulatory enzymes, namely the concerted model (MWC) and the sequential model (KNF). However, the complex interactions of isoleucine and valine with the enzyme can be interpreted adequately in terms of the subunit interactions which are fundamental to both models.

Let us assume, as a working model, that the enzyme is an oligomeric protein containing n subunits, two for simplicity (there is no example to date of a regulatory enzyme with a single polypeptide chain which exhibits the properties of allosteric regulatory enzymes).

Hatfield and Umbarger (1970) provide tentative evidence for a two-subunit TD from B. subtilis. The enzyme may exist in at least two forms in equilibrium: taut (T) and relaxed (R); the existence of intermediate or hybrid forms cannot be determined from our data. The binding of ligands to the enzyme displaces the equilibrium toward that conformation to which the ligand binds best.

The enzyme has at least two binding sites for threonine, and at least two separate sites for binding isoleucine. The enzyme is active in the pH range 7.0 to 10.0; at the lower pH, the T conformation is favoured, in which form the enzyme is assumed to be least active. The T form also has the highest affinity for isoleucine, and this amino acid can displace the equilibrium to favour the T form. Threonine and valine are positive effectors, and thus favour the most active (R) form of the enzyme. Threonine is considered as rather slowly shifting the equilibrium from the T to the R conformation (homotropic cooperative effects of threonine), to which form threonine has the highest affinity. At high pH, the enzyme is already in the R form, so no cooperative effects of threonine are observed. Valine is postulated to act as an activating ligand by binding at a threonine site, in effect functioning as a substrate analog and eliminating threonine cooperative effects at low pH. The inhibitory effects of valine are believed to be caused by valine being able also to bind at a site identical with, or overlapping, an isoleucine site.

This model is based on the concerted MWC model, bearing in mind that the MWC model is a special case of the more general KNF sequential model. The following discussion will attempt to show how the results described in this section support this model for the TD from

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S. pombe.

4.1.19.3. Allosteric sites

The fact that at pH 10.0, concentrations of isoleucine as high as 10 mM cause only about 5% inhibition while catalytic activity is still strong (table 4.1.12) suggests that dissociable active groups on the enzyme are involved in the binding of isoleucine but not in the catalytic process. That is, the isoleucine binding site is distinct from the threonine binding site ("allosteric" in MWC terms). Clearly, in those cases where treatment of TD with heat or mercurials can eliminate isoleucine sensitivity without affecting catalytic activity (Changeux, 1961; Maeba and Sanwal, 1966), one must conclude that separate binding sites for threonine and isoleucine exist on the enzyme. TD from S. pombe could not be desensitized by these procedures (section 4.1.18), as is the case for the enzyme from bakers' yeast (Cennamo et al., 1964; de Robichon-Szulmajster, 1968). Magee has since succeeded in isolating thiaisoleucine-resistant mutants which possess TD's with catalytic activities close to wild type level and which are up to 100-fold less sensitive to isoleucine (Betz et al., 1970). This is strong evidence that the bakers' yeast enzyme has distinct sites for threonine and isoleucine.

Intuitively, one would suspect that a regulatory enzyme which is "competitively" inhibited by a compound which has no steric or structural relationship with the substrate would have separate binding sites for each ligand. In "classical" enzymology, competitive inhibition is shown with steric analogs of the substrate, and it is assumed that the substrate and inhibitor compete for the catalytic

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site. But with regulatory enzymes, the absence of structural relationship between the highly specific inhibitor and the substrate renders unlikely a competition for the catalytic site. The specificity of threonine as substrate and isoleucine as retroinhibitor have been amply demonstrated for the S. pombe TD. It was precisely through such reasoning that the term "allosteric" was coined to describe the effects of such unrelated ligands on regulatory enzymes.

Definitive proof of distinct and separate sites for threonine and isoleucine on the S. pombe TD must await the isolation and study of TD desensitized mutants. However, the fact that high pH causes almost total loss of feedback sensitivity but leaves considerable residual enzymic activity is good evidence for two distinct sites.

#### 4.1.19.4. Isoleucine effects

The pH at which TD is assayed has distinct effects on both activity and interaction with ligands. A pH optimum in the range 9.0 (fig. 4.1.1) makes the S. pombe TD more like the bacterial enzymes, e.g. B. subtilis (Hatfield and Umbarger, 1970), S. typhimurium (Burns and Zarlengo, 1968) and E. coli (Changeux, 1964), and a threonine dehydrase assayed in Neurospora extracts (Yanofsky and Reissig 1953). On the other hand, isoleucine is most inhibitory at pH 7.4, and its effect decreases sharply with increasing pH. In contrast, TD from bakers' yeast is optimally active at pH 8.0-8.2 (Cennamo et al., 1964; de Robichon-Szulmajster and Magee, 1968), and is most sensitive to isoleucine at pH 7.15. A similar effect of pH on enzyme activity and feedback sensitivity was observed with the aspartate transcarbamylase of bakers' yeast (Kaplan et al., 1967).

In view of the effect of pH on isoleucine inhibition, it seems reasonable to propose that at low pH (7.0-7.4), S. pombe TD exists predominantly in the T form, while higher pH values favour the R (most active) form.

Isoleucine was shown to exhibit strong homotropic cooperative effects in its interactions with TD (see fig. 4.1.3, for example). These effects were most pronounced at lower pH, and the affinity of TD for isoleucine was shown to decrease with increasing pH (section 4.1.3). In terms of the model, isoleucine must bind optimally to the T form, which form tends to predominate at lower pH.

Low concentrations of isoleucine did not significantly activate S. pombe TD, no matter whether the threonine concentration used was saturating or lower (fig. 4.1.6). In contrast, the enzyme from S. cerevisiae shows a stimulation of up to 125%, as deduced from curves (Betz et al., 1971). This effect led these authors to postulate an isoleucine stimulatory site on the TD molecule, in addition to the regular inhibition site.

#### 4.1.19.5. Valine effects

As in other system (S. typhimurium, Burns and Zarlengo, 1968; E. coli, Changeux, 1963; S. cerevisiae, de Robichon-Szulmajster and Magee, 1968, to mention only three), valine reverses isoleucine inhibition of S. pombe TD while displaying cooperative effects.

Valine has been shown to act as a positive effector: it exerts positive heterotropic effects by activating TD activity at low threonine concentrations, with consequent normalization of the threonine saturation

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curves at lower pH (fig. 4.1.8). Double reciprocal plots suggested that valine exerts its activating effect by lowering the  $K_m$  for threonine. In the T form of the enzyme (e.g. at lower pH), subunit interactions are greatest ( $n = 1.5$  for threonine) whereas in the R form (which predominates at pH 9.0), the structure of the enzyme loosens and  $n$  approaches 1.0 (fig. 4.1.9). Valine appears to convert TD to the R form where the enzyme has highest affinity for threonine and lowest affinity for isoleucine ( $n$  values for threonine approach 1.0: fig. 4.1.9).

What conclusions do these data allow? As in S. pombe, low valine stimulates and normalizes the threonine saturation curves of TD from R. spheroides (Datta, 1966). This finding led Datta to propose that valine may indeed be acting as a substrate analog by occupying a threonine site (or a site closely associated with it) and eliminating cooperative interactions between threonine molecules. Although there is little steric relationship between valine and threonine, it is nevertheless conceivable that valine may bind at a threonine site on the S. pombe enzyme, but it is surprising that in such circumstances there is not more inhibition by valine. When the homotropic effects of threonine are strongest (pH 7.0-7.4), valine activates the enzyme and normalizes the saturation curves; at high threonine levels, the threonine and threonine + valine curves have the same  $V_{max}$  (fig. 4.1.8 and 4.1.12). Reciprocal plots of these data verify that the antagonism between valine and threonine is competitive in nature, suggesting that valine may bind at some of the catalytic sites. In effect, it then acts as a substrate analog and activates TD by eliminating the strong subunit interactions reflected in the cooperative effects of threonine.

This interpretation of the mechanism of action of a positive effector is invoked by Gerhart and Pardee (1963) to explain the

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activation of E. coli ATCase by maleate and succinate. The nucleotide dCDP was concluded to act analogously in the activation of E. coli (deoxy)-thymidine kinase (Okazaki and Kornberg, 1964).

Valine alone at high concentrations only slightly inhibited TD activity, and in this capacity as well, exhibited cooperative effects (fig. 4.1.11). Valine inhibition is strongest at pH 9.0 (figs. 4.1.8 and 4.1.12) where the affinity for isoleucine is lowest. Although valine activates at very low threonine levels, the inhibition it causes at this pH is non-competitive in nature, because its presence lowers the  $V_{max}$  reached at saturating threonine; this suggests that valine exerts its inhibitory effects on a site separate from the threonine sites. This site could be an isoleucine site, which might, under these conditions (i.e. high pH) bind valine. This is similar to the model of TD for R. spheroides (Datta, 1966), and was not ruled out for the B. subtilis enzyme (Hatfield and Umbarger, 1970). Returning to R. spheroides, valine shows a dual effect on TD: concentrations up to 0.5 mM stimulate TD activity about 135% at low threonine levels, while 10 mM valine causes up to 75% inhibition at all substrate levels (Datta, 1966). Concentrations of valine greater than 0.5 mM render the threonine saturation curve more sigmoidal than with threonine only, an effect similar to that caused by isoleucine. Datta thus concludes that valine may occupy a site identical with, or overlapping the isoleucine site.

The dual binding capacity of the inhibitor site could be an intrinsic property of the R conformation of the enzyme, or might be due to conformational changes induced by the high pH. If valine had a higher affinity for a threonine site than for an isoleucine site,

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then the two effects of valine at high pH could be rationalized. For example, in fig. 4.1.12, valine at 1 mM activates up to 8 mM threonine; up to this concentration of substrate, valine can bind to a threonine site. At about 8 mM threonine, the enzyme should be entirely in the R form, operating maximally, and valine no longer stimulates activity. As the increasing threonine concentration precludes valine from binding at active sites, it becomes inhibitory by binding to the inhibitor (isoleucine) site (s).

The alternative concept that valine binds at specific sites distinct from those for isoleucine and threonine cannot be ignored or ruled out. In B. subtilis, valine was shown to be a non-competitive inhibitor (up to 15% inhibition was observed), suggesting that valine binds at an "activator" site separate from the catalytic site (Hatfield and Umbarger, 1970). A similar valine activator site was proposed for E. coli TD (Changeux, 1963). Valine, on the other hand, does not appear to be inhibitory to the bakers' yeast enzyme (de Robichon-Szulmajster and Magee, 1968).

Proof of one model over another must await total total purification of the S. pombe enzyme, and the isolation and study of selected mutants. This model is intended as provisional; if it stimulates further enquiry and research, then it shall have accomplished its aim.

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#### 4.1.19.6. Interactions of isoleucine and valine with threonine

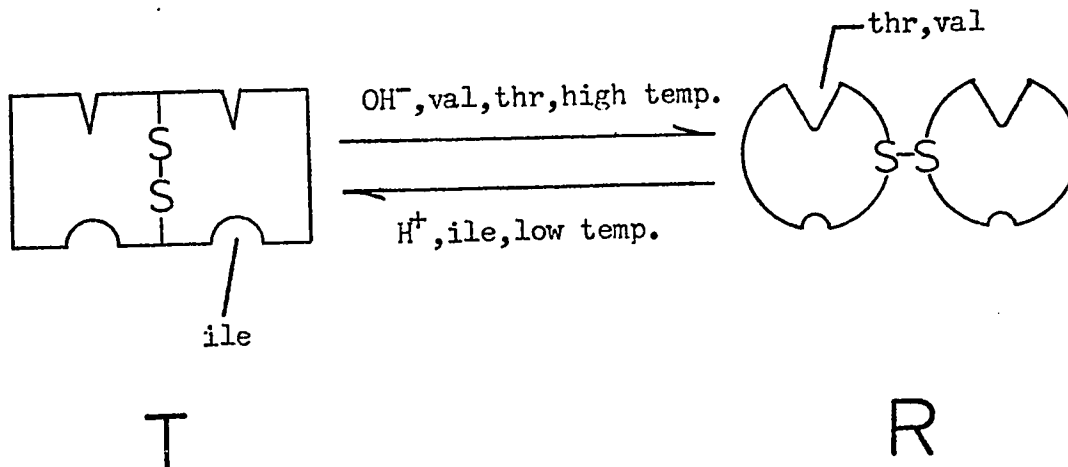
The allosteric properties of S. pombe TD are readily observed when the effects of isoleucine and valine on activity are examined in the presence of increasing concentrations of threonine. It was shown that the  $K_m$  of TD for threonine decreased with increasing pH (section 4.1.8). Thus, low pH favours the T conformation (low affinity for threonine, cooperative homotropic effects), and high pH favours the R form (increased affinity for threonine, loss of homotropic effects).

At the routine pH for assay, the threonine saturation curve is only slightly sigmoidal. With increasing concentrations of isoleucine, the curves become increasingly sigmoidal (fig. 4.1.16); isoleucine shifts the equilibrium  $T \rightleftharpoons R$  towards the T conformation, at low substrate levels, decreasing the affinity of the enzyme for threonine and increasing subunit interactions. Isoleucine inhibition is pseudocompetitive (fig. 4.1.18), and as the threonine concentration is increased, isoleucine inhibition is overcome, i.e. threonine displaces the  $T \rightleftharpoons R$  equilibrium to the form with higher affinity for threonine and decreasing affinity for isoleucine. The nature of this displacement of equilibrium, whether concerted or gradual (sequential with hybrid states of the enzyme) cannot be determined at least from the present data.

Valine reverses isoleucine inhibition and normalizes the saturation curve in the presence of isoleucine (fig. 4.1.16). Valine activates at low threonine levels as well, as the curves in the presence of the valine + isoleucine are more hyperbolic than that with

threonine alone. The competitive nature of the antagonism between isoleucine and valine is similar to observations reported for TD from R. spheroides (Datta, 1966) and B. subtilis (Hatfield and Umbarger, 1970). This result suggests two possibilities. Valine binding at a threonine site can reverse isoleucine inhibition (by analogy with threonine) through a pseudocompetitive type of relation, or valine can also bind at an isoleucine site and displace isoleucine simply by mass action, by virtue of the fact that higher levels of valine are required than of isoleucine for the effect.

With available evidence the first is most appealing. It has already been postulated that valine can bind at a threonine site to achieve its activation effects, and that, in so doing, it favours the R conformation of the enzyme which has highest affinity for threonine and lowest affinity for isoleucine. Various aspects of this model for the TD from S. pombe are summarized in the following scheme:



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Some evidence in support of the conclusions reached about the binding of threonine, isoleucine and valine to S. pombe TD exists in the literature. Freundlich and Umbarger (1963) examined the effects of analogs of the threonine, isoleucine and valine on TD from S. typhimurium. These analogs could be separated into groups on the basis of their ability to act like isoleucine, by increasing stability and inhibiting activity, or like threonine by overcoming isoleucine effects on activity and stability. Group I compounds, including isoleucine and leucine, caused inhibition of activity and increased the sigmoidicity of the threonine saturation curve as well as protecting the enzyme against inactivation at 0 C. These workers proposed that all these compounds bind at the inhibitor site. Compounds in group II (including threonine and valine) antagonized the effects of group I compounds, and stimulated enzyme activity at low threonine concentrations (normalized the substrate curves). These compounds were regarded as binding to the enzyme at the substrate site. They concluded that all of the many compounds tested could be considered to bind at either the isoleucine or threonine sites.

A third (activator) site on E. coli TD for valine was postulated by Changeux (1963) and Monod et al. (1963) because valine was unable completely to overcome isoleucine inhibition. Freundlich and Umbarger (1963) showed (as we have for TD from S. pombe) that valine exerts two effects on the Salmonella enzyme: 1) it activates at low threonine; and 2) it inhibits at higher concentrations of threonine (i.e. the saturation curves cross). However, at low threonine concentrations, they showed that valine could overcome isoleucine inhibition completely. Also, the other compounds of Group II could cause total reversal of isoleucine inhibition. From their similarity in action to threonine in this respect, the simplest view was that these compounds

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combine at the threonine site. In addition,  $Hg^{2+}$  at concentrations which desensitize TD to isoleucine does not antagonize the activation caused by valine or other Group II compounds. However the possibility of a third site is not ruled out.

In another paper, Umbarger et al. (1965) discuss the inhibitory effects of high concentrations of valine. They point out that the fact that  $Hg^{2+}$  can overcome the inhibitory effect of valine as it does for isoleucine, allows one to conclude that valine inhibition is due to its ability to bind to the enzyme at the isoleucine site.

#### 4.1.19.7. Temperature effects

The influence of temperature on activity and regulatory properties of TD from S. pombe can readily be interpreted in terms of conformational changes, strengthening the concept that TD is a subunit regulatory enzyme.

TD activity rises with temperature up to 37 C, increasing temperature displacing the equilibrium to favour the R conformation. The decrease in isoleucine inhibition with increasing temperature is then a necessary consequence, for the R form has much lower affinity for isoleucine than has the T form. At temperatures above 37 C, protein denaturation presumably occurs with concomitant loss of activity and regulatory properties. The increase in the experimental  $E_A$  for TD when isoleucine is present would be due to the increased energy which is required for threonine to convert the T form (which predominates when isoleucine is present) to the more highly active R form. Excess valine present with isoleucine would favour the R form by virtue of its antagonistic effects with isoleucine, so that the  $E_A$  observed in the presence of isoleucine + valine would be expected

to be that measured with thr only.

#### 4.1.19.8. Effects of leucine on TD activity

It was shown that leucine can cause significant inhibition of TD from S. pombe (up to 40% at 10 mM, fig. 4.1.13), and that at low concentration, it partially relieves inhibition caused by isoleucine. The simplest interpretation, given these facts, is that leucine is acting as a valine analog. However, unlike valine, leucine does not activate TD: the saturation curve in the presence of leucine is a hyperbola with lowered  $V_{max}$  (fig. 4.1.14), and the threonine curve in the presence of isoleucine is made only somewhat less sigmoidal by leucine. It may be inferred that leucine does not bind to a threonine site, as was postulated for valine in the activation process.

Leucine may bind at the isoleucine site. This notion is supported by the fact that the antagonism between leucine and isoleucine is competitive in nature (see fig. 4.1.15 B). Moreover, the concentration range where leucine can reverse isoleucine inhibition is of the same order of magnitude as the isoleucine concentration for inhibition. It was shown (fig. 4.1.13) that the ability of valine to reverse inhibition by isoleucine was increased by the presence of leucine; leucine, at low concentrations, may act synergistically with valine to further reduce inhibition by isoleucine via competition for isoleucine sites between leucine and isoleucine.

Relatively high levels of leucine are required for inhibition (i.e. 10 mM produces 40% inhibition), and the slight cooperative effects of threonine are unaffected (fig. 4.1.14). This same high concentration of leucine makes the threonine curve in the presence

of isoleucine + leucine only less sigmoidal than that with threonine and isoleucine alone; this would be expected if leucine were competing with isoleucine, which amino acid would have the advantage because it can produce cooperative effects.

Leucine inhibition of TD activity has been observed in some other systems, e.g. E. coli (Changeux, 1964), B. licheniformis (Leitzmann and Bernlohr, 1968) and bakers' yeast (Cennamo et al., 1964). In bakers' yeast, the leucine inhibition curve is sigmoidal and inhibition of activity up to about 90% is achieved by 20 mM leucine; along with other compounds, isoleucine and leucine stabilized this enzyme against inactivation by dilution. Umbarger and Brown (1958) report as well that in E. coli, leucine is a competitive inhibitor of TD activity and has the kinetics of a bimolecular reaction. Freundlich and Umbarger (1963) proposed that leucine binds at the inhibitor (isoleucine) site, because leucine, like isoleucine, increased the sigmoidicity of the substrate curve and protected the enzyme against inactivation. These effects of leucine and isoleucine were reduced by high pH and treatment with mercurials.

#### 4.1.19.9. Threonine-dependent transitions of TD

When TD is extracted from S. pombe, it may be considered to be poised in an "inactive" or "active" state, depending on the concentrations of isoleucine and valine contaminating the crude extract, the pH of the extraction buffer, the stabilizing compounds present, etc. At pH 8.0, the routine pH for assay, TD activity gave linear plots with time at all substrate concentrations, indicating that the enzyme is largely in the active (R) state under these conditions, and that even low concentrations of threonine can shift the equilibrium  $T \rightleftharpoons R$  completely enough to give essentially optimal enzymic activity.

The effects of isoleucine and valine on TD in rate-time plots can be adequately interpreted in terms of the model. A linear relationship exists between activity and time when threonine alone is present. When enzyme is preincubated in isoleucine, the amino acid will bind to the enzyme and "pull" it to the T conformation. When threonine is subsequently added, a finite time period elapses while the first few molecules of threonine are bound and displace the equilibrium in favour of the R state; at this point, the enzyme can bind threonine more efficiently, and at the same time, its affinity for isoleucine is decreasing. The result is a new, inhibited steady-state rate is established, which rate depends upon the isoleucine level present. The same sort of lag would be expected if the enzyme is preincubated with both isoleucine and valine, as isoleucine binds with greater affinity than either threonine or valine (comparing  $K_i$ 's for isoleucine and valine, and the  $K_m$  for threonine: much less

isoleucine is required to saturate its site than threonine or valine for their sites). The addition of threonine, then, starts the shift of the enzyme's conformation to the R state, with valine facilitating the process. The steady-state rate achieved is essentially that in the presence of substrate only, i.e. isoleucine inhibition is overcome. Preincubation in valine alone has no effect because the enzyme would be completely in the R form when substrate is added and is therefore immediately optimally active.

Some of these responses of TD to its effectors might be an example of the hysteretic enzyme concept of Frieden (1970). Hysteretic enzymes are those that respond slowly (in terms of some kinetic characteristic) to rapid changes in ligand concentrations. These slow changes are manifest by a lag in the response of the enzyme to changes in the ligand level. Such a hysteretic response allows a time-dependent buffering of some metabolites, which effect may be of importance in pathways which utilize common intermediates or in which there are multiple branch points.

The time-dependent lag when threonine is added to an enzyme-isoleucine complex can thus be considered a hysteretic response. Similar results (a gradual decrease in reaction velocity) were observed when isoleucine and threonine were added simultaneously to enzyme, when enzyme was added to threonine and isoleucine, and when threonine was added to enzyme-isoleucine complex, indicating that the activating effect of valine is exerted slowly. However, the sudden addition of isoleucine or valine to a running reaction (enzyme-threonine complex) resulted in a rapid deceleration or acceleration, respectively, of reaction velocity. Thus there appears to be a lack of

a hysteretic response when sudden changes in isoleucine or valine are applied to an in-course reaction.

No attempts were made to discover the nature or type of hysteretic response observed here, i.e. whether it is due to isomerization of enzyme states or molecular weight change as a result of aggregation of subunits.

The TD from B. subtilis is considered to be a hysteretic enzyme (Hatfield and Umbarger, 1968, 1970; Hatfield et al., 1970; Hatfield, 1971); it was shown for this enzyme that these slow responses are a consequence of a slow, ligand-induced isomerization rate between active and inhibited states of the enzyme. In contrast, the enzyme from E. coli appears not to be hysteretic (Hatfield, 1971): the enzyme reacted immediately to sudden increases in the concentration of either isoleucine or valine, but did show a slow response only when abnormally high concentrations of threonine were added to the enzyme-isoleucine complex.

These results for the S. pombe enzyme are tentative; they are, however, reproducible with both crude extracts and partially purified enzyme. Further proof will be obtained when such experiments are performed with pure enzyme, and using the coupled assay which allows continuous monitoring of changing reaction velocities. It should also be possible to magnify the hysteretic responses in such a system by varying parameters such as threonine and isoleucine concentrations, preincubation times, pH, etc. The lag observed when threonine is added to enzyme-isoleucine complex should lengthen when assay temperature is lowered; if this should be the case, it may be possible to study isomerization states of the enzyme (if they exist).

4.1.19.10. Nature of *S. pombe* TD

TD from *S. pombe* can best be described as a modulator dependent cooperative (MDC) system in the terminology of Sanwal (1970), at least at its pH optimum for activity. It shows normal kinetics at pH 9.0 except in the presence of the negative effector, isoleucine. It is not, however, strictly an MDC system, for such systems always give hyperbolic rate-concentration plots in the absence of inhibitors. This is not always the case for the *S. pombe* enzyme, for it yields sigmoidal saturation plots in the absence of effectors at low pH (7.0, 7.4) and very slightly sigmoidal plots at pH 8.0; these are the properties ascribed to a modulator independent cooperative (MIC) system. Perhaps a better term to describe this TD system at lower pH would be "modulator influenced cooperative", because other factors are involved in the cooperative response, such as low pH which favours the T conformation which binds isoleucine optimally.

It was never tested whether the enzyme would give a hyperbolic saturation plot when saturated with isoleucine so that all the enzyme is in the T state. It would be difficult to test this aspect of the system experimentally, as the enzyme was shown to be inhibited up to 100% by isoleucine in the pH range 7.0 to 9.0. In any case, it exhibits most of the properties of a true MDC system at the pH optimum for activity: the enzyme exists, at pH 9.0, primarily in the R state which has high affinity for substrate. In unsaturating concentrations of inhibitor, the enzyme becomes distributed into R and T states, and the response to substrate is sigmoidal because threonine binds with different affinities to the two states. Isoleucine, in addition, was shown to exhibit pseudocompetitive inhibition with respect to threonine.

The inferred existence of hybrid states of the enzyme implies the KNF sequential model. This only seems to emphasize that steady state kinetic results allow only educated guesses about the behaviour of regulatory enzymes, and do not allow choice between one model or another. Such conclusions can only be reached after physico-chemical studies on binding of effectors and subunit isomerizations have been accomplished on the purified protein.

## 4.2. ACETOHYDROXYACID SYNTHETASE

### 4.2.1. Requirements for activity

In a preliminary experiment to determine whether or not the S. pombe AHAS shows the same requirements as the enzyme from other systems, a crude extract from S. pombe was prepared as described for TD (4.2.1.a). The reaction mixture was prepared as described (3.2.2) except that Tris-HCl buffer (80  $\mu$ moles / ml, pH 6.5) was substituted for  $PO_4$  buffer. The results are presented in table 4.2.1. It can be seen that the S. pombe AHAS requires thiamine pyrophosphate (TPP) and flavin adenine dinucleotide (FAD). Divalent cations have a small stimulatory effect, and manganese ion can substitute for magnesium ion. Routinely, the AHAS assay mixture was made up to contain TPP, FAD and  $Mg^{2+}$ . It may be noted here that the AHAS from bakers' yeast was not stimulated by any of these co-factors (Magee and de Robichon-Szulmajster, 1968 ).

Table 4.2.1 Requirements of S. pombe AHAS.

Conditions	$\mu$ moles acetolactate formed in 30 min
Complete assay mixture	0.139
— enzyme	—
— pyruvate	—
— TPP	0.081
— FAD	0.087
— $Mg^{2+}$	0.117
complete ( $Mn^{2+}$ instead of $Mg^{2+}$ )	0.124

#### 4.2.2. Effect of pH on AHAS activity

The effects of pH on the AHAS activity in a crude extract of S. pombe are illustrated in fig. 4.2.1. The pH profile shows no evidence of more than one acetolactate synthetase, which functions optimally at pH 6.3-6.6. Very little AL-synthesizing activity was detected above pH 7.5. Moreover, the synthetase is sensitive to inhibition by L-valine in its optimal pH range: at pH 6.5, 10 mM valine caused 73% inhibition. This fact indicates that the activity being measured is indeed that of the biosynthetic enzyme.

In all microorganisms investigated so far, the biosynthetic AHAS is optimally active in the alkaline pH range (i.e. pH 7.2-8.0). Several organisms possess a second AL-forming enzyme at pH 6.0 (E. coli and N. crassa, Radhakrishnan and Snell, 1960; A. aerogenes, Halpern and Umbarger, 1959; P. radiatus, Satyanarayana and Radhakrishnan, 1963); in all cases, this pH 6.0 enzyme is insensitive to valine.

To verify that extracts of S. pombe do not possess a pH 8.0 AHAS, the experiment described in fig. 4.2.2 was performed. Again, no peak of AHAS activity was observed in the alkaline pH range. S. pombe thus appears to be unique in that its biosynthetic AHAS is optimally active at pH 6.5.

For routine assays, then, AHAS activity in extracts of S. pombe was assayed at pH 6.5; additives such as pyruvate, L-valine or other amino acids, were all adjusted to this pH prior to addition to the reaction mixture.

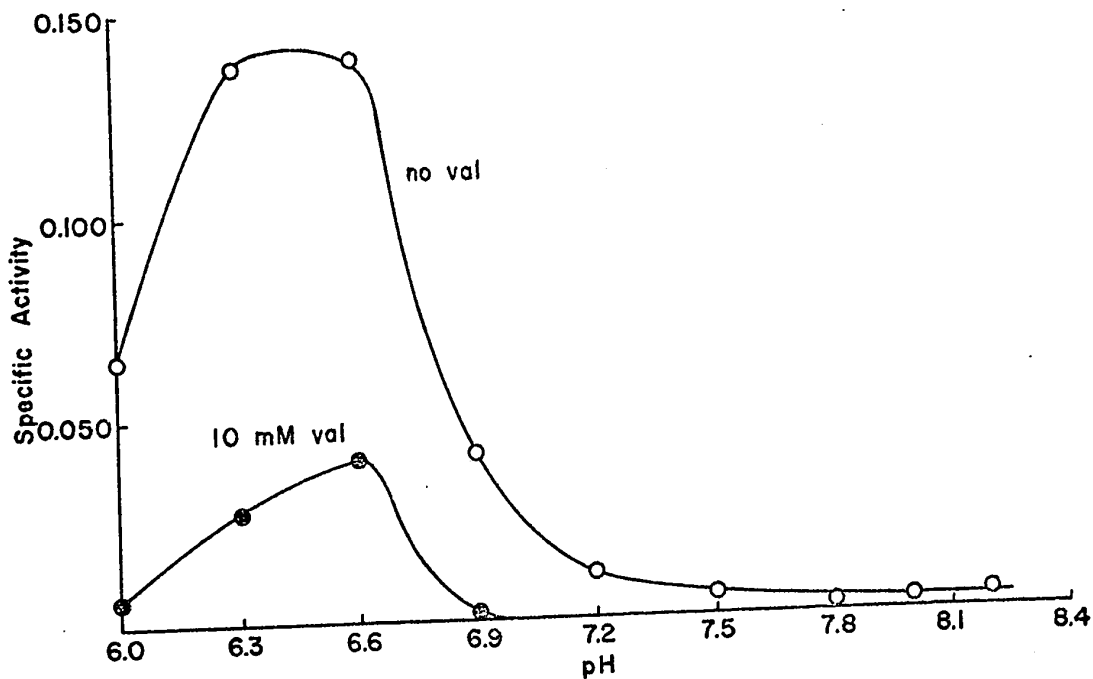


Fig. 4.2.1. Effect of pH on ANAS activity and valine sensitivity. Enzyme source was crude extract (45.0 mg protein / ml) made in 0.1 M potassium phosphate buffer, pH 7.5, containing 20% glycerol. Activity was assayed in the standard assay mixture (50  $\mu$ l for 30 min) at the pH values indicated. Feedback sensitivity was measured in the presence of 10 mM L-valine. AL formed was determined as outlined in methods (section 3.2.2).

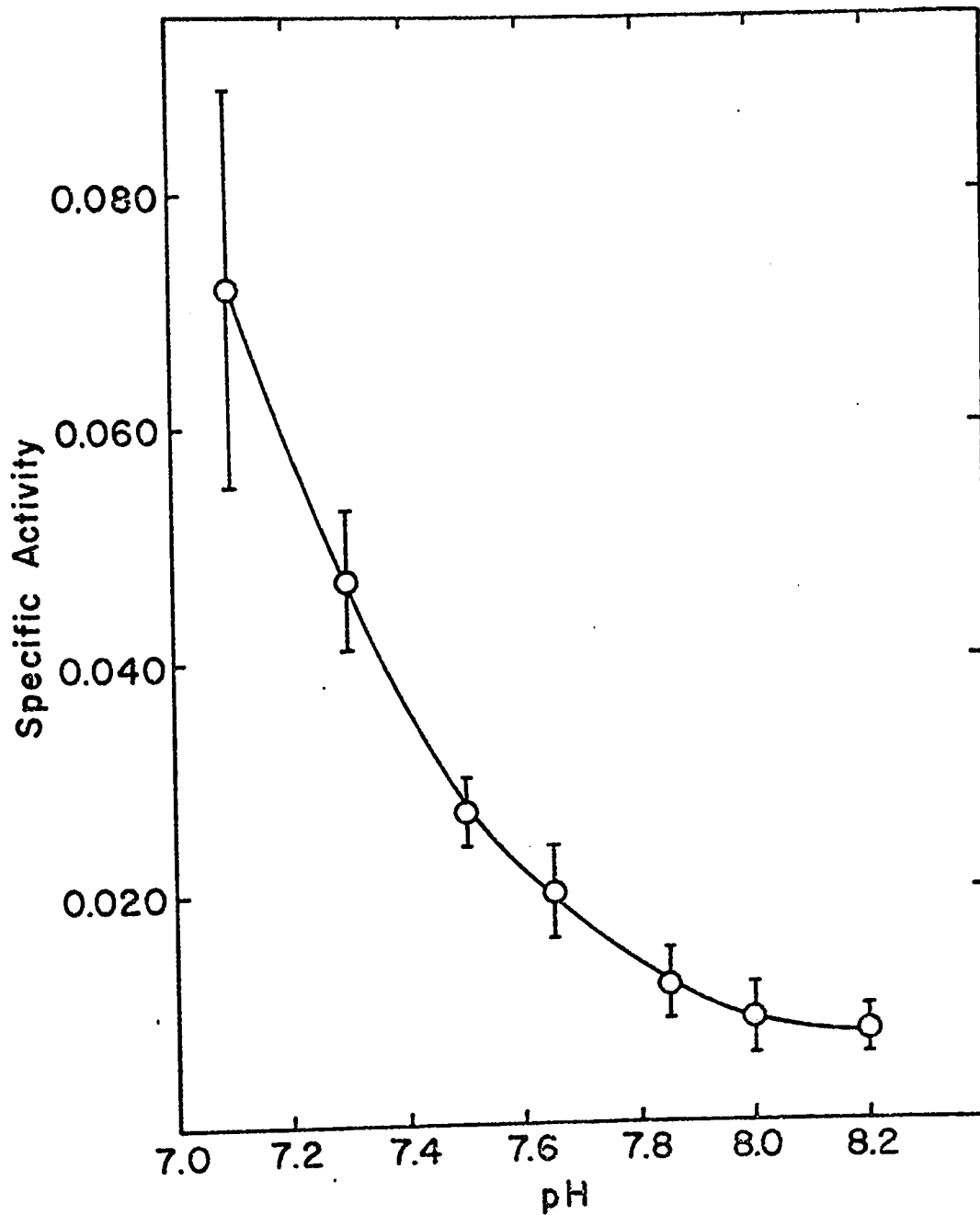


Fig. 4.2.2. AHAS activity in alkaline pH range. Crude extract (36.2 mg protein / ml) prepared as described in fig. 4.2.1 was used. Activity was assayed (50  $\mu$ l for 30 min) in quadruplicate reactions at the pH values indicated; results are expressed as specific activities with standard deviations from the mean.

#### 4.2.3. AHAS activity in *S. cerevisiae*

As a check on the method of assay being used, the AHAS activity of *S. cerevisiae* strain D237-10b (3.1.2) was measured in the pH range 7.0-8.2. Bakers' yeast is known to possess a biosynthetic synthetase which functions optimally between pH 7.0-7.5 (Magee and de Robichon-Szulmajster, 1968a). The results of such an experiment are presented in fig. 4.2.3. A pH optimum of 7.65 was obtained for the biosynthetic AHAS of this strain, in good agreement with the results of Magee and de Robichon-Szulmajster (1968a). It may be concluded that the results obtained with the enzyme from *S. pombe* are not an artifact of the assay procedure.

#### 4.2.4. Effect of valine on AHAS activity

Valine is the known feedback inhibitor of the biosynthetic AHAS in a number of microorganisms (Umbarger, 1969). It was observed in an earlier section (4.2.2) that the enzyme from *S. pombe* is sensitive to 10 mM L-valine in the pH range for optimal catalytic activity. In the experiment described here, AHAS activity was assayed in increasing levels of L-valine; from fig. 4.2.4, it can be seen that enzyme activity is maximally inhibited (ca. 75%) by concentrations of valine greater than 0.8 mM. The concentration of valine which gave 50% inhibition was determined by inspection to be 0.3 mM.

The data presented in table 4.2.2 show that the inhibitory effect of valine is specific towards AL formation; valine affects only the total amount of acetoin measured in the differential assay, not the levels of free acetoin.

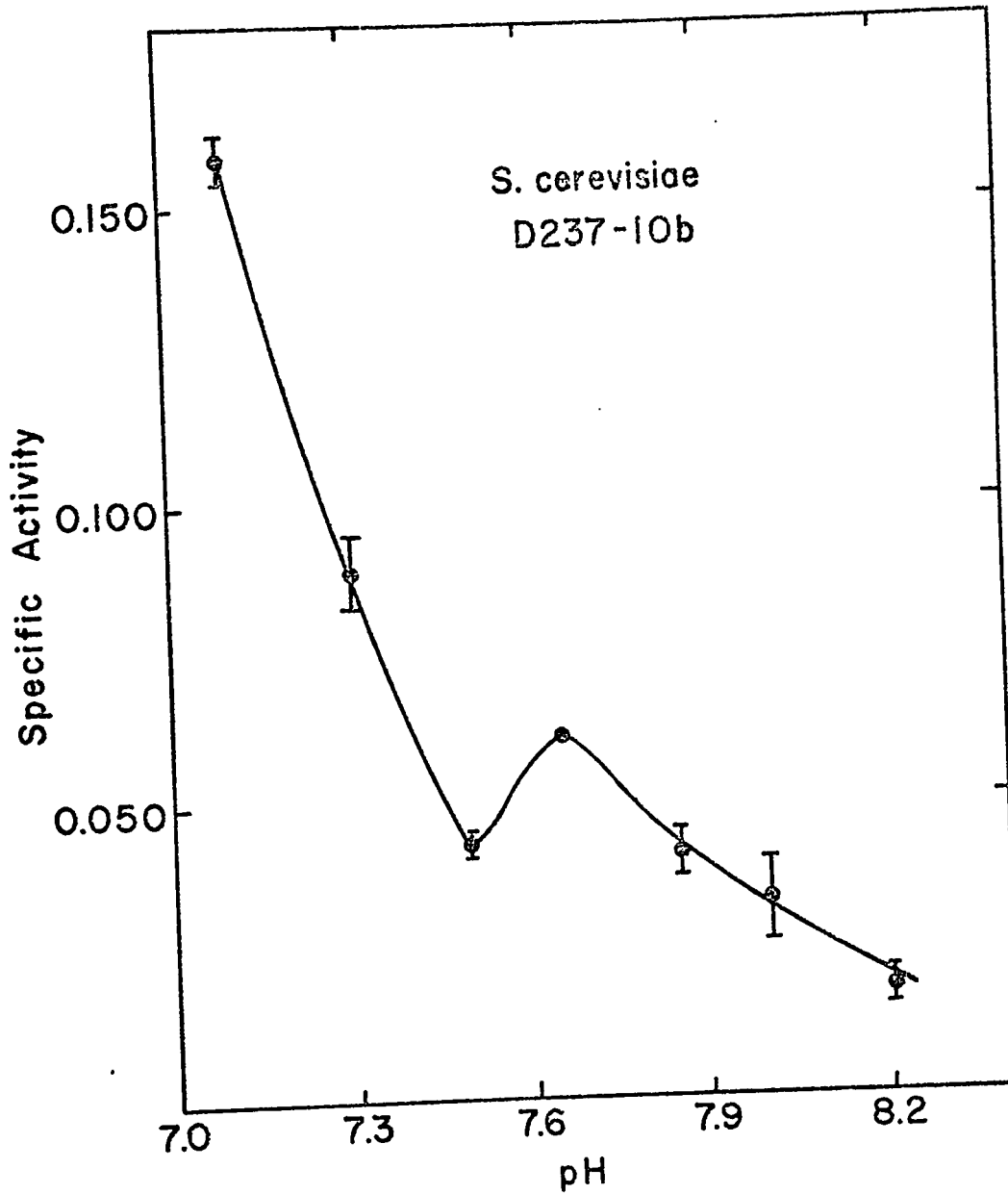


Fig. 4.2.3. Activity of *S. cerevisiae* AHAS in alkaline pH range. Cells of *S. cerevisiae* strain D237-10b were grown and harvested as described (section 3.1.2); a crude extract was prepared as for *S. pombe* (fig. 4.2.1). AHAS activity was assayed in triplicate at the pH values indicated, using the standard reaction mixture; 50  $\mu$ l (1.6 mg protein) were incubated at 30 C for 30 min. Activities are presented with standard deviations from the mean.

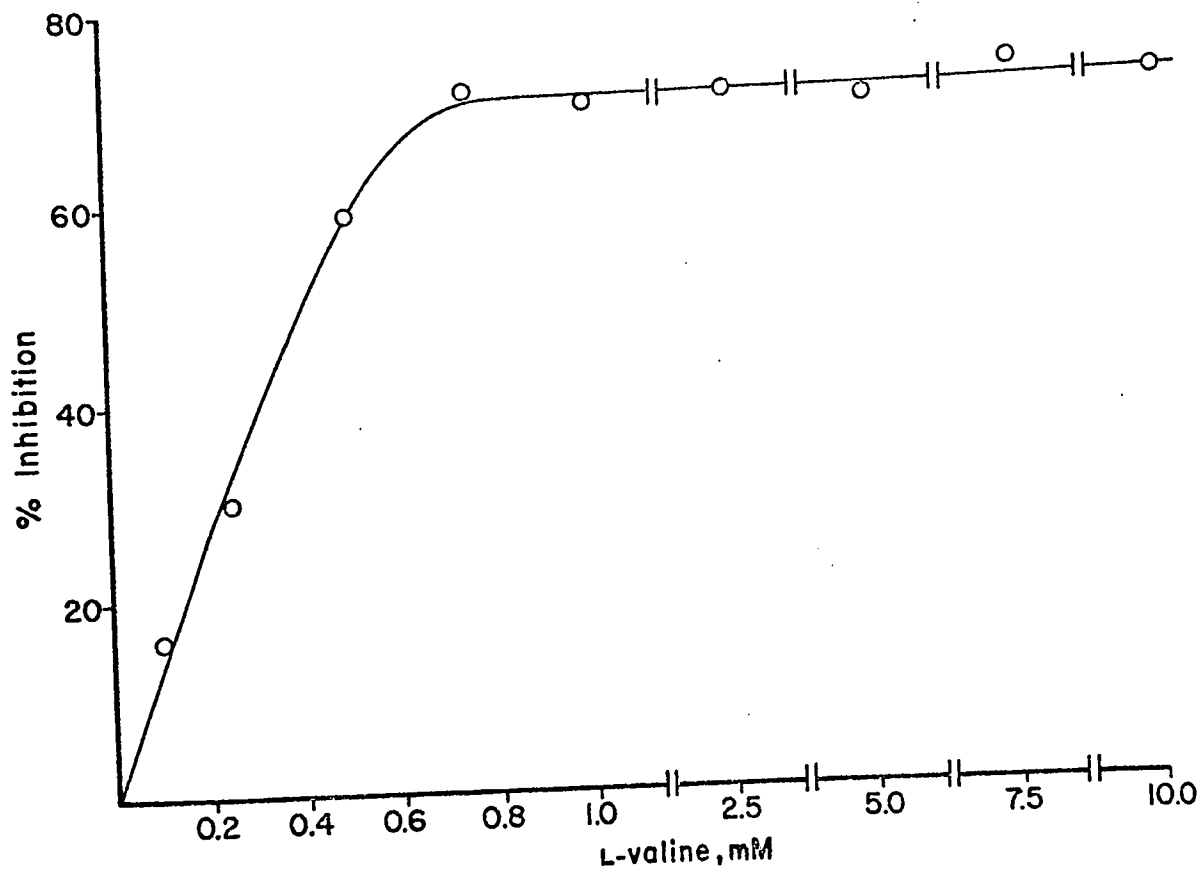


Fig. 4.2.4. Influence of valine concentration on *S. pombe* AHAS. Crude extract, from cells grown on glycerol as carbon source, was assayed at pH 6.5 in reaction mixtures containing increasing concentrations of L-valine, as indicated.

Table 4.2.2. Specificity of valine inhibition of S. pombe AHAS

L-valine mM	OD <sub>540</sub> mp			% inhibition
	Acetoin Total	Free	$\alpha$ -acetolactate	
none	0.240	0.073	0.167	
0.5	0.145	0.067	0.078	59.3
10.0	0.126	0.069	0.057	73.3

#### 4.2.5. Partial purification of S. pombe AHAS

##### 4.2.5a Preparation of crude extract

In the course of experiments to achieve at least a partial purification of the AHAS from S. pombe, it was found that the enzyme was considerably more stable when extracted into P-G buffer\* which had been supplemented with 1 mM DTT, 5 mM Mg SO<sub>4</sub>, 2 mM TPP and 20  $\mu$ g / ml FAD. The latter three compounds were prepared in a concentrated stock solution and diluted 100-fold upon addition to the buffer; this stock solution was stable for at least a month stored at -20 C.

The cells from 2 500-ml cultures were smashed as previously described (4.2.1.a); the resultant extract was centrifuged at 31,000 x g for 20 min, at 4 C. The supernatant was then subjected to further purification steps, or filtered through a small column (0.9 x 15 cm) of Sephadex G-25 for immediate use. At this stage, the enzyme retained its activity for about 12 hrs, stored at 4 C.

\* 0.1 M potassium phosphate, 20% glycerol

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#### 4.2.5b Protamine sulfate treatment

Solid PS, to give a final concentration of 0.2% (w/v) in the CE, was dissolved in a volume of extraction buffer  $1/10$  the volume of the CE. This concentrated PS solution was added slowly by drops to the CE, which was continuously stirred in ice. The mixture was stirred a further 15 min after addition of PS, then centrifuged as before; the supernatant should be clear at this stage. Although this step usually resulted in a small loss of activity ( $< 10\%$ ), the specific activity of the supernatant was somewhat higher.

#### 4.2.5c Ammonium sulfate fractionation

The PS supernatant was brought to 35% saturation with ammonium sulfate by the addition of 1.96g salt / 10 mls solution; the salt was added slowly in small amounts to the continuously-stirred supernatant at 4 C. After all the  $(\text{NH}_4)_2 \text{SO}_4$  had been added, stirring was continued for a further 20 min; the solution was centrifuged as before and the supernatant retained.

The supernatant was then made 50% saturated with  $(\text{NH}_4)_2 \text{SO}_4$  by further addition of 0.87 g salt / 10 mls solution. The mixture was stirred for 20 min at 4 C and centrifuged. The pellet, which contained most of the AHAS activity, was dissolved in a small volume of extraction buffer (usually 1 ml) and the volume of the dissolved  $(\text{NH}_4)_2 \text{SO}_4$  precipitate was recorded.

#### 4.2.5d Filtration through Sephadex G-25

For this step, Sephadex G-25 or G-50 work equally well; G-25 was used most often because of the ease in pouring a column and its greater flow rate.

The gel was used in a small column (0.9 x 15 cm) and was pre-equilibrated with the buffer and additives used for extraction and throughout the purification. 1.0 ml of enzyme solution from the  $(\text{NH}_4)_2 \text{SO}_4$  step was applied to the column and eluted with the same buffer. The enzyme is diluted about two-fold in this step, but retains all of its original activity.

The enzyme at this stage of purification is about 5-8 fold purified over activity in the crude extract, and is relatively stable at 4 C. It is inhibited by valine to the same extent as is activity in the CE, and can be frozen overnight and thawed again for use without significant loss in activity.

Table 4.2.3 shows the results of a typical purification of AHAS following the above outlined procedure. A greater yield of AHAS protein can be obtained by using cells grown in minimal medium supplemented with 5 mM L-isoleucine; under these growth conditions, the synthetase is derepress approximately two-fold over the level in cells grown on unsupplemented minimal medium (section 4.6.3).

Table 4.2.3 Partial purification of AHAS from *S. pombe*

Fraction	Volume mls	Total protein	Total activity	Specific activity $\mu$ moles AL/min/mg P	Yield %	Purification
crude extract	17.6	739.2	69.34	0.094	100	
$(\text{NH}_4)_2 \text{SO}_4$ , 35-50%	1.8	68.6	41.62	0.607	60.0	
Sephadex G-50	4.1	66.8	41.00	0.614	59.1	6.5

#### 4.2.6. Nature of acetolactate formed by *S. pombe* AHAS

Juni and Heym (1956) reported that pyruvic oxidase preparations from pigeon breast muscle, and *E. coli* extracts both form a racemic mixture of  $\alpha$ -acetolactate. In order to determine whether the AL formed by the AHAS from *S. pombe* is the biologically active isomer, acetolactate decarboxylase (AD) from *A. aerogenes* was added to a reaction mixture in which an extract of *S. pombe* had synthesized AL. The bacterial AD is specific for the dextrorotatory isomer of AL (Juni, 1952a), and will decarboxylate only one-half of a synthetic (racemic) preparation of AL.

AD was prepared from *A. aerogenes* as described in section 3.1.3. A crude extract of *S. pombe* was prepared as described (4.2.5a); 50  $\mu$ ls of this extract were added to 1.0 ml of standard AHAS reaction mixture containing 25  $\mu$ moles pyruvate instead of the usual 500  $\mu$ moles. Low substrate was used, along with a long incubation time (30 min), in order that there be as little pyruvate as possible left in the reaction mixture when the reaction was stopped with 0.1 ml 2.5 N NaOH. Heated AD preparation (0.4 ml) was then added to the control tube (without pyruvate) and stopped reaction tubes; the tubes were incubated an additional 20 min, and these reactions were stopped by addition of 0.2 ml 1 M Zn (OH)<sub>2</sub> and the volumes were adjusted to 2.0 mls with water. Acetoin was determined in 0.2 ml aliquots of these solutions with and without acid treatment (section 3.2.2).

The results are presented in table 4.2.4. It can be seen that after 20 min incubation with AD, only 1.5  $\mu$ moles AL were detected, as compared with 6.3  $\mu$ moles which were originally formed by the *S. pombe* AHAS. Thus it appears that at least 75% of the AL formed by the fission yeast

AHAS is of the biologically-active form.

Table 4.2.4. Decarboxylation of S. pombe acetolactate by A. aerogenes

AL decarboxylase

Conditions	μmoles acetoin		acetolactate μmoles
	-acid	+acid	
AL formed by <u>S. pombe</u> AHAS in 30 min	12.1	18.4	6.3
After 20 min in presence of <u>A. aerogenes</u> extract	14.9	16.4	1.5

4.2.7. Test for acetolactate decarboxylase in S. pombe

In addition to its role as a valine precursor, acetolactate also serves as an intermediate in acetoin biosynthesis in A. aerogenes (Juni, 1952a) and in N. crassa (Radhakrishnan and Snell, 1960). Moreover, the synthesis of acetoin is favoured at pH ca. 6.0. Although it had been demonstrated that AL is not an intermediate in acetoin formation in S. cerevisiae, which does not possess AL decarboxylase (Juni, 1952b), it was necessary to determine if this enzyme was present or not in extracts of S. pombe. If this yeast does not have AL decarboxylase activity, then it may be reasonably assumed that the AL formed by AHAS at pH 6.5 is directed towards valine biosynthesis.

A crude extract from S. pombe was prepared as before (4.2.5a).

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AL decarboxylase activity was assayed as previously described (3.2.8): 50  $\mu$ ls of CE were incubated at 30 C for 10, 20 and 30 min. The reaction was stopped and the free acetoin was determined as described (3.2.7).

Table 4.2.5. Lack of acetolactate decarboxylase in S. pombe

Conditions of assay	OD <sub>540 m<math>\mu</math></sub>		
	10min	20min	30min
complete reaction mixture	0.176	0.179	0.193
RM minus enzyme	0.177	0.188	0.189
RM minus AL	0.000	0.001	0.004

Table 4.2.5 shows the results obtained under three different assay conditions. It can be seen that the same amount of acetoin was formed in the presence as in the absence of extract. Moreover, the amount of acetoin formed is not proportional to incubation time. The acetoin measured must therefore arise from spontaneous decarboxylation of AL in the acid milieu of the reaction mixture.

As the method of assay used here is sensitive enough to pick up the activity if it existed, it may be concluded that S. pombe has no AL decarboxylase activity. Similar results were obtained whether cells grown on glucose or glycerol were used. The possibility that the enzyme is present in S. pombe cells but is unstable and deteriorates during preparation of the CE cannot be excluded.

4.2.8. Levels of AHAS in glucose - and glycerol - grown cells

It was reported by Coukell and Polglase (1969) that the AHAS from E. coli B is subject to catabolite repression when the cells are grown in high concentrations of glucose as carbon source; the enzyme is de-repressed about 2-fold when the cells are grown on low concentrations of glucose or on any concentration of a poor energy source such as glycerol. Moreover, the nature of the carbon source had a greater effect on the formation of AHAS than had valine + isoleucine + leucine + pantothenate.

S. pombe was tested for growth on various carbon sources other than sugars (e.g. Krebs cycle intermediates, asparagine, acetate) in an effort to determine whether the yeast cells could be made to produce higher amounts of AHAS, and if, under these conditions, the enzyme might be more stable for kinetic studies. The only compound other than glucose which could serve as sole carbon source was found to be glycerol.

Growth on glycerol was considerably slower (generation time doubled) and, as can be seen from table 4.2.6, the cells produced only half as

Table 4.2.6. AHAS levels in cells grown on different carbon sources

Carbon source	Specific activity $\mu\text{moles AL/min/mg P}$
glucose	$0.234 \pm 0.037$
glycerol	$0.122 \pm 0.043$

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much AHAS under these conditions (specific activities given with standard deviations from the mean). This effect of glycerol was not specific for AHAS: TD levels were about 35% lower, and DH activity was about 25% lower in similar extracts. No enzymes other than these were tested from glycerol-grown cells, except pyruvate decarboxylase (which will be discussed in detail in a later section).

#### 4.2.9. Effect of substrate concentration on AHAS activity

The production of AL by AHAS as a function of pyruvate concentration was investigated using extracts of cells grown in glucose and glycerol. The substrate saturation curves obtained are presented in fig. 4.2.5. While the synthetase from glycerol-grown cells exhibited a hyperbolic response to increasing pyruvate concentrations (Hill  $n = 1.1$ ), the enzyme from glucose-grown cells gave a pronounced sigmoidal saturation curve, the  $n$  value for which was 1.9 (fig. 4.2.7a). The data obtained with the glycerol extract gave a linear double reciprocal plot (inset of fig. 4.2.5), from which an apparent  $K_m = 7 \times 10^{-2}M$  could be estimated. When the data obtained with the glucose extract were rearranged for a double reciprocal plot, a good linear plot was obtained when  $1/V$  was plotted against  $1/S^2$ . From these results, it appeared that the carbon source on which the cells were grown had a profound effect on the kinetics of the AHAS from these cells.

The apparent  $K_m$  of S. pombe AHAS for pyruvate was determined to be  $4.8 \pm 1.5 \times 10^{-2}M$ ; this value is the average of 11 separate determinations, obtained with glycerol extracts and partially purified glucose preparations. This value is higher than the  $K_m = 8.6 \times 10^{-3}M$  shown by

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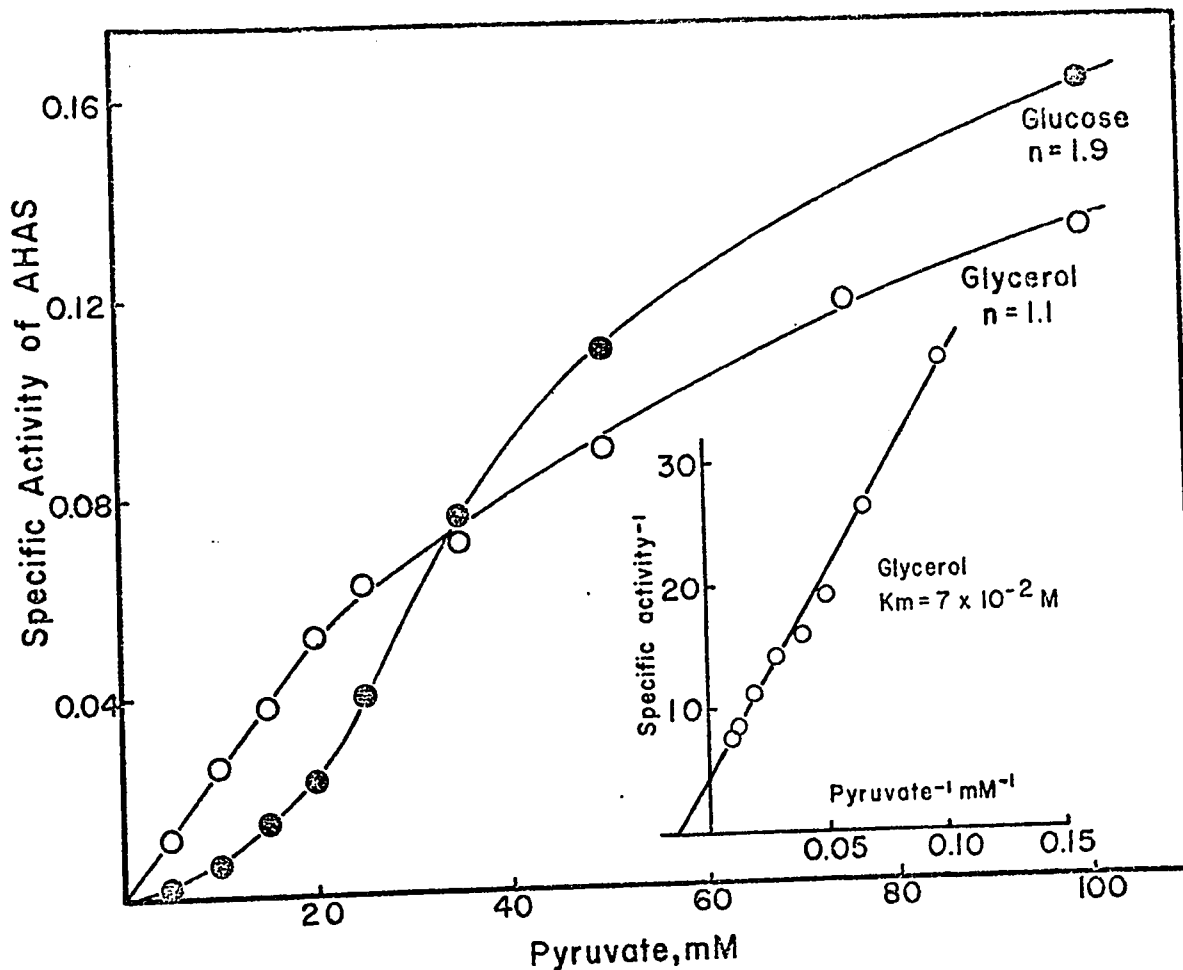


Fig. 4.2.5. Pyruvate saturation curves for AHAS extracted from glucose- and glycerol-grown cells. Crude extracts used were prepared as described in section 4.2.5a; 50  $\mu$ l of each extract were incubated 30 min in reaction mixtures containing pyruvate ranging in concentration from 5-100  $\mu$ moles / ml. AL formed was determined as acetoin (section 3.2.2). Inset shows Lineweaver-Burk transformation of results obtained with glycerol extract.

the bakers' yeast AHAS (Magee and de Robichon-Szulmajster, 1968a).

All attempts to normalize the saturation curve obtained with extracts from glucose-grown cells were unsuccessful. The amino acids L-isoleucine, L-valine, L-leucine and L-threonine, added singly and in combination, had no effect. In an attempt to determine whether thiol groups were involved, the enzyme was preincubated in  $Hg^{2+}$ , with no effect on the pyruvate curve; inclusion of  $Hg^{2+}$ , in the reaction mixture was similarly ineffective. This may indicate that the sigmoidicity of the saturation curve may not be a function of the enzyme protein (e.g., subunit interactions), but rather may be due to limitation of substrate or cofactor(s) by another enzyme. Aldehydic inhibitors of pyruvate decarboxylase (which enzyme requires TPP and  $Mg^{2+}$ , and which has optimal activity in the acid pH range) were tried: acetaldehyde, propionaldehyde, furfuraldehyde (Juni, 1961), and all were found to have no effect on the sigmoidicity of the substrate curve. Finally, a crude extract from glucose-grown cells was heated at 100 C for 5 min and the denatured protein was removed by centrifugation; when aliquots of this boiled extract were added to an assay system containing AHAS from glycerol-grown cells, a hyperbolic saturation curve was still obtained, indicating that no small heat-stable factor in the glucose extract is involved in modifying the kinetics of AHAS towards pyruvate.

4.2.10. Subjection of cells to change in carbon source and its effect on AHAS activity

If the altered kinetics of AHAS with respect to substrate concentration is a function of the carbon source used in the growth medium, then addition of glucose to a culture utilizing glycerol as a C-source should cause the kinetics of substrate saturation of the AHAS in those cells to change from non-cooperative to cooperative. The results of an experimental test of this hypothesis are shown in fig. 4.2.6. The substrate curve obtained with the extract from the glycerol cells was a rectangular hyperbola with a Hill coefficient = 1.2 (fig. 4.2.7B). The data for the enzyme from the cells which had received glucose gave a sigmoid saturation plot with a Hill  $n=1.6$  (fig. 4.2.7B). Thus, after only 4 hrs in glucose, the kinetics of AHAS were cooperative, although these effects were not as pronounced as those shown by the enzyme from cells grown only on glucose.

4.2.11. Excretion of free acetoin by *S. pombe*

In fermenting yeast cells (e.g. growing on glucose), pyruvate can be enzymatically transformed, via several pathways, to acetaldehyde (and lactic acid), acetoin (acetylmethylcarbinol) and acetate (Fruton and Simmonds, 1958). In yeast, acetoin is a by-product of pyruvate decarboxylase (Juni, 1952b), and this enzyme is optimally active at pH 6.0 and requires  $Mg^{2+}$  and TPP (Barman, 1969). It was therefore decided to investigate the excretion of acetoin into the growth medium by *S. pombe*, and to determine whether the formation of acetoin was influenced by the C-source used in the medium. Acetoin was determined as described (3.3.2)

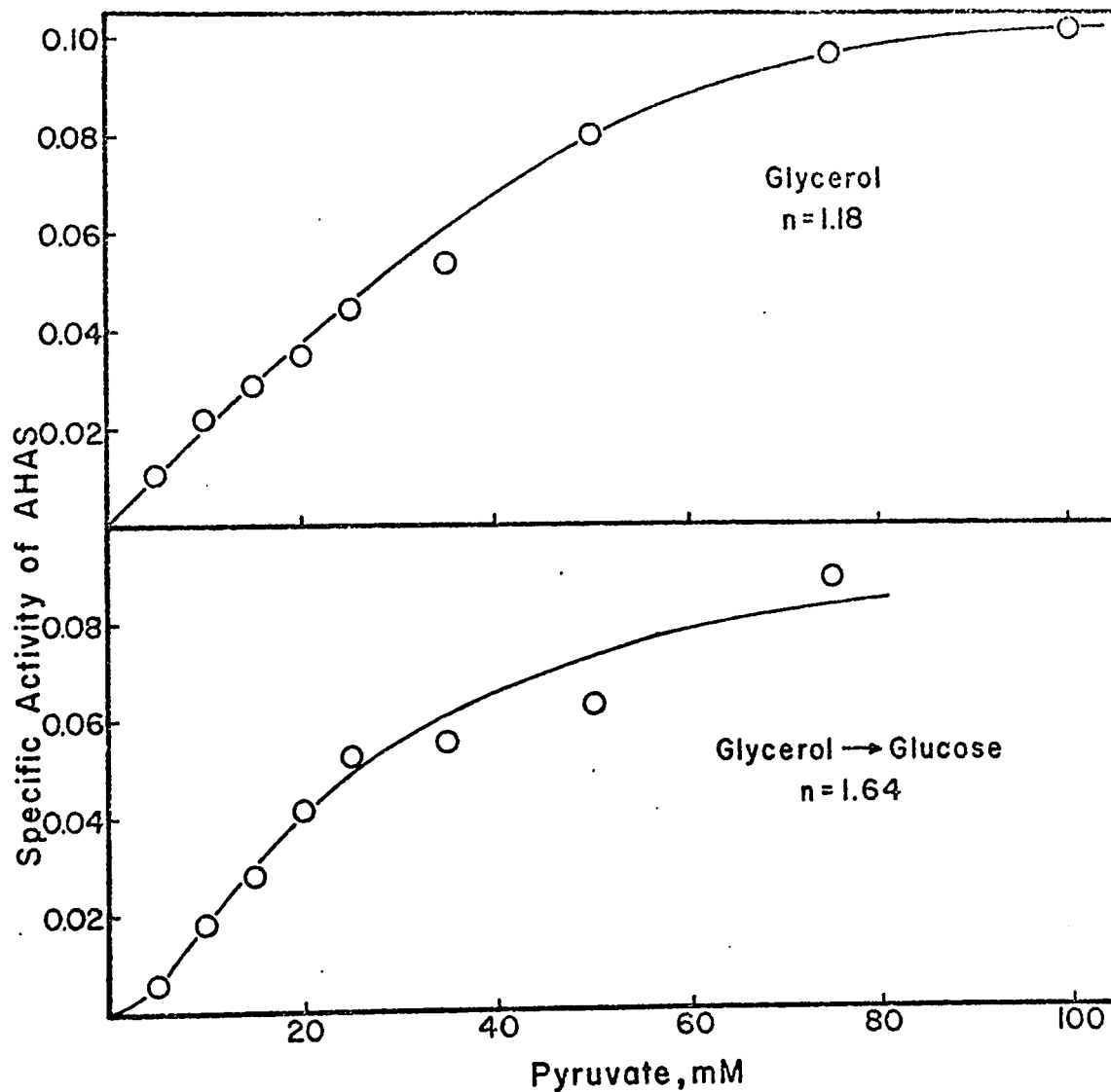


Fig. 4.2.6. Influence of carbon source change on AHAS activity. Cells of *S. pombe* were cultured in 1 l EM with 4% glycerol as carbon source, to OD<sub>660</sub> reading of 190 units. The culture was divided in two: one half was grown in glycerol medium an additional 5 hrs to 226 Klett units. To the other half of the culture was added 50 ml 20% glucose and the cells were grown an additional 4 hrs to 214 Klett units. The cultures were harvested and crude extracts prepared as before. AHAS activity of the extracts was assayed in the standard reaction mixture supplemented with pyruvate ranging from 5-100  $\mu$ moles / ml; 20  $\mu$ l of glucose extract and 50  $\mu$ l of glycerol extract were incubated for 30 min. AL was determined as acetoin in the Westerland test (section 3.2.2).

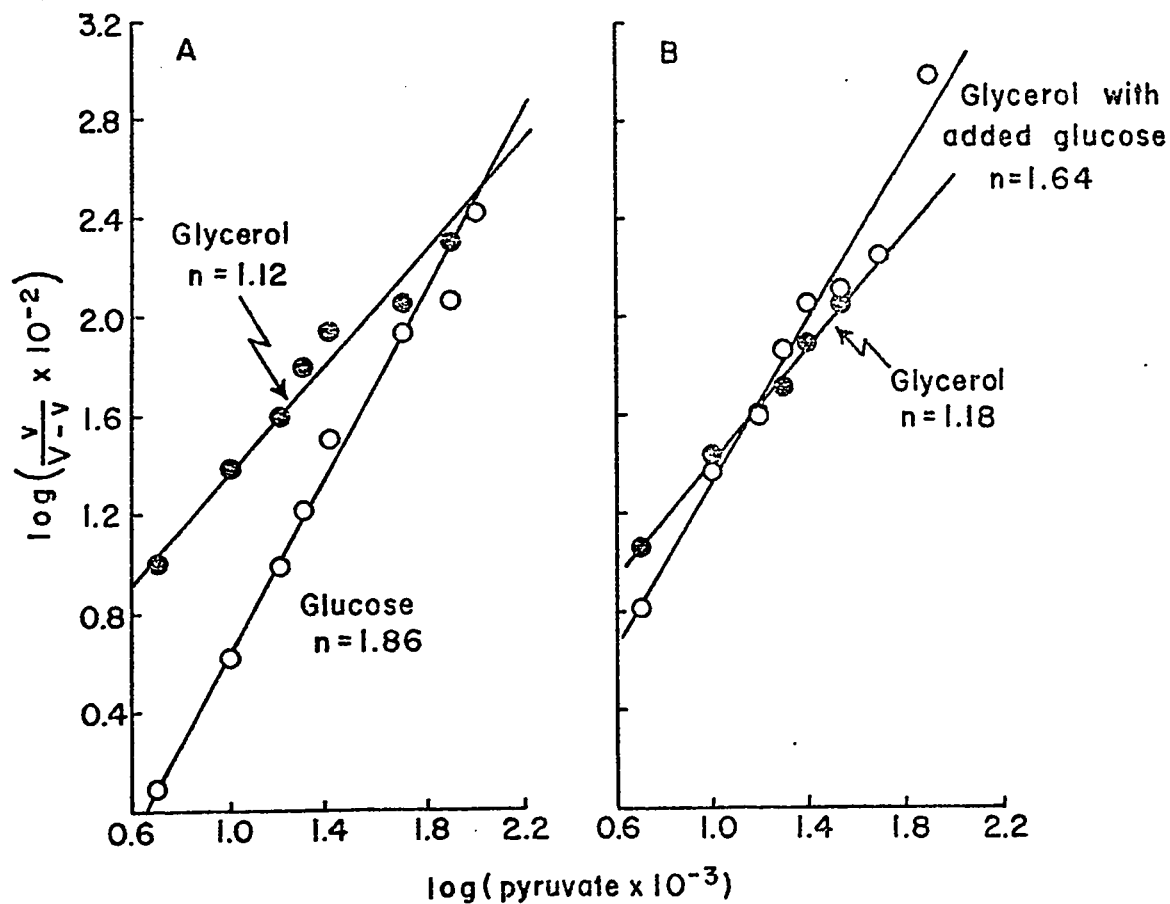


Fig. 4.2.7. Effect of substrate saturation on AHAS activity, as shown by the empirical Hill plot. A: data taken from results in fig. 4.2.5; B: data from fig. 4.2.6.

in culture fluids after harvesting of the cells, and the pooled results from many experiments are presented in table 4.2.7. It can be seen that glucose-grown cells excrete about 13 times as much acetoin as do glycerol-grown cells.

Table 4.2.7. Excretion of free acetoin by S. pombe

Carbon source	$\mu$ moles acetoin excreted per ml culture fluid
Glucose	54.1
Glycerol	4.3

4.2.12. AHAS activity in cells grown on glucose + acetoin

After observing that glucose-grown cells excrete large quantities of acetoin into the growth medium, it seemed reasonable to determine whether excess acetoin added to the growth medium would have any effect on the AHAS activity of those cells. From fig. 4.2.8, it can be seen that the AHAS extracted from cells grown in glucose medium supplemented with acetoin exhibited a hyperbolic pyruvate saturation curve; the Hill value of 1.2 indicates slight cooperative effects of pyruvate, but this may be due to scatter of the points as a result of the dilutions involved in the assay procedure (fig. 4.2.9).

The activity of AHAS from cells grown in glucose medium supplemented with excess acetoin is similar to that of glycerol-grown cells with

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Fig. 4.2.8. AHAS activity in cells grown in glucose minimal medium supplemented with excess acetoin. Acetoin, to give a final concentration of 0.05 M in the medium, was added to the 20% glucose solution to be used, and this final solution was filter-sterilized immediately prior to addition to the medium. The cells from an overnight culture were harvested and an extract prepared from them as usual. AHAS was assayed in the standard reaction mixtures with the pyruvate concentration varied as indicated. Inset shows Lineweaver-Burk transformation.

Fig. 4.2.9. Effect of exogenous acetoin on AHAS activity, as shown by the empirical Hill plot. Data are from fig. 4.2.8.

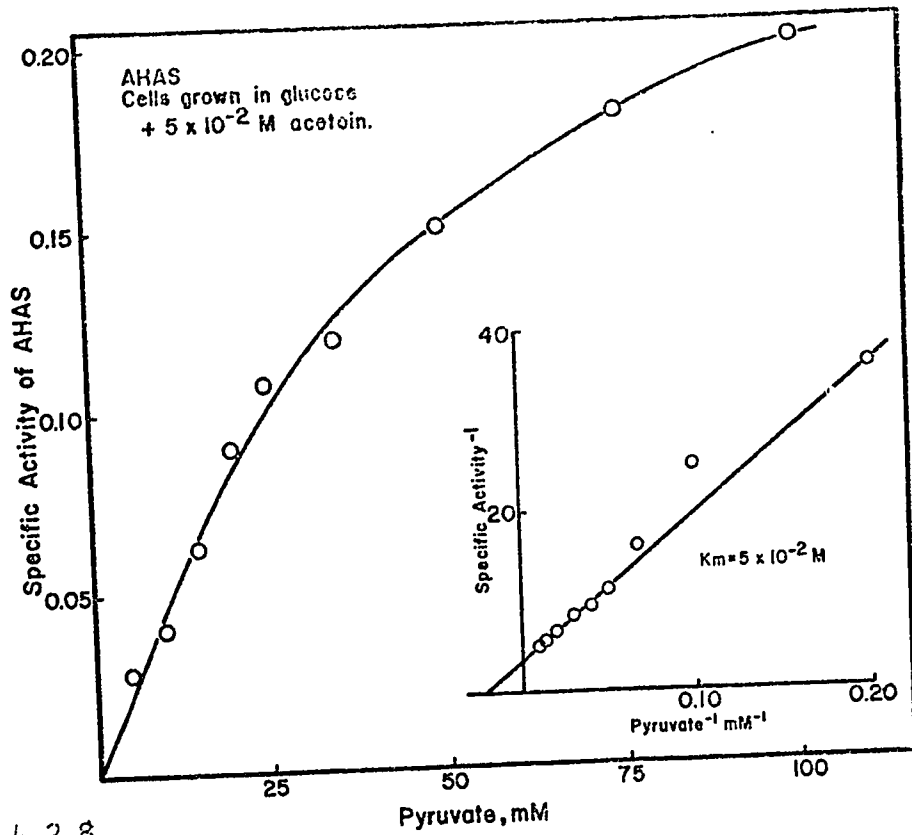


Fig. 4.2.8

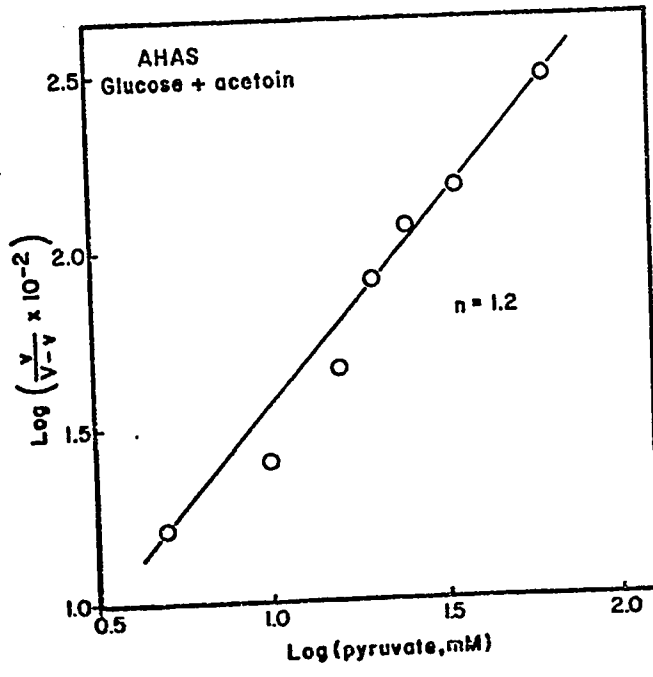


Fig. 4.2.9.

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respect to pyruvate saturation kinetics and Hill coefficient, i.e. slight or no cooperative effects of pyruvate. In addition, the  $K_m$  of  $5 \times 10^{-2}M$  is in good agreement with that obtained for the enzyme from glycerol-grown cells (e.g.  $7 \times 10^{-2}M$ , fig. 4.2.5); the specific activity of 0.244 of this synthetase preparation, in the presence of saturating pyruvate, is double that of AHAS from glycerol cells (table 4.2.6). It may be concluded that acetoin is somehow involved with the activity of AHAS, at least in glucose-grown cells.

#### 4.2.13. Effect of acetoin on AHAS activity in vitro

In view of the results described in the previous section, the effect of acetoin added to the reaction mixture was tested. Fig. 4.2.10 shows that, despite the scatter of points for the acetoin curve, acetoin in vitro does not normalize the pyruvate saturation curve. Acetoin does not, therefore, exert its effect at the enzymic level; rather, its effect is produced in the intact cell and it must then act by permitting more efficient use of substrate or inhibiting some competing element at the molecular level.

#### 4.2.14. Studies on pyruvate decarboxylase (PD)

It was suggested by Juni (personal communication), that the sigmoidal response of AHAS from glucose-grown cells to increasing pyruvate concentration might be due to competition, at low substrate levels, for the common substrate (pyruvate) between AHAS and pyruvate decarboxylase. Acetoin synthesis is mediated by this enzyme (PD), and in addition the enzyme functions in the same pH range as AHAS and requires two of the

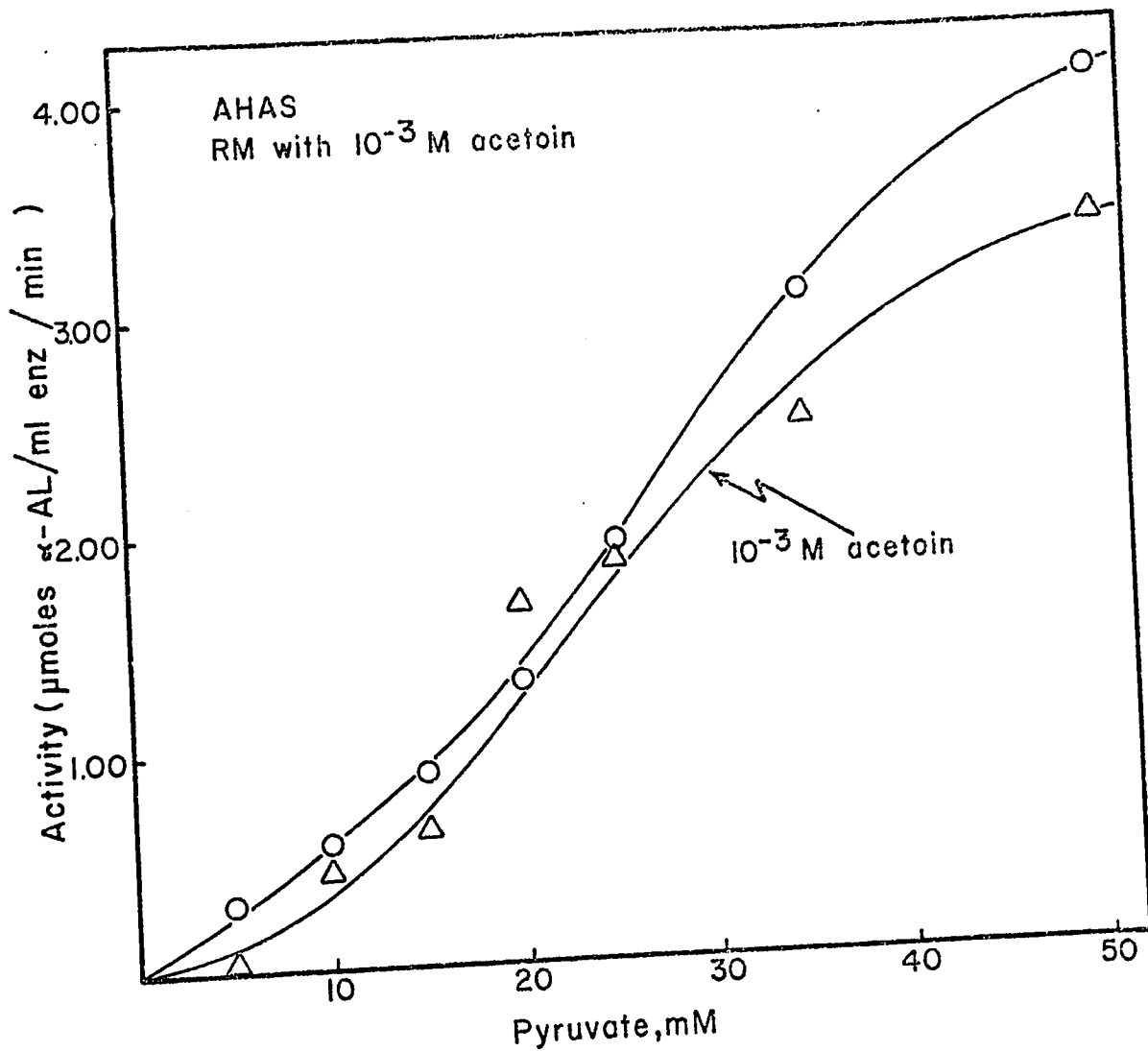


Fig. 4.2.10. AHAS kinetics and effect of acetoin included in the reaction mixture. Crude extract (20 $\mu$ l for 30 min) was assayed in standard assay mixture supplemented with acetoin in a final concentration of 1 mM; pyruvate level was varied from 5-50  $\mu$ moles / ml.

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same cofactors (TPP and  $Mg^{2+}$ ) for activity. This competition would be strong in vitro at low pyruvate concentrations, and the enzyme able to function most efficiently at low substrate concentration would act as a drain of pyruvate with respect to the activity of the other. The concept of regulation through enzyme competition has been reviewed by Holzer (1961), where examples are given for the regulation of carbohydrate metabolism in yeast by competition between enzymes for the same substrate as well as for the same coenzyme systems. It was to follow up these leads that the following studies on PD were undertaken.

#### 4.2.14.1. Effect of enzyme concentration

For pyruvate decarboxylase studies, it was found that a 1/50 dilution of a crude extract from glucose-grown cells gave activities of PD which could be assayed in the 0 - 0.500 OD range of the spectrophotometer scale. Fig. 4.2.11 shows that PD in such a preparation gave a linear response of activity with enzyme concentrations up to 100  $\mu$ ls diluted CE (2.5 mg protein).

#### 4.2.14.2. PD levels in cells grown under different conditions

The activity of PD in extracts of cells of S. pombe grown on glucose or glycerol was investigated. Extracts from both types of cells were prepared as above; the glycerol extracts were routinely diluted 1/25 in buffer containing  $Mg^{2+}$  and TPP prior to assay, because of the lower activity of PD in these cells. From the results shown in table 4.2.8, it can be seen that cells grown on glucose

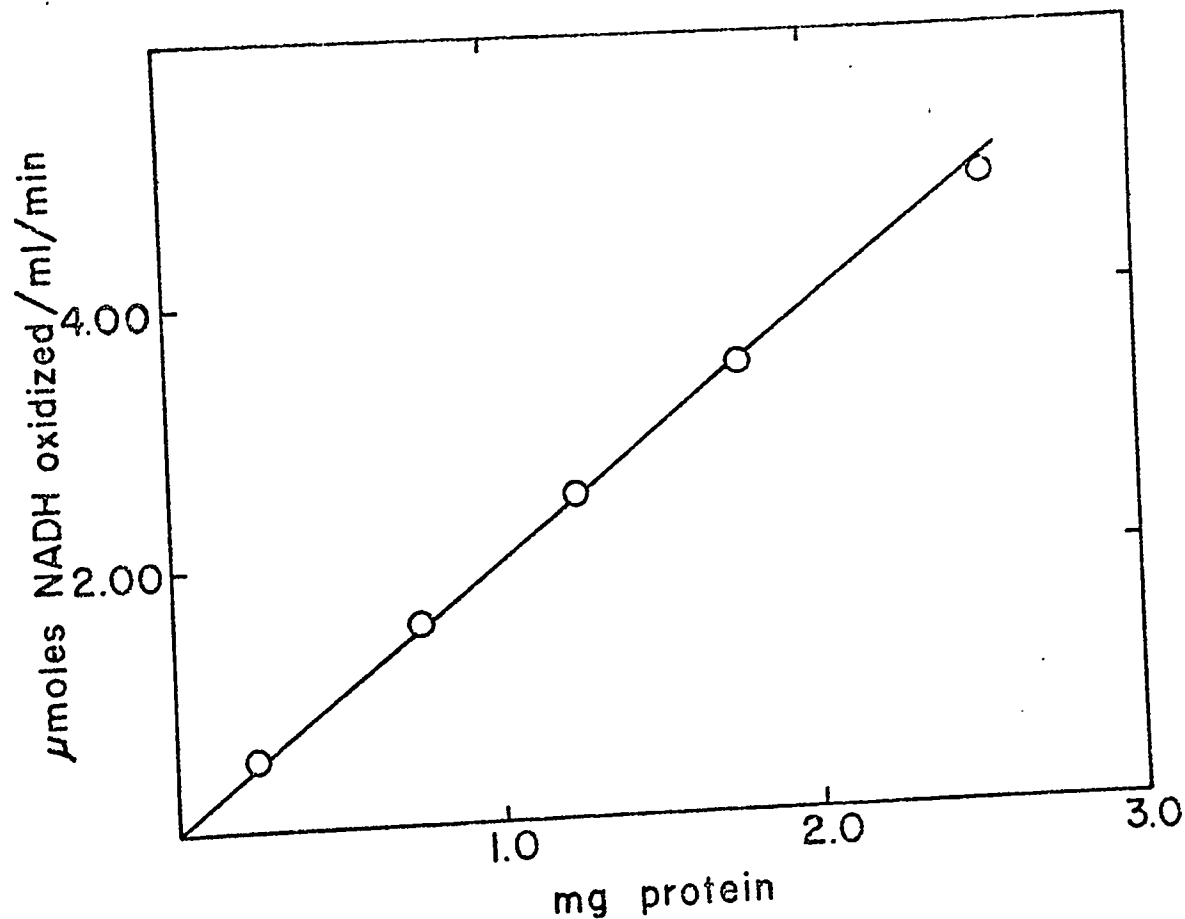


Fig. 4.2.11. Activity of pyruvate decarboxylase (PD) in S. pombe. Crude extract was prepared as for AHAE (section 4.2.5a); the extraction buffer was at pH 6.5, and FAE was omitted. PD activity was assayed as described in section 3.2.6, using 10, 30, 50, 70 and 100  $\mu$ l of a 1/50 dilution of crude extract.

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contain over twice as much PD activity as do cells grown on glycerol (specific activities expressed with standard deviations from the mean; each mean is the average of PD determinations from 6 extracts).

Table 4.2.8. Pyruvate decarboxylase activity in S. pombe

Carbon source for growth	Specific activity $\mu\text{moles NADH oxidized/min/mg P}$
glucose	$1.49 \pm 0.31$
glycerol	$0.61 \pm 0.16$

4.2.14.3. Effect of pyruvate on PD activity;  $K_m$  values

Typical pyruvate saturation curves for PD isolated from glucose- and glycerol-grown cells are illustrated in fig. 4.2.12. PD exhibits normal Michaelis-Menten kinetics, regardless of the carbon source for cell growth. The apparent  $K_m$  of PD from glucose-grown cells is 1.4 mM (average of 3 determinations), while that of glycerol-grown cells is 4.2 mM. It is not known whether this difference in  $K_m$  for PD from the two types of cells is significant: the  $K_m$  for glycerol-grown cells was determined only once. If this difference is significant, several explanations can be advanced; these will be mentioned in the discussion to follow.

PD is present in larger amounts in glucose-grown cells than in

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Fig. 4.2.12. Pyruvate saturation kinetics of PD. Activity was assayed with crude extracts from glucose-grown (29.4 mg protein / ml) and glycerol-grown (27.5 mg protein / ml) cells; 50  $\mu$ ls of 1/50 diluted glucose extract, and 100  $\mu$ ls of 1/25 diluted glycerol extract, were incubated in the standard assay mixtures (section 3.2.6) in which the pyruvate concentrations were varied as indicated. Reactions were monitored in a Gilford 2400 recording spectrophotometer, and enzyme activities were calculated from the slopes of the recorder plots. All activities were corrected for endogenous NADH oxidase activity. Insets show Lineweaver-Burk transformations.

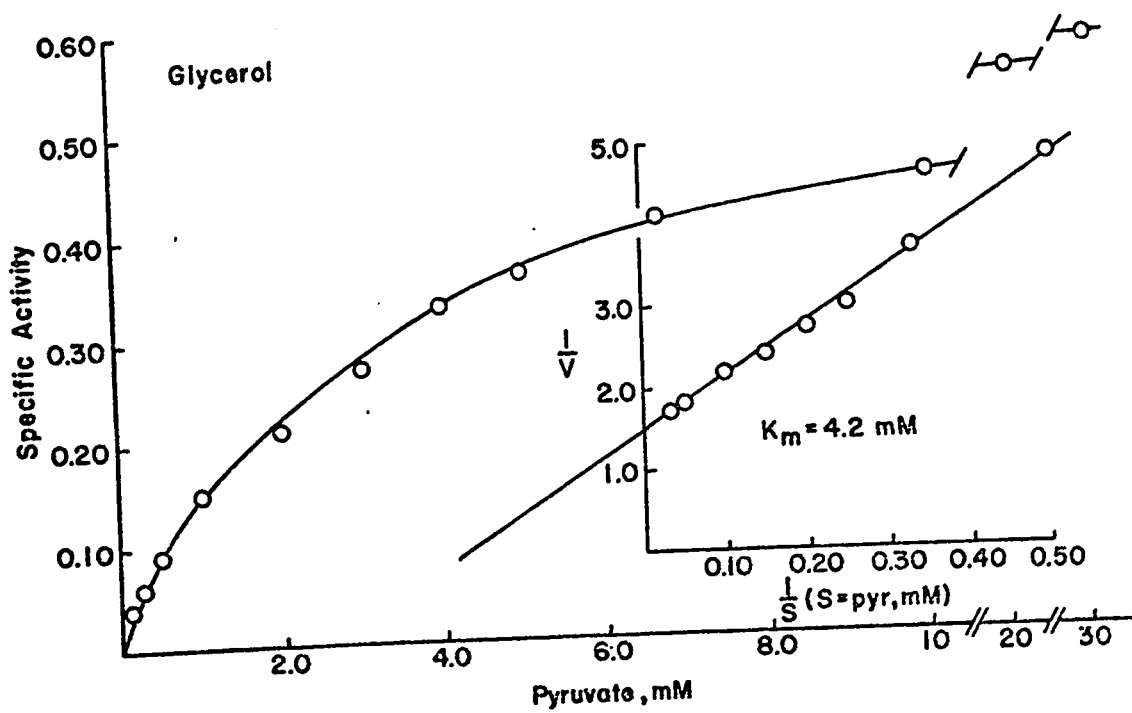
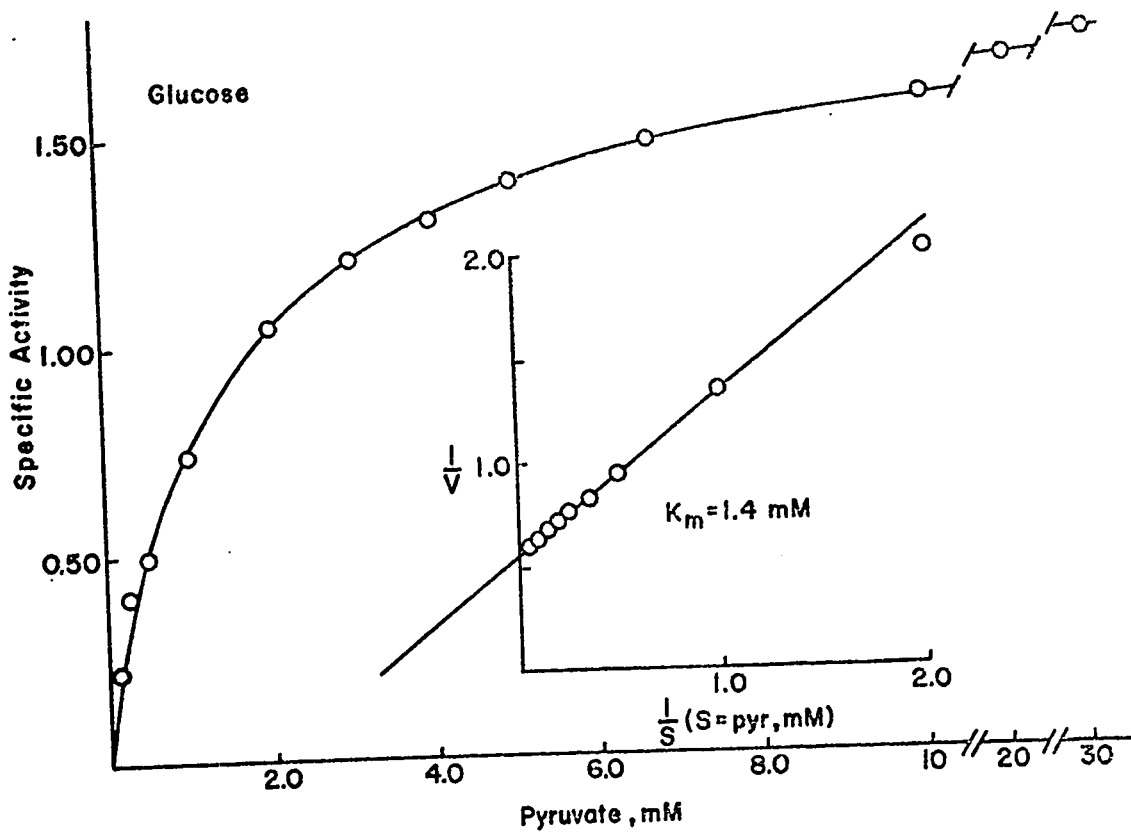


Fig. 4.2.12.

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glycerol-grown cells, and the  $K_m$  values for PD appear to indicate that PD has a higher affinity for pyruvate than does AHAS. By inspection of the PD saturation curves in fig. 4.2.12, it can be seen that the enzyme is effectively saturated when the pyruvate concentration is 30 mM. In contrast, AHAS saturation curves (in the majority of cases) are not completely saturated even at 100 mM pyruvate (see, for example, fig. 4.2.5). Thus the interpretation of Juni for the sigmoidal kinetics exhibited by AHAS from glucose-grown cells is supported by the experimental findings on PD and AHAS.

#### 4.2.15. Relation between AHAS and PD during partial purification of AHAS

If the sigmoidal saturation kinetics observed with the synthetase from glucose-grown cells are indeed brought about through a competition with PD for pyruvate, at low concentrations, then if AHAS can be separated from PD, the synthetase should exhibit normal Michaelian kinetics. To test this hypothesis, AHAS extracted from cells grown in HM supplemented with 5 mM L-isoleucine was purified according to the scheme in section 4.2.5. The precipitates from the two  $(\text{NH}_4)_2 \text{SO}_4$  fractionation steps were each dissolved in 1.0 ml of buffer solution used throughout the purification procedure. These two fractions along with the crude extract, PS supernatant and the supernatant from the 35-50%  $(\text{NH}_4)_2 \text{SO}_4$  step were all assayed for AHAS and PD activities. For the PD assays, all fractions were diluted 1/50 as before, and 50 and 100  $\mu\text{l}$ s of these dilutions were assayed in the Gilford 2400 recording spectrophotometer. The results of this experiment are presented in table 4.2.9. The overall purification

Table 4.2.9. Relative activity of AHAS and PD during purification

Enzyme	Specific Activity*				
	CE	PSN	AS-1(0-35%)	AS-2(35-50%)	SN
AHAS	0.578	0.780	0.355	2.513	0.564
PD	1.33	2.15	0.32	0.26	2.92

\*AHAS:  $\mu$ moles  $\alpha$ -AI/min/mgP

PD:  $\mu$ moles NADH oxidized/min/mgP

of AHAS was 5-fold over activity in the crude extract. Since the cells were grown in the presence of ile, which condition causes derepression of the AHAS (section 4.6.3), the specific activity in the CE is about double that of an ordinary extract (see table 4.2.6). It can be seen that while most of the AHAS activity is recovered in the 35-50% AS fractionation step, the PD activity is still mostly in the supernatant from 50%  $(\text{NH}_4)_2 \text{SO}_4$  saturation. In effect, a partial separation of AHAS and PD has been achieved by  $(\text{NH}_4)_2 \text{SO}_4$  fractionation.

The supernatant containing the PD activity was brought to 75% saturation with  $(\text{NH}_4)_2 \text{SO}_4$ , and the resultant precipitate was recovered by centrifugation and resuspended in 1.0 ml of buffer. The specific activity of this fraction was 3.97  $\mu$ moles NADH oxidized/min/mg P, representing about a 3-fold enrichment of PD activity over that of the crude extract.

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4.2.16. Saturation kinetics of partially purified AHAS alone and recombined with partially resolved PD

A substrate saturation curve was obtained using the partially purified AHAS from glucose-grown cells described in section 4.2.15. From curve A of fig. 4.2.13, it can be seen that the partially purified enzyme exhibits normal saturation kinetics. Curve B shows the effect of combining this AHAS fraction with the PD fraction which was partially resolved from the synthetase as described in section 4.2.15. The substrate curve obtained in this case showed pronounced sigmoidicity, which probably reflects competition for the pyruvate at low concentrations by the two enzymes present. A puzzling feature of these results is the greater amount of AL formed in the presence of the two enzymes than with AHAS alone. It may be that under these conditions (i.e. recombination of enriched enzymes) and at higher pyruvate concentrations, the intermediate formed between decarboxylated pyruvate (acetaldehyde) and TPP (HETDP), which is common to both enzymes, is available for use by the synthetase to form additional AL instead of being used by the decarboxylase to form acetoin. Another possibility is that the AHAS might be stabilized by the increased protein concentration of the combined fractions, so that it would be more active in the synthesis of its product, AL.

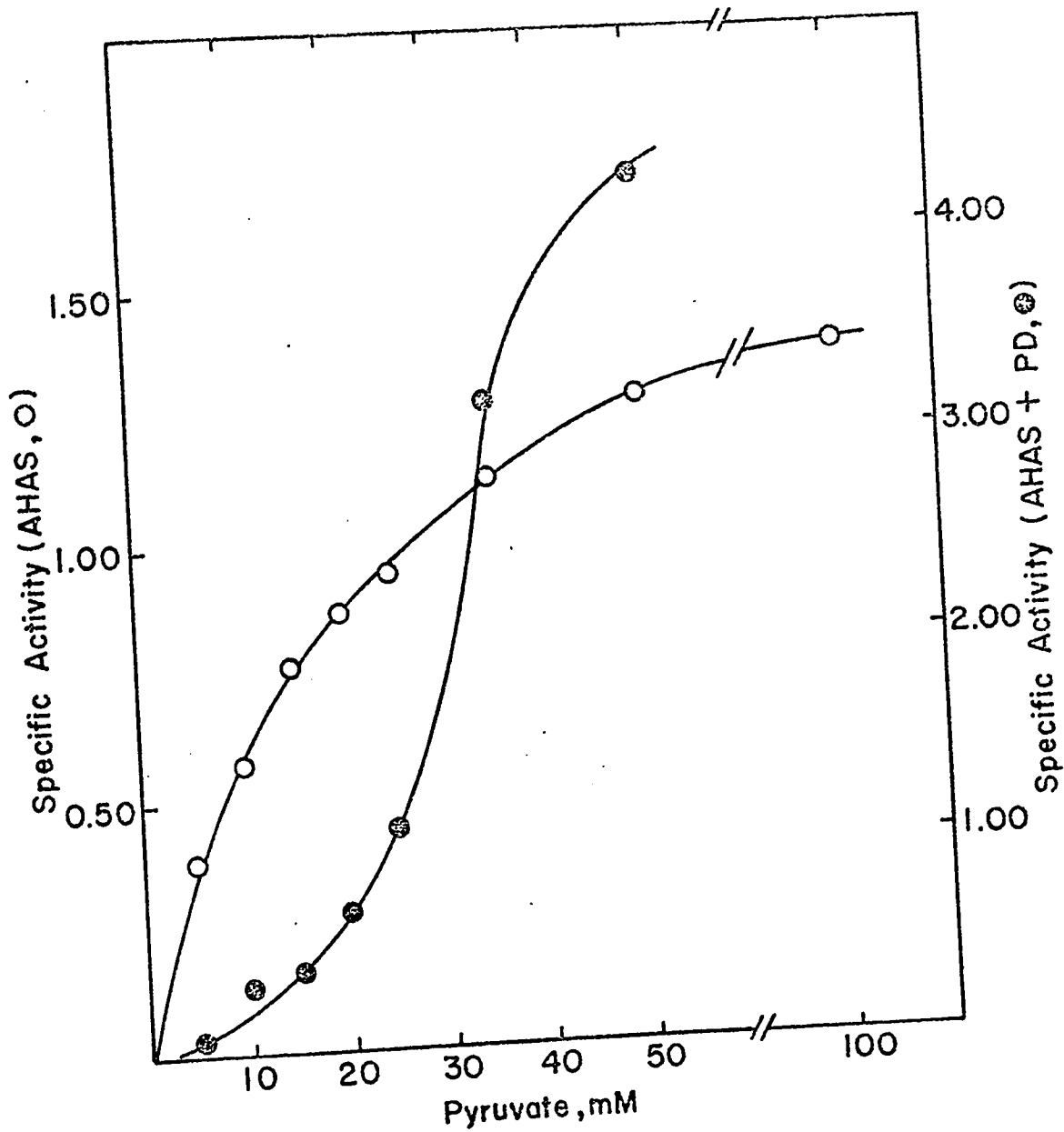


Fig. 4.2.13. Recombination of partially purified AHAS and PD. The G-25-filtered  $(\text{NH}_4)_2\text{SO}_4$  fraction of AHAS and the 50-75%  $(\text{NH}_4)_2\text{SO}_4$  fraction of PD from the purification described in section 4.2.15 were used. For the AHAS saturation curve, the G-25 eluate was diluted 1:1 with buffer and 20  $\mu\text{l}$ s were assayed for 20 min in reaction mixtures containing the pyruvate levels indicated. The second curve (●—●) was made by assaying 20  $\mu\text{l}$ s of a v/v mixture of the AHAS and PD fractions for 20 min in a similar set of assay mixtures. AL formed was determined as acetoin (section 3.2.2).

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#### 4.2.17. Further studies on valine inhibition

In an earlier section (4.2.4), it was shown that valine is an effective inhibitor of AHAS activity, although it does not inhibit more than 75-80%

##### 4.2.17.1. Valine inhibition studies

Inhibition by L-valine was investigated using extracts from both glucose- and glycerol-grown cells. More emphasis was placed on the effects of low concentrations of valine. Fig. 4.2.14 shows that the curves obtained with both extracts are sigmoidal, an indication of homotropic cooperative effects of valine; the Hill values obtained were 1.8 for glucose and 1.4 for glycerol extracts (lower figure). In this experiment, the enzyme in the extract from glucose cells was inhibited 65% by 1 mM valine, while the glycerol extract was inhibited 76% by the same valine level. Increasing the valine levels to 10 mM did not routinely produce greater inhibition.

##### 4.2.17.2. Kinetics of valine inhibition

Acetolactate formation in E. coli extracts at pH 8.0 was shown to be competitively inhibited by L-valine (Umbarger and Brown, 1958). Valine inhibition of AHAS is non-competitive in nature in A. aerogenes (Halpern and Umbarger, 1959), Salmonella (Bauerle et al., 1964) and S. cerevisiae (Magee and de Robichon-Szulmajster, 1968b).

Figs. 4.2.15 and 4.2.16 illustrate the results of experiments designed to determine the type of inhibition exerted by L-valine on S. pombe AHAS. In both cases, valine inhibits the synthetase reac-

Fig. 4.2.14. Valine inhibition of AHAS from glucose- and glycerol-grown cells. Crude extracts were used; the extract from glycerol cells was filtered through Sephadex G-25 immediately prior to assay. 50  $\mu$ ls of glucose extract and 100  $\mu$ ls of glycerol G-25 eluate were incubated 30 min in reaction mixtures supplemented with the indicated concentrations of L-valine; pyruvate concentration was 500  $\mu$ moles / ml. Lower figure: data from upper figure rearranged in Hill plots.

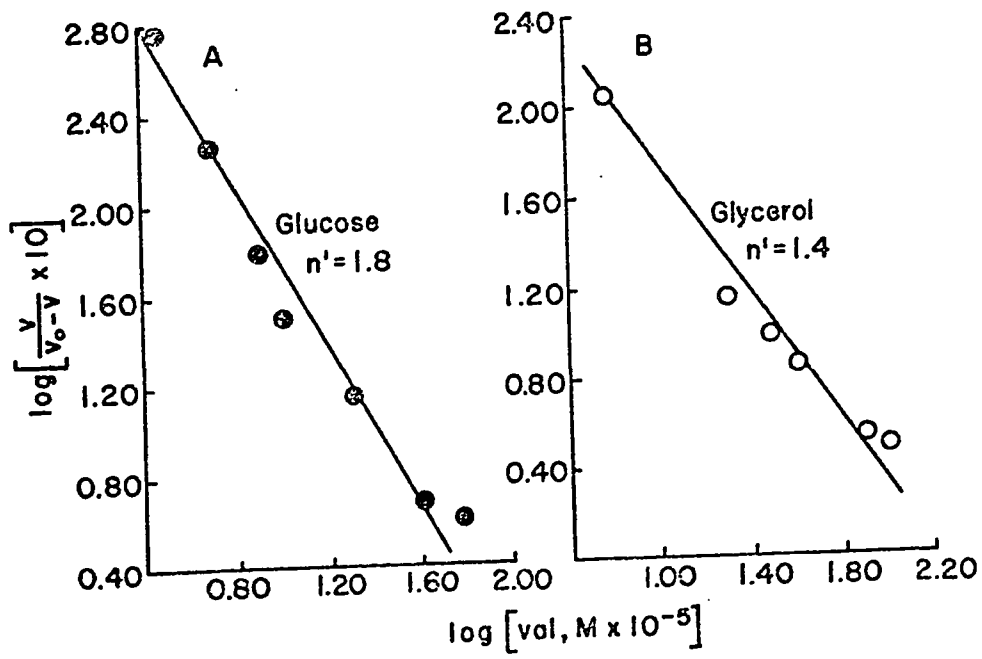
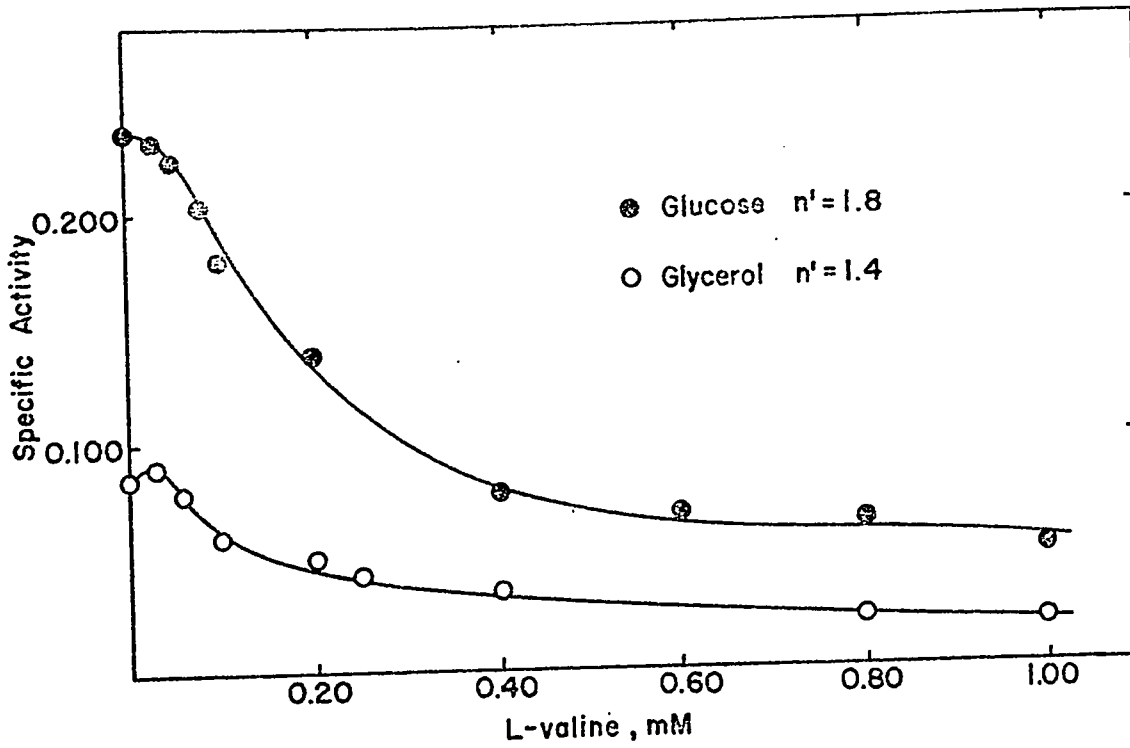


Fig. 4.2.14.

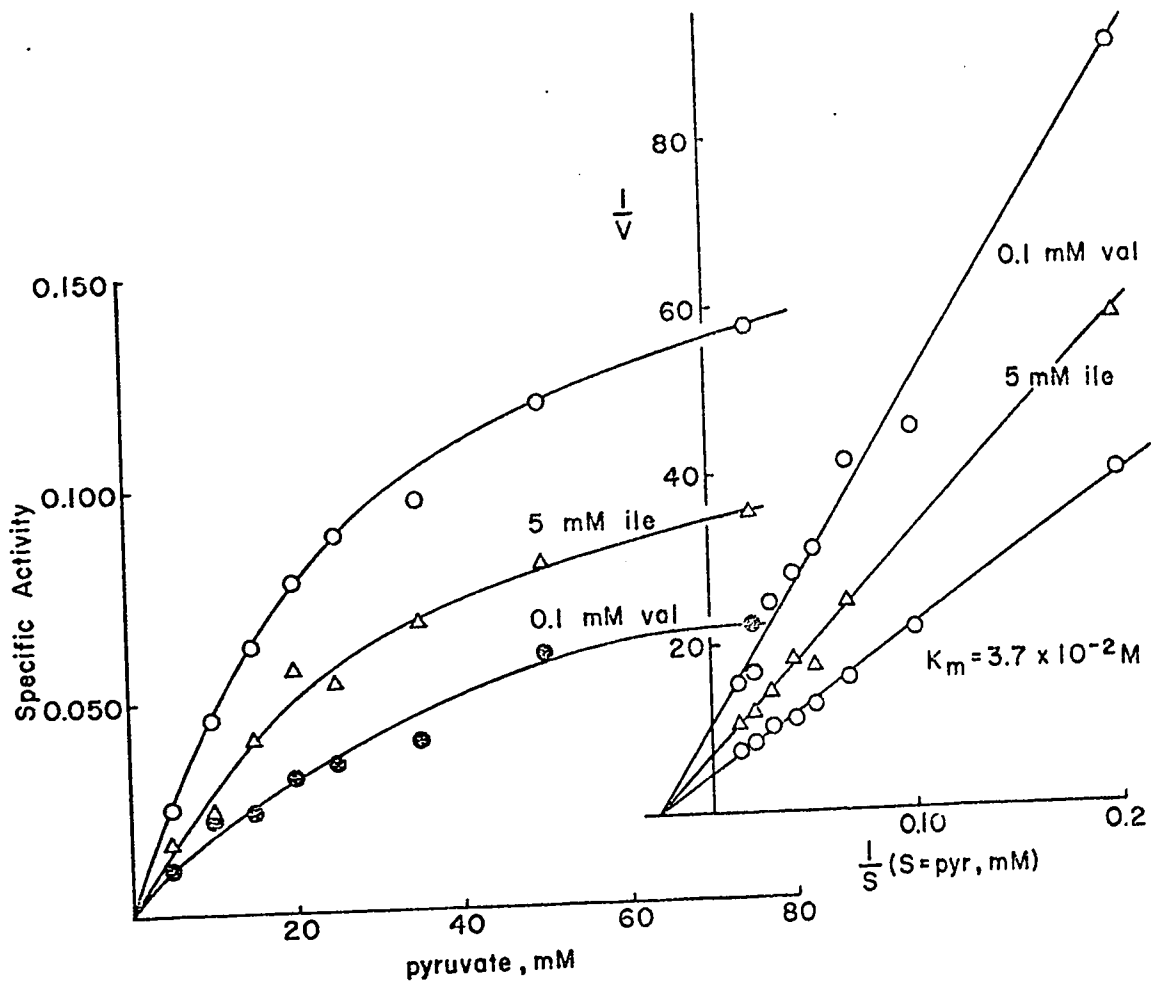


Fig. 4.2.15. Kinetics of valine inhibition of AHAS. Enzyme source was extract from glycerol-grown cells (11.3 mg protein / ml); 50 pl were incubated for 30 min in standard reaction mixture with varying pyruvate concentrations (○—○), supplemented with 0.1 μmole / ml L-valine (●—●) or 5 μmoles / ml L-isoleucine (△—△). Inset shows Lineweaver-Burk transformations.

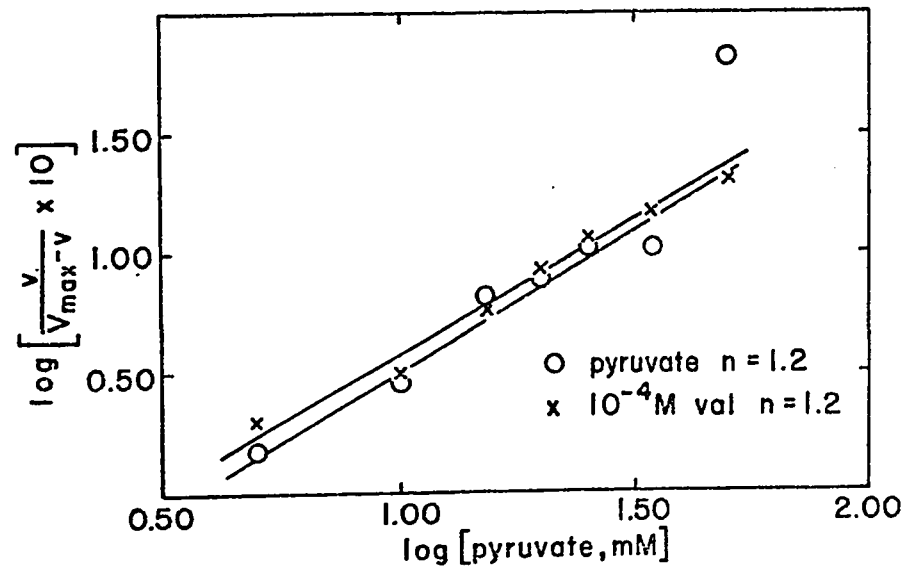
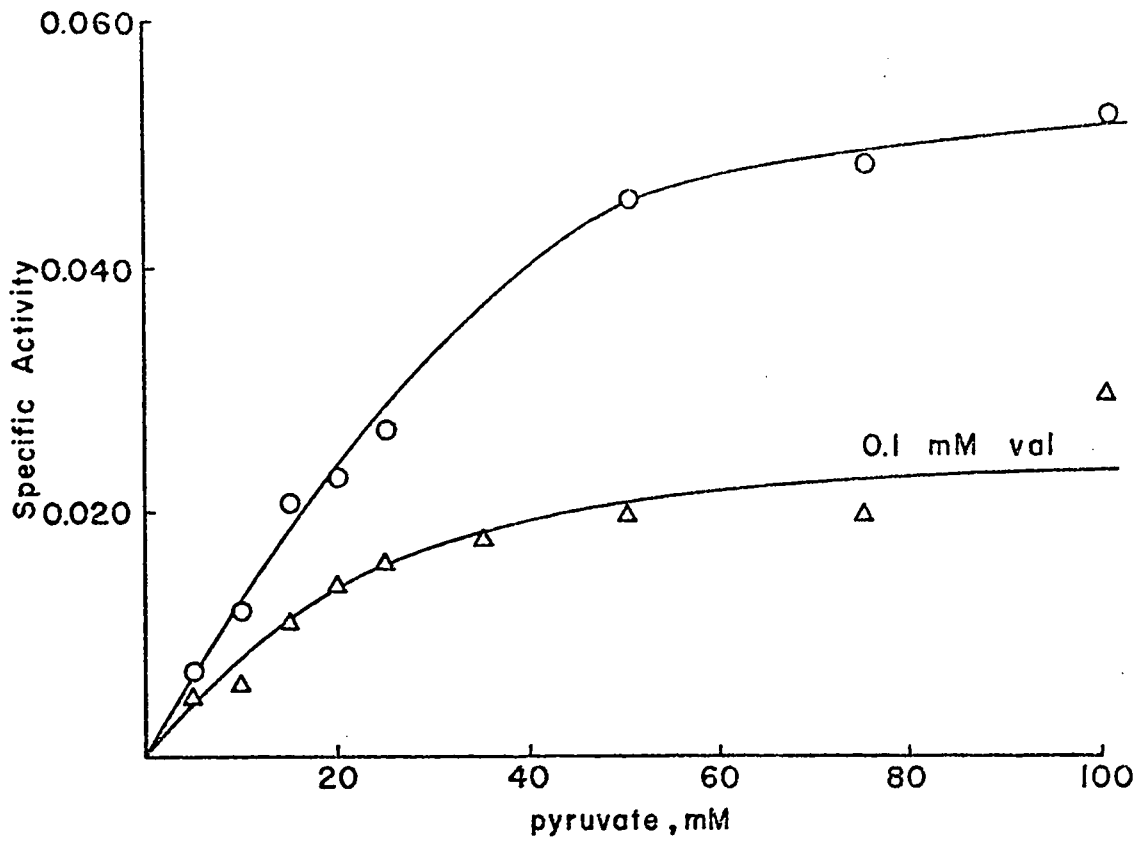


Fig. 4.2.16. Kinetics of valine inhibition of partially purified AHS. Enzyme was extracted from glucose-grown cells and purified as described (section 4.2.5); 20  $\mu$ ls (0.39  $\mu$ g protein) were assayed for 30 min in reaction mixtures containing varying pyruvate concentrations as indicated. Valine sensitivity was measured in the presence of 0.1  $\mu$ mole L-valine / ml. Lower figure: same data rearranged for Hill plot.

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tion but does not cause cooperative effects between substrate molecules; it appears only to lower the  $V_{max}$ . For the glycerol extract, the Hill coefficients both in the presence and absence of valine are 1 (fig. 4.2.17); for the enzyme purified from glucose-grown cells,  $n = 1.2$  whether valine is present or not (fig. 4.2.16, lower chart). This suggests that there might be cooperative effects involved; the "high" Hill values may simply be due to scatter of the points about the saturation curve.

Double reciprocal plots show that L-valine causes linear non-competitive inhibition of AHAS activity in S. pombe: the  $K_m$  for pyruvate is unchanged in the presence of valine (fig. 4.2.15 inset, and fig. 4.2.18).

#### 4.2.17.3. Determination of $K_i$ for valine

Dixon (1953) devised a simple way in which to calculate  $K_i$  directly for an inhibitor in an enzyme-substrate system. If activity is measured in varying concentrations of inhibitor at two different substrate levels, straight lines may be obtained by plotting  $1/v$  against inhibitor concentration for each substrate level. The point of intersection of the lines gives a value of  $K_i$  directly.

The results of such an experiment with the AHAS - valine system from S. pombe are presented in fig. 4.2.19. The point of intersection gives an apparent  $K_i$  for valine of 0.1 mM.

The effect of L-valine on an untreated crude extract from glucose-grown cells is shown in fig. 4.2.20; valine inhibited enzyme activity 70% at 75 mM pyruvate, and increased the sigmoidicity of the saturation curve. If  $1/v$  was plotted against  $1/s^2$ , a good linear fit

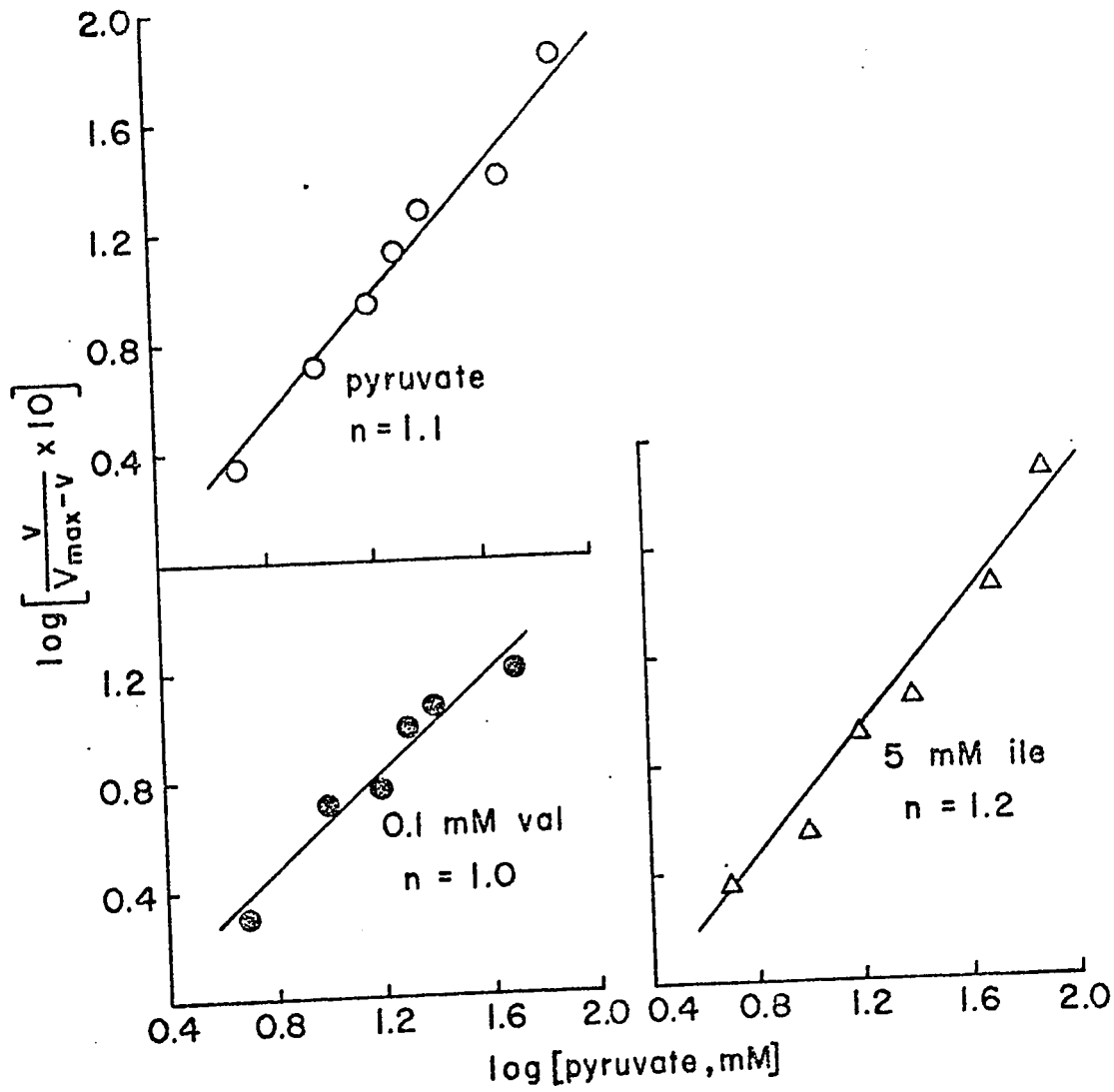


Fig. 4.2.17. Inhibition kinetics of AHAS as shown by the Hill plot. Data taken from results shown in fig. 4.2.15.

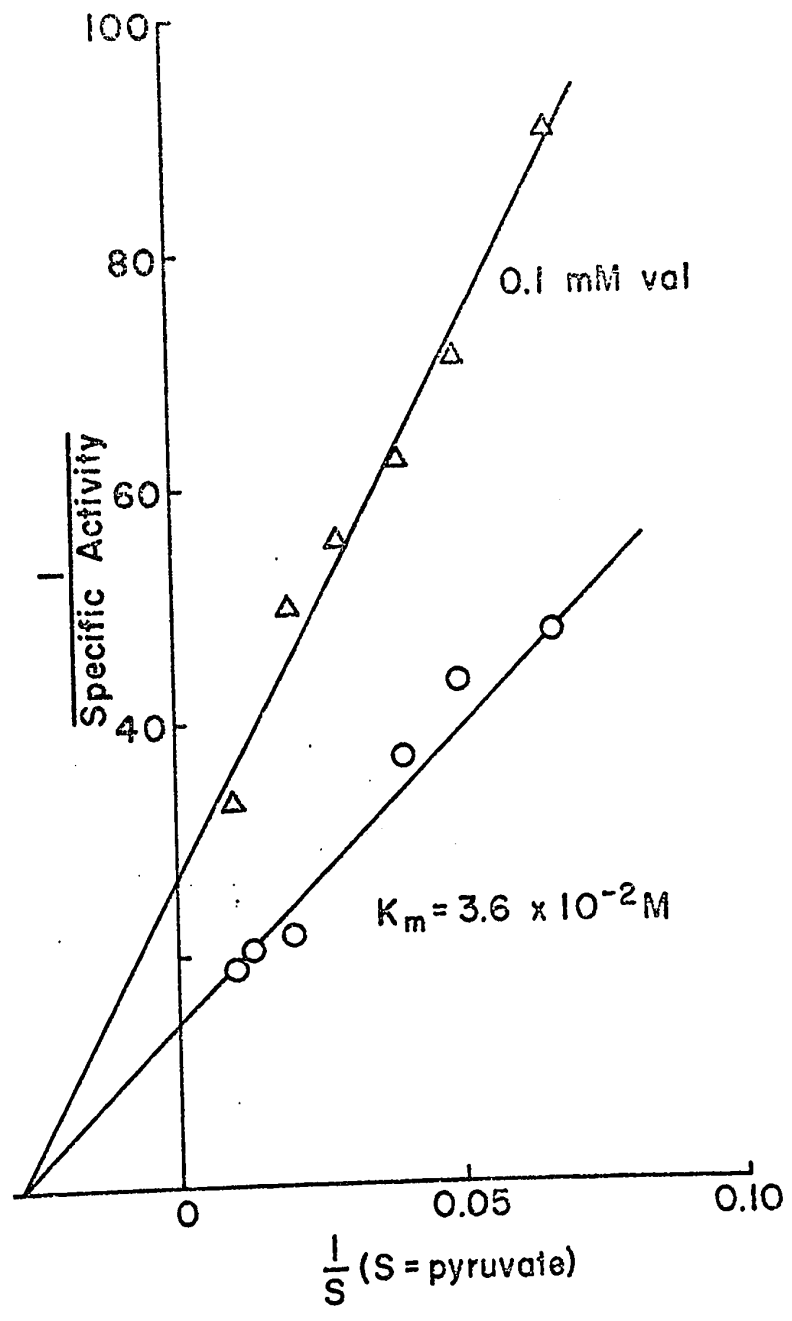


Fig. 4.2.18. Nature of valine inhibition of partially purified AHAS. Lineweaver-Burk transformation of data given in fig. 4.2.16.

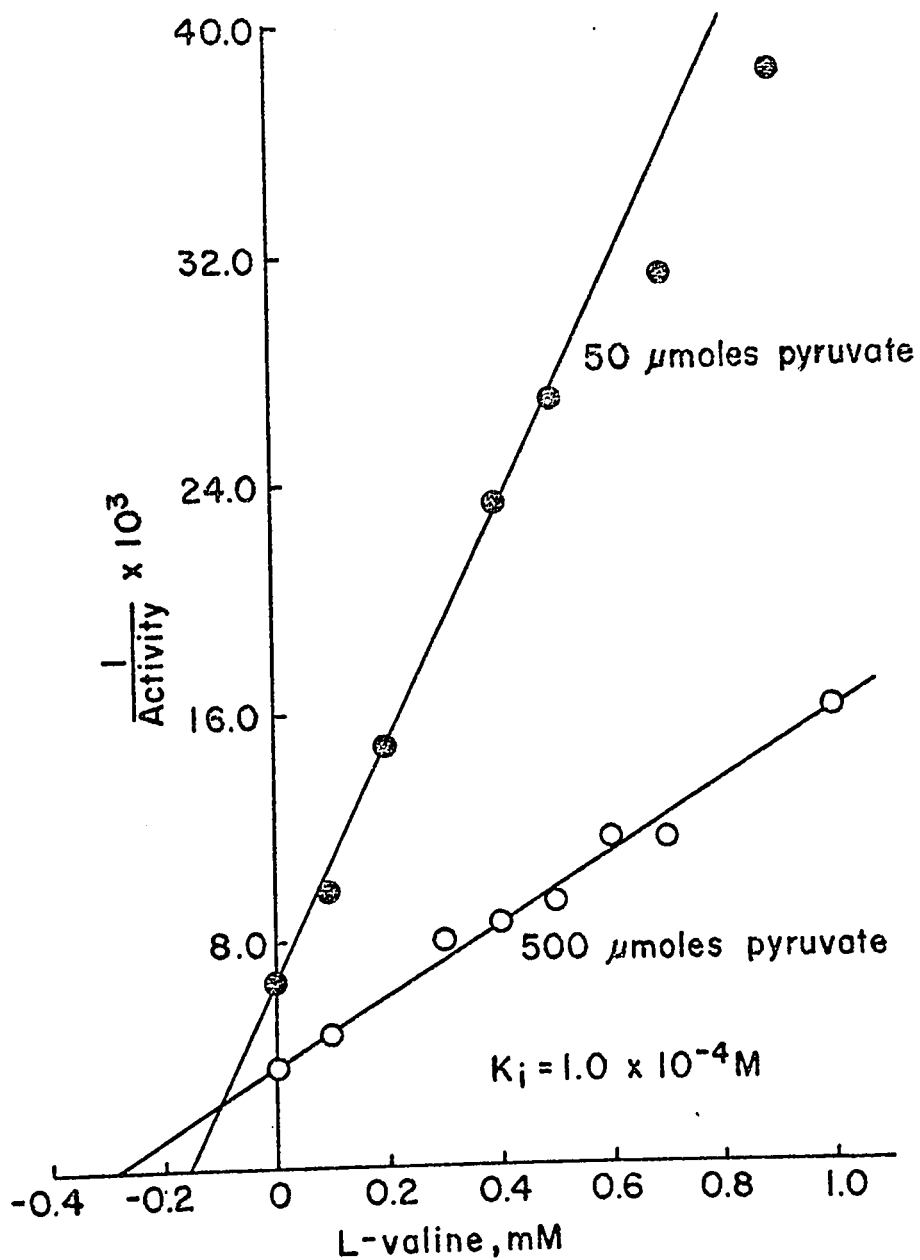


Fig. 4.2.19. Determination of  $K_i$  for valine. AHAS from glucose-grown cells was extracted into phosphate-glycerol buffer; 50  $\mu\text{ls}$  of the extract were assayed for 30 min in varying concentrations of L-valine, at two substrate levels: 50 and 500  $\mu\text{moles / ml}$  pyruvate. The data were plotted according to Dixon (1953).

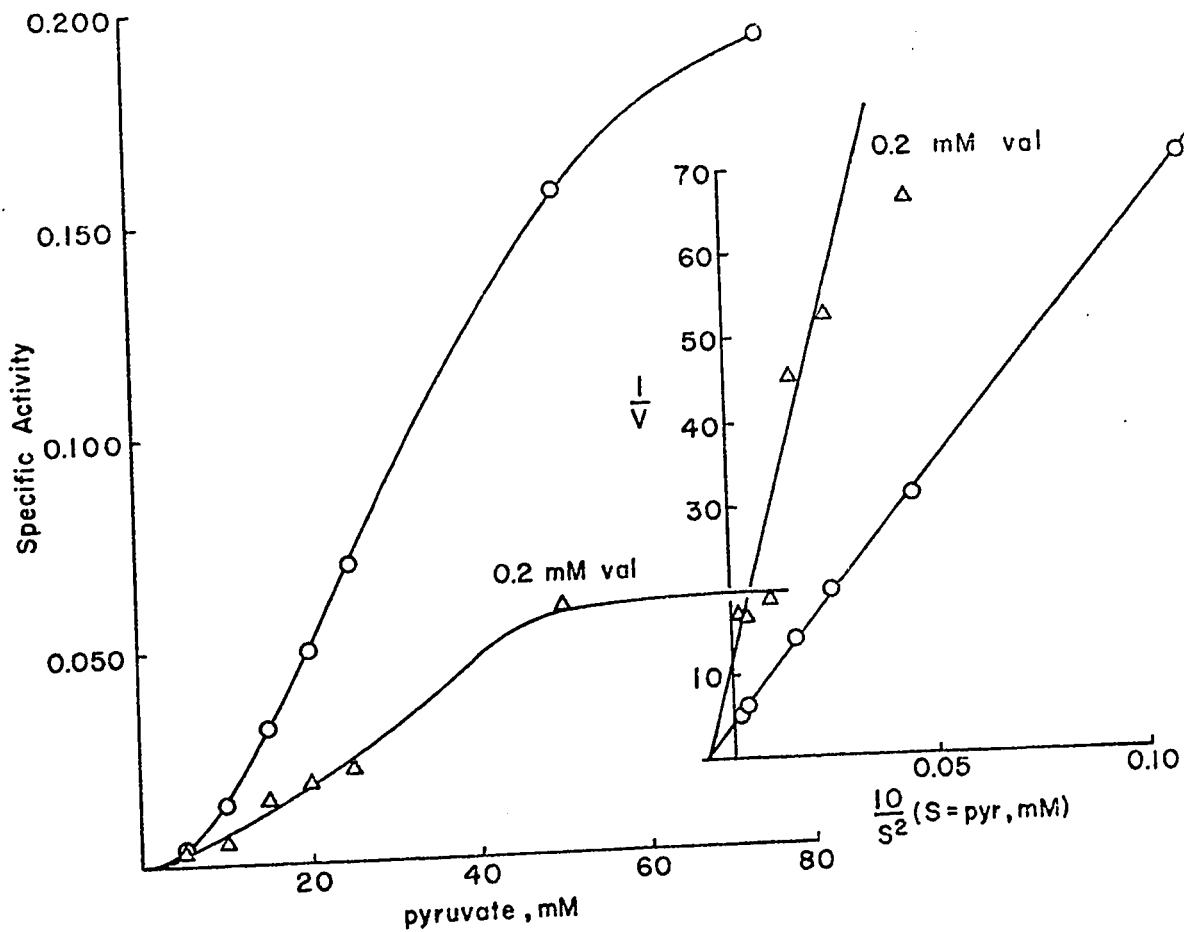


Fig. 4.2.20. Non-competitive inhibition by valine of AHAS in a crude extract from glucose-grown cells. 50  $\mu$ ls (1.1 mg protein) extract were incubated for 30 min in reaction mixtures containing increasing concentrations of pyruvate. Valine sensitivity was assayed in the presence of 0.2  $\mu$ moles / ml L-valine. Inset shows plot of reciprocal velocity vs reciprocal of square of pyruvate concentration.

was obtained, and, again, valine inhibition is non-competitive (inset, fig. 4.2.20).

#### 4.2.18. Effect of isoleucine and leucine on AHAS activity

Bauerle et al. (1964) report that 20 mM isoleucine causes 42% inhibition of AHAS activity in S. typhimurium. In P. aeruginosa, 56-63% inhibition was caused by 10 mM isoleucine (Marinus and Loutit, 1969), while in bakers' yeast, Magee and de Robichon-Szulmajster (1968b) show that isoleucine has negligible effects on AHAS.

The effect of L-isoleucine on AHAS from S. pombe is shown in fig. 4.2.21. Isoleucine caused inhibition of synthetase activity (35% at 75 mM pyruvate) and, like valine, increased the sigmoidicity of the saturation curve. In fig. 4.2.15, 5 mM isoleucine caused similar inhibition of AHAS from a "glycerol" extract; it appears to have affected the  $V_{max}$ , for, from the double reciprocal plot, the  $K_m$  is unchanged. Isoleucine is a non-competitive inhibitor, and probably binds at the valine site. In fig. 4.2.17, the corresponding  $n$  value is 1.2, but no conclusion may be drawn from this value.

Isoleucine was unable to reverse inhibition caused by valine, as was found for the Salmonella AHAS (Bauerle et al., 1964).

Leucine was without effect on the AHAS saturation curve and caused negligible inhibition of enzyme activity (about 5%). Both leucine and threonine were unable to reverse valine inhibition, and valine + isoleucine + leucine together caused no more inhibition of activity than did valine alone.

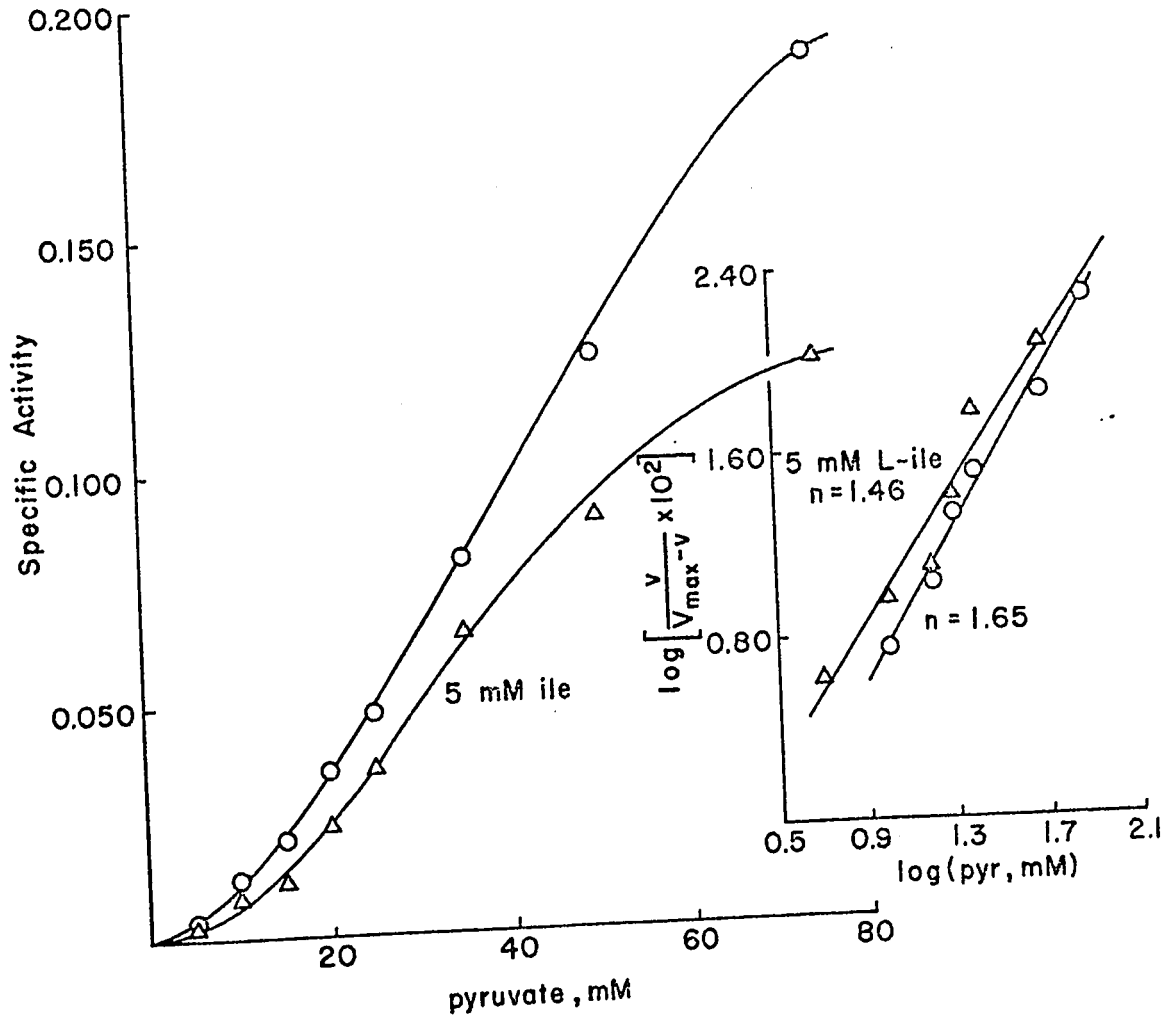


Fig. 4.2.21. Inhibition of AHAS by L-isoleucine. A crude extract prepared from glucose-grown cells was assayed for AHAS activity in reaction mixtures containing increasing levels of pyruvate; 50  $\mu$ ls of enzyme (1.8 mg protein) were incubated for 30 min. Isoleucine sensitivity was determined in the presence of 5  $\mu$ moles / ml L-isoleucine. Inset shows data rearranged in Hill plot.

#### 4.2.19. Effect of $\alpha$ -aminobutyrate on AHAS activity

$\alpha$ -Aminobutyric acid (AB) is a known analog of valine (Radhakrishnan and Adelberg, 1964). These authors isolated AB-resistant mutants of E. coli which were derepressed for AHAS; these mutants were shown to be affected in an operator locus which controls the synthesis of AHAS only. More recently, Kisumi et al. (1971) reported AB-resistant mutants of S. marcescens which have derepressed levels of AHAS and which excrete valine; several of these mutants have an AHAS which is insensitive to feedback inhibition by valine.

$\alpha$ -Aminobutyrate was found to be somewhat inhibitory to growth of S. pombe when it was included in high concentrations in the medium (generation time was increased from 3.3 hrs to 5.8 hrs by 0.1 M L- $\alpha$ -aminobutyrate). Tests were made to determine whether AB could cause false feedback inhibition of AHAS in extracts of S. pombe. An experiment was performed to determine the  $K_i$  for AB using the Dixon method (section 4.2.17.3), and typical results are presented in fig. 4.2.22. AB does exhibit false feedback inhibition, but the concentration required to produce 70% inhibition (20 mM AB) is about 200 x that of L-valine for the same effect. The Dixon plot (lower figure) of the same data confirms the lesser effectiveness of AB as inhibitor (the  $K_i$  for AB is  $1.5 \times 10^{-3}$  M, about 15 times the  $K_i$  for valine).

In S. typhimurium, AB at 20 mM caused 50% inhibition of AHAS (Bauerle et al., 1964), whereas in P. aeruginosa, 1 mM AB caused 31% inhibition (Marinus and Loutit, 1969).

Attempts to isolate mutants of S. pombe resistant to AB failed, probably because AB does not increase the generation time of wild type cells sufficiently to allow AB-resistant colonies to be readily picked off plates supplemented with the compound.

Fig. 4.2.22. False feedback inhibition of AHAS by  $\alpha$ -aminobutyrate. Extract from glycerol-grown cells served as source of AHAS; 50  $\mu$ ls (2.0 mg protein) were incubated for 15 min in standard reaction mixture containing increasing concentrations of L- $\alpha$ -aminobutyrate, at two substrate levels: 50 and 500 mM pyruvate. The results were plotted according to Dixon (1953) (lower figure).

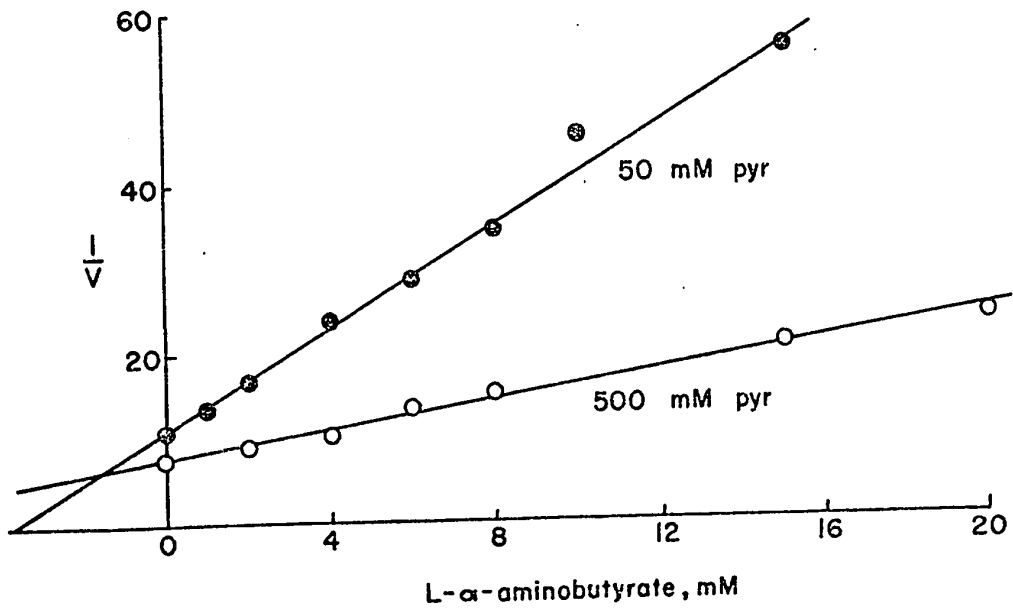
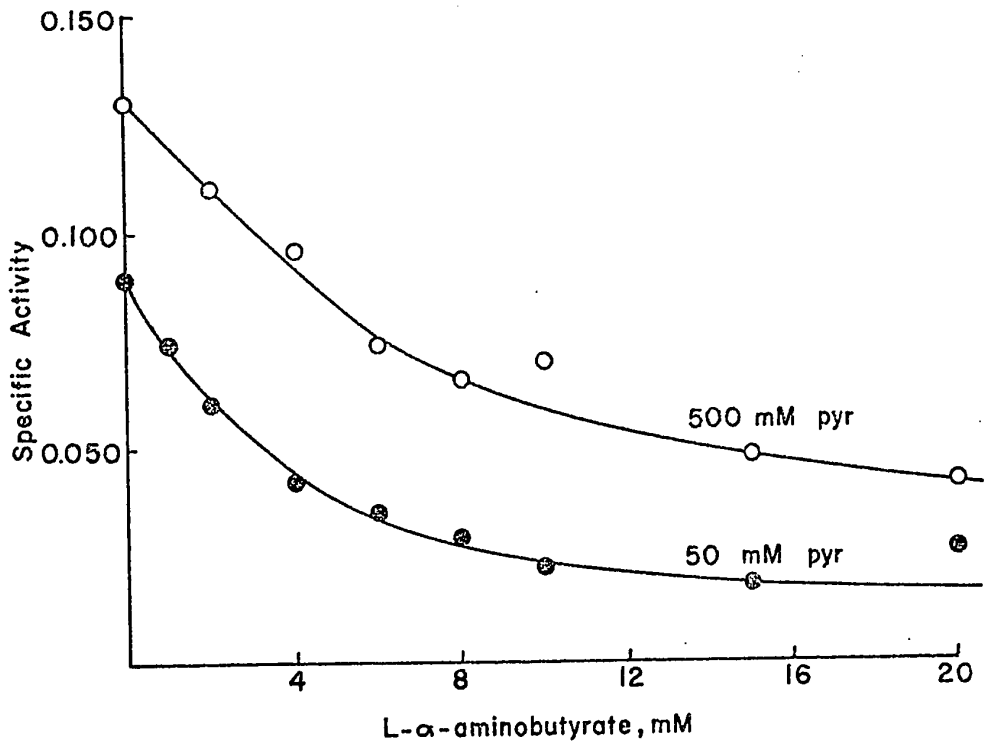


Fig. 4.2.22.

#### 4.2.20. Desensitization of *S. pombe* AHAS

The sensitivity of AHAS of Salmonella (Bauerle et al., 1964) to valine can be removed by heat,  $Hg^{2+}$  and urea treatments. In bakers' yeast, Magee and de Robichon-Szulmajster (1968b) present evidence that the AHAS loses 50% of its activity and is totally desensitized to valine inhibition after only 3 min at 35 C. However, this desensitization does not occur when the enzyme is so treated in permeabilized cells. In P. aeruginosa, valine inhibition was removed by treatment with mercurials or by storage at 4 C (Marinus and Loutit, 1969).

$Hg^{2+}$  desensitization of S. pombe synthetase was carried out using a crude extract prepared from glycerol-grown cells. An aliquot of the CE was incubated at 30 C for 5 min with 0.4 mM  $Hg Cl_2$ . A portion of this aliquot was assayed; DTT was immediately added to the remainder, to a final concentration of 1 mM, and the sample was incubated an additional 10 min. Controls of extract incubated for 5 and 15 min were run simultaneously. Fifty  $\mu$ ls of all samples were assayed for 15 min with and without 0.2 mM L-valine, and the acetolactate formed was determined as acetoin in the Westerfeld procedure (3.2.2). The results of the experiment are presented in table 4.2.10. Incubation with mercury inhibited enzyme activity about 60% and completely eliminated sensitivity to valine, suggesting that  $Hg^{2+}$  affects the enzyme in different ways, or is acting at different sites on the enzyme. DTT restored both activity and valine sensitivity. It appears, therefore, that valine has a binding site on the enzyme which is distinct from the active site.

Table 4.2.10. Effect of  $\text{Hg}^{2+}$  on activity and valine sensitivity of AHAS

Treatment	Specific activity		% inhibition by valine
	no valine	0.2 mM valine	
None	0.139	0.045	67.6
0.4 mM $\text{Hg Cl}_2$	0.055	0.054	1.8
0.4 mM $\text{Hg Cl}_2$ + 1 mM DTT	0.120	0.051	57.5

4.2.21. Summary of AHAS results

AHAS from S. pombe has been partially-purified from crude extracts, and some of its kinetic and regulatory properties have been investigated. In contrast to the enzyme from bakers' yeast, the S. pombe synthetase requires TPP, FAD and  $\text{Mg}^{2+}$  for maximal activity. The enzyme is optimally active at pH 6.5, and AL formation is maximally inhibited (to 80%) by valine at this pH. The AL formed at this pH appears to be of the biologically-active isomer. Valine is a non-competitive inhibitor of AHAS activity ( $K_i = 0.1$  mM), and exhibits homotropic cooperative effects.

The synthetase from cells grown on glycerol as carbon source, and in partially purified extracts, shows normal Michaelian kinetics with respect to pyruvate ( $K_m = 4.8 \times 10^{-2}M$ ). The enzyme in crude extracts of glucose-grown cells exhibits sigmoidal saturation kinetics; this property was shown to be due to the presence in these extracts of high levels of

pyruvate decarboxylase, which probably competes with AHAS for pyruvate, the common substrate. Preliminary studies on PD show that this enzyme is present at levels 2-fold higher than those of glycerol-grown cells, and that it exhibits normal kinetics with a  $K_m$  value for pyruvate of 1.4 mM. The PD and AHAS can be partially separated by ammonium sulfate fractionation.

Valine and isoleucine cause a decrease in the  $V_{max}$  of AHAS without affecting the  $K_m$ . Valine inhibition is not augmented when leucine and/or isoleucine are present, nor is it reversed by isoleucine or leucine.  $\alpha$ -Aminobutyrate was shown to be an effective false feedback inhibitor of AHAS activity ( $K_i = 1.5 \times 10^{-3}M$ ), although under ordinary conditions, it has little effect on the growth of S. pombe.

The synthetase was shown to be desensitized to valine inhibition by treatment with  $Hg^{2+}$ ; enzymic activity was concomittantly decreased about 60%. Almost full activity and sensitivity to valine were restored after incubation with Cleland's reagent. The AHAS from S. pombe appears to be an allosteric regulatory enzyme.

#### 4.2.22. Discussion of AHAS results

The AHAS from S. pombe can be considered an allosteric regulatory enzyme, at least in its behaviour in vitro.

The biosynthetic synthetase from S. pombe is unique among those studied in other systems because it has a pH optimum in the mildly acidic range (fig. 4.2.1). Several microorganisms, e.g. A. aerogenes (Halpern and Umbarger, 1959), E. coli and N. crassa (Radhakrishnan and Snell, 1960; Caroline et al., 1969), possess two distinct AL synthetases. The enzyme

functioning at pH 6 is involved in the conversion of pyruvate to acetoin (via the intermediate acetolactate), which is excreted into the medium. The second enzyme, which functions optimally around pH 8, is the first enzyme of the valine biosynthetic pathway, and is subject to end-product inhibition by valine and to repression by valine and by valine + isoleucine + leucine + pantothenate (Freundlich et al., 1962). In addition to the above organisms, the following possess only a biosynthetic AHAS which operates optimally at pH 7.2-8.0: S. cerevisiae (Magee and de Robichon-Szulmajster, 1968a); P. aeruginosa (Marinus and Loutit, 1969); Penicillium chrysogenum (Goulden and Chattaway, 1969). Phaseolus radiatus (Satyanarayana and Radhakrishnan, 1963) was shown to possess only the acidic AL synthetase which was insensitive to valine.

There seems little doubt that the AHAS assayed at pH 6.5 in extracts of S. pombe is indeed the biosynthetic enzyme, in view of the nature of the product it forms and its inhibition by valine. In addition, in a later section, it will be shown that the enzyme is repressible by valine and by a combination of the three branched chain amino acids.

#### 4.2.22.1. AHAS is an allosteric enzyme

From the kinetic data presented, the AHAS can be considered to be an allosteric enzyme as described by Monod et al. (1963). Valine sensitivity is strongly affected by changes in pH (fig. 4.2.1), as is, for example, the AHAS from bakers' yeast (Magee and de Robichon-Szulmajster, 1968b) and S. typhimurium (Bauerle et al., 1964). The fact that small molecules other than valine can affect enzyme activity (e.g. isoleucine, leucine,  $\alpha$ -aminobutyrate) is consistent with

allosteric theories. The fact that it can be desensitized to valine inhibition while remaining enzymically active suggests separate and distinct binding sites for pyruvate and valine. Additionally, valine inhibition is non-competitive (figs. 4.2.15 and 4.2.18). One would expect that valine, which bears no structural resemblance to pyruvate whatever, would bind at a site other than the catalytic site.

#### 4.2.22.2. Interactions with substrate and inhibitors

The S. pombe enzyme exhibits normal Michaelian kinetics when extracted from cells grown on glycerol (fig. 4.2.5) or when partially purified from extracts of glucose-grown cells (fig. 4.2.13). The  $K_m$  of  $4.8 \times 10^{-2}M$  is close to that of other systems except for S. cerevisiae ( $K_m = 8.5 \times 10^{-3}M$ , Magee and de Robichon-Szulmajster, 1968a) and S. typhimurium ( $K_m = 5.7 \times 10^{-3}M$ , Bauerle et al., 1964). Valine is a true non-competitive inhibitor (section 4.2.17). From these data, the S. pombe enzyme conforms to a negative "V system" as defined by Monod et al. (1965). Valine presumably has maximum affinity for the inactive state of the enzyme.

The MWC model also predicts the following: a) an allosteric effector (a specific ligand which has different affinities towards the two states of the enzyme) should exhibit cooperative homotropic interactions, and b) in V systems, where the effector does not modify the apparent affinity of the substrate, the latter should not exhibit homotropic cooperative interactions (Monod et al., 1965). The criteria in (b) are satisfied as just described; the Hill values of 1.1 - 1.2 obtained for pyruvate should not be given too much significance considering the inherent potential for experimental error in the AHAS

assy procedure. That valine exhibits homotropic cooperative effects was demonstrated in fig. 4.2.14, satisfying the requirement in (a) above.

The enzyme from bakers' yeast could not be so easily classified, as no cooperative effect with inhibitor could be shown (Magee and de Robichon-Szulmajster, 1968b). The enzymes from E. coli and S. typhimurium do not show cooperative binding of either substrate or inhibitor (Umbarger, 1969).

The AHAS could not be inhibited more than 80% even with valine levels as high as 10 mM. In bakers' yeast, the enzyme could not be inhibited more than 65%, although the enzyme in benzene-permeabilized cells was shown to be almost 100% valine-inhibitable (Magee and de Robichon-Szulmajster, 1968b). AHAS activity (as well as TD, DH and IR activities) can be successfully assayed in toluene-permeabilized cells of S. pombe; under these conditions, the synthetase could not be completely inhibited by valine. It may be that the evolutionary significance of the small valine-resistant AHAS residual activity is that it would still provide a small amount of acetohydroxybutyrate required for isoleucine biosynthesis even in the presence of saturating intracellular valine pools, and averting an artificial isoleucine requirement. As pointed out by Umbarger (1969), there is little evidence obtained with growing cells of E. coli that the valine sensitivity of AHAS is physiologically significant. The Carnegie Group (Roberts et al., 1955) carried out studies on the biochemical activities of E. coli B growing in minimal salts medium containing  $^{14}\text{C}$ -glucose and varying amino acid supplements. They found that  $^{14}\text{C}$  from the glucose appeared in valine even when the medium was supplemented with excess cold

valine; the same result was obtained for alanine, glutamic acid and glycine. That the rate of synthesis of valine was hardly affected was shown by the fact that radioactive valine appearing in the culture fluid was of the same order of magnitude as that normally synthesized. In contrast, when lysine, isoleucine, leucine and cystine were present as supplements in  $^{14}\text{C}$ -glucose medium, only an amount of the respective  $^{14}\text{C}$ -amino acid considerably less than the normal amount synthesized appeared in the medium. Thus, exogenous isoleucine was effective in quenching its synthesis in vivo, whereas valine appeared to be unable to do so. Nevertheless, valine inhibition is a real and reproducible phenomenon in vitro.

#### 4.2.22.3. Biosynthesis of acetoin in *S. pombe*

Extracts from bacteria which produce acetoin were shown by Juni (1952a) to contain two enzymic components involved in the biosynthesis of acetoin. One component acts on pyruvic acid to form  $\alpha$ -acetolactic acid, and the second component decarboxylates AL, forming acetoin. In yeast, however, AL is not an intermediate in acetoin formation (Juni, 1952b) because yeast do possess an acetolactate decarboxylase. This was shown to be true for *S. pombe* (section 4.2.7). Juni (1961) proposed that bakers' yeast synthesizes acetoin from pyruvate by the action of pyruvate decarboxylase (PD) in a two-site mechanism. In the first step, pyruvate combines with TPP as an enzyme-bound complex and is decarboxylated to hydroxyethyl TPP (HETDP) ("activated acetaldehyde"). The activated acetaldehyde is postulated to be transferred to a second site, from which the aldehyde unit can reversibly dissociate

to yield free acetaldehyde. Acetoin is formed as a side reaction by the condensation of enzyme-bound HETDP with acetaldehyde. As described in section 3.2.6, PD activity is assayed by measuring the acetaldehyde formed from pyruvate with alcohol dehydrogenase coupled with NADH.

Pyruvate decarboxylase of S. pombe exhibited normal Michaelian kinetics with pyruvate (fig. 4.2.12). The  $K_m$  values of the enzyme were somewhat different depending on the carbon source used for growth: several explanations can be advanced. The two  $K_m$ 's are different because of statistical or methodological artifact: 1) not enough determinations were made using glycerol extracts; 2) some component in glycerol extracts may in some way be interfering with PD activity. The differences could be of a genetic nature, involving perhaps a derepression or induction of PD in glucose-grown cells, or a repression in cells which are adapting for growth on glycerol. The different  $K_m$ 's might also arise through some method whereby glycerol (or glucose) cause a perturbation in enzyme structure or conformation so that the  $K_m$  may be increased or decreased, respectively. The problem can only be resolved by more  $K_m$  determinations on extracts from glycerol cells and on partially purified enzyme.

According to Magee and de Robichon-Szulmajster (1968a), the acetoin forming system of bakers' yeast (presumably PD activity) does not follow classical Michaelis-Menten kinetics (in plots of  $V$  vs  $V/S$ , a linear relationship is obtained only with the pyruvate concentration squared). The  $K_m$  for pyruvate for acetoin formation was found to be  $1.4 \times 10^{-2}M$ , or about 10 x larger than the  $K_m$  for PD in S. pombe.

The biological function of acetoin synthesis is to divert the acidic products of glucose catabolism to neutral products (Halpern

and Umbarger, 1959). Yeast cells growing on glucose as a carbon source catabolize glucose to pyruvate, which is then decarboxylated to "active" acetaldehyde under anaerobic conditions (which obtain essentially when yeast are grown on glucose without direct or forced aeration). The "active" acetaldehyde can then be reduced to ethanol, via alcohol dehydrogenase, with the regeneration of  $\text{NAD}^+$ . The "active" acetaldehyde, as we have seen, is also an intermediate (along with pyruvate or acetaldehyde) in acetoin synthesis, a reaction mediated by pyruvate decarboxylase. Thus cells fermenting glucose would generate large amounts of pyruvate, some of which is utilized for the synthesis of amino acids, and for  $\text{NAD}^+$  generation; of the rest, some is converted to acetoin and products derived therefrom, and the rest is excreted. In experiments reported by Trevelyan and Harrison (1954), it was shown that the concentration of intracellular pyruvate increased in close relation with the fermentation rate of yeast growing in glucose medium, until the glucose supply was exhausted. They also determined that the excretion of pyruvate into the medium increased with fermentation rate. Upon exhaustion of glucose, there was a rapid fall in intracellular pyruvate; the excretion of pyruvate into the medium also stopped, and the level of extracellular pyruvate began slowly to decline. Holzer (1968) observed that alcohol dehydrogenase is partially repressed by glucose to about  $1/5$  the activity found in yeast incubated in acetate as a carbon source.

Cells of S. pombe oxidizing glycerol as a carbon and energy source grow more slowly than do cells utilizing glucose (generation time about 2 times longer). Glycerol utilization apparently involves free diffusion across the cell membrane, then phosphorylation by an

inducible glycerokinase to L- $\alpha$ -glycerophosphate; this compound is then oxidized to dihydroxyacetone phosphate by an aerobic  $\alpha$ -glycerophosphate oxidase (Sols, 1968). In the (aerobic) oxidation of glycerol, pyruvate is not generated in such large amounts as from glucose, so that the amino acid syntheses and gluconeogenesis intermediates from the Krebs cycle probably drain pyruvate constantly; pyruvate decarboxylase could be present in much lower amounts as there would be less pyruvate available for acetoin synthesis. In addition, alcohol dehydrogenase would be present at its normal level since there is no glucose present to repress its activity.

These aspects of yeast metabolism would explain why, in S. pombe, levels of PD in glucose-grown cells are 2-fold higher than those of glycerol-grown cells, and excretion of acetoin is greater in the former.

It is of interest to note that in the industrial cultivation of bakers' yeast, the yeast is transferred stage by stage from anaerobic fermentation conditions to vigorously aerated conditions of cultivation. The decarboxylase activity in such cells was found to decrease to 1/6 of the original value by the time they reached the strictly aerobic stage of growth (Suomalainen and Oura, 1959). These workers did not investigate whether the cause of the decrease in enzyme activity was diminished enzyme synthesis (repression), an induction of the enzyme, or a competition for cocarboxylase.

#### 4.2.22.4. Relationship between PD and AHAS

That the irregular kinetics exhibited by the AHAS in extracts from cells grown on glucose are due to a competition between AHAS and PD when levels of the common substrate, pyruvate, are low, is supported by several lines of evidence. The sigmoidal kinetics of these AHAS preparations do not seem to be a property of the AHAS protein itself, because effectors like isoleucine, valine and leucine, or treatment with  $Hg^{2+}$ , or additions of boiled extract, all failed to normalize the pyruvate saturation curve. Known inhibitors of pyruvate decarboxylase (aldehydes) had no effect on acetolactate formation (4.2.9).

When PD is physically removed from the AHAS preparations from glucose-grown cells by ammonium sulfate fractionation, the synthetase shows normal kinetics (section 4.2.16). The synthetase presumably has all the pyruvate in reaction mixtures for its own use under these conditions, so that its kinetics with respect to pyruvate resemble those exhibited by the glycerol enzyme. The recombination of the separated enzyme fractions (fig.4.2.13) resulted in a return to the sigmoidal kinetics with respect to AL formation by AHAS. The  $K_m$  values for pyruvate for the two enzymes appear to indicate that PD has a higher affinity for pyruvate than does AHAS. In addition, PD is saturated by levels of pyruvate substantially lower than that required to saturate AHAS (section 4.2.14).

The results of the experiment described in section 4.2.10 ("carbon source shift" experiment) can be interpreted simply in terms of the competition hypothesis. Upon transfer of the cells to fresh

glucose medium, their metabolism would shift immediately to a fermentative type because glucose is more easily and preferentially utilized when present along with other carbon sources (diauxie, Monod, 1947). In E. coli, glycerol utilization is prevented when glucose is present, because fructose 1,6-diphosphate (a metabolite of glucose) is a feedback inhibitor of glycerol kinase (Zwaig and Lin, 1966). A rise in PD activity would be expected to accompany this change in metabolism, and this increased activity of PD would account for the appearance of the sigmoidal kinetics shown by the AHAS extracted from these shifted cells. No studies were made, during the course of this experiment, on the levels of PD in the two cultures.

An interesting observation is the effect of acetoin on AHAS. Acetoin added exogenously to medium containing glucose caused the kinetics of AHAS of cells grown in this medium to be Michaelian (fig. 4.2.8), whereas acetoin in the reaction mixture had no effect on the synthetase kinetics (fig. 4.2.10). It is possible that excess acetoin added to the growth medium may have caused repression or inhibition of PD, although experiments designed to test this hypothesis were unsuccessful. That acetoin has no effect on AL formation in vitro suggests that acetoin does not inhibit PD activity.

The relative simplicity of the competition hypothesis is a strong feature in its favour. Nevertheless, other models are possible. For instance, it is possible that two AHAS synthetases are present in S. pombe, the elaboration of one or the other being dependent upon the carbon source used for growth. Another possibility is the existence of some modifier protein (enzyme) which can modify the synthetase under different growth conditions, by analogy with the ATP: glutamine

synthetase adenylyltransferase enzyme described by Kingdon et al.  
(1967), which catalyzes alterations in the regulatory properties of  
glutamine synthetase.

#### 4.3. ISOMEROREDUCTASE (IR)

It has been shown in a previous section (3.2.3) that cell-free extracts from S. pombe are capable of converting  $\alpha$ -acetolactate and  $\alpha$ -aceto- $\alpha$ -hydroxybutyrate to  $\alpha,\beta$ -dihydroxyisovalerate and  $\alpha,\beta$ -dihydroxy- $\beta$ -methylvalerate, respectively. These conversions are catalyzed by the  $\alpha$ -aceto-hydroxy acid isomeroreductase (IR), which is the third enzyme in the isoleucine pathway, and the second enzyme of the valine pathway. There is evidence that the one enzyme serves for both pathways (Umbarger et al., 1960; Kiritani et al., 1966).

In this section, data will be presented which help to characterize the IR from S. pombe and allow some comparison with the enzyme isolated from other organisms.

##### 4.3.1. Requirements for activity

For this experiment, cells of S. pombe were smashed as previously described, except that the enzyme was extracted into Veronal buffer, pH 8.0, containing 1 mM DTT. IR activity was assayed in the reaction mixture described in 3.2.3, except that Tris-HCl buffer (0.1 M, pH 7.8) was used instead of phosphate (phosphate buffers were avoided in this experiment in order to keep  $Mn^{2+}$  salts in solution). With AHB as substrate, 50  $\mu$ l of crude extract were used for assay; 100  $\mu$ l crude extract were used with AL serving as substrate. The results are presented in table 4.3.1. The activity of the enzyme is 4-5 times greater with AHB as substrate than when AL is provided. This has also been found for the enzymes from E. coli (Umbarger et al., 1960), S. typhimurium (Arfin and Umbarger, 1969) and Pheseolus radiatus (Satyanarayana and Radhakrishnan, 1965).

Table 4.3.1. Requirements of *S. pombe* IR

Conditions	Specific activity*	
	AHB	AL
complete	0.030	0.007
- substrate	0.001	0.001
- Mg <sup>2+</sup>	0.010	0.007
- NADPH	0	0
complete (Mn <sup>2+</sup> instead of Mg <sup>2+</sup> )	0.001	0.002

\*  $\mu$ moles NADPH oxidized/min/mg protein

Mg<sup>2+</sup> appears to be a cofactor in the reaction, at least with AHB as substrate; Mn<sup>2+</sup> is not a satisfactory substitute for Mg<sup>2+</sup>. A similar requirement for Mg<sup>2+</sup> was demonstrated for the IR of *E. coli* and *N. crassa* (Radhakrishnan et al., 1960), *Salmonella* (Arfin and Umbarger, 1969) and *S. cerevisiae* (Strassman et al., 1960).

#### 4.3.2. Effect of pH on IR activity

The effect of pH on the activity of IR was investigated in the range 6.4-8.8, using AL and AHB as substrates. The results in fig. 4.3.1 show a rather broad plateau of IR activity in the range pH = 7.2-8.0 with AL as substrate. A sharper peak of activity was attained with AHB as substrate, which occurred at pH 7.6. Assays for IR activity in *S. pombe* extracts were subsequently routinely assayed at pH 7.6.

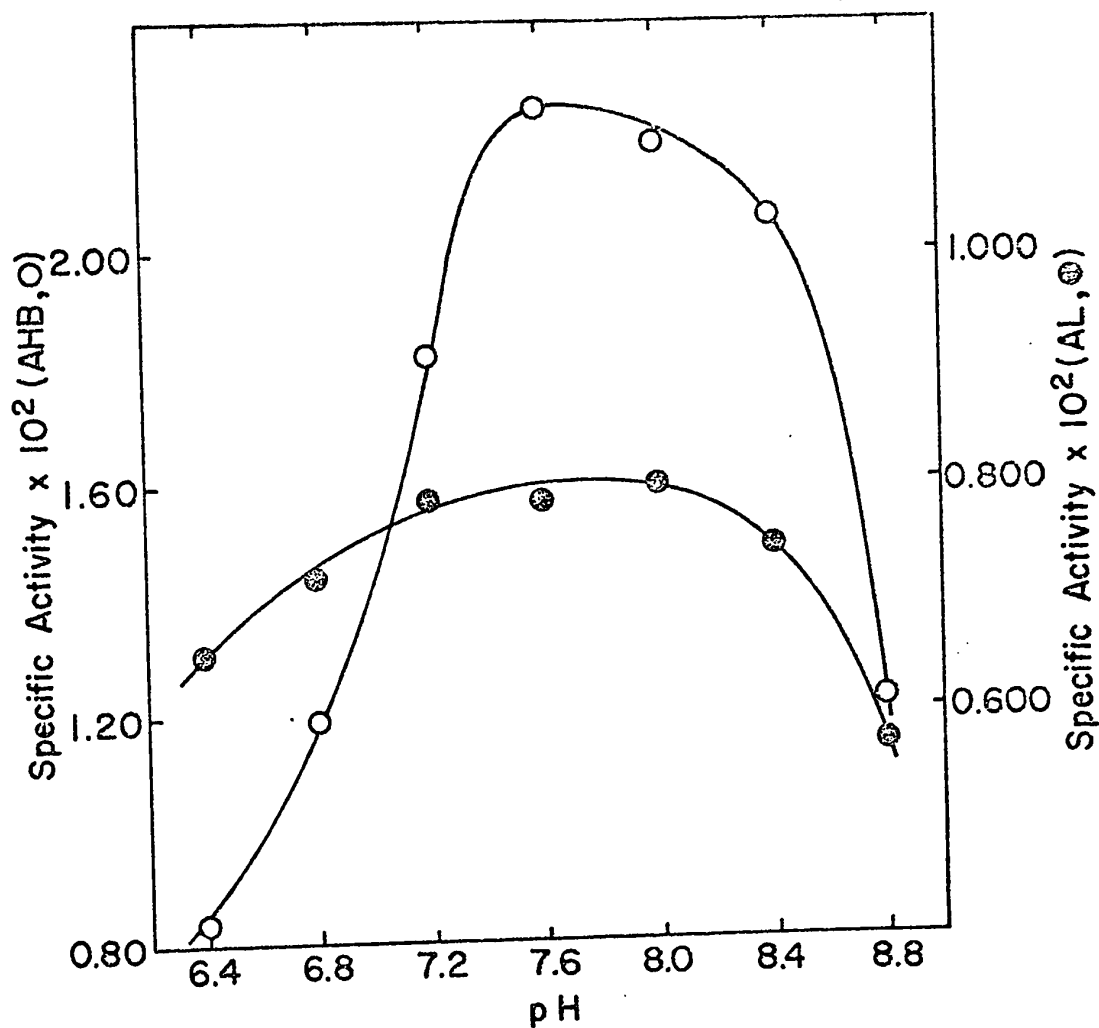


Fig. 4.3.1. Influence of pH on IR activity from *S. pombe*. A crude extract was prepared as described in section 4.2.1a, using 0.1 M potassium phosphate buffer, pH 7.5, supplemented with 20% glycerol and 1 mM DTT. Activity was assayed in the standard reaction mixture (section 3.2.3), using tris- $\text{PO}_4$  buffer adjusted to the pH's indicated. CE was diluted 1:1, and 100  $\mu\text{l}$  were incubated with AHB (○—○) and AL (⊗—⊗). Specific activity is umoles NADPH oxidized/min/mg protein.

The pH optimum of IR from S. pombe compares with that of other systems except Phaseolus, which is optimally active at pH 8.6 (Satyanarayana and Radhakrishnan, 1965). No attempts were made to determine if the IR reaction is reversible at higher pH, as was the enzyme from S. typhimurium (Arfin and Umbarger, 1969).

#### 4.3.3. Substrate saturation of IR and $K_m$ values

The  $K_m$  values of the two substrates for IR from several species are listed in table 4.3.2. It can be seen that, for the two bacterial species, the  $K_m$  for AL and AHB are essentially identical. The enzymes from the two eucaryotes listed have different  $K_m$  values for AL and AHB. No values could be obtained for the enzyme from bakers' yeast. It was of interest, therefore, to investigate the kinetics of substrate saturation of the IR from S. pombe, and to characterize the enzyme with respect to its  $K_m$  values.

The results are presented in fig. 4.3.2. With both substrates, the saturation kinetics yield normal rectangular hyperbolas and linear double reciprocal plots. It can be seen that the IR from S. pombe has an affinity about 4 times greater for AHB as substrate than for AL. This difference is similar to the difference in enzyme activity noted when AL or AHB was used as substrate (4.3.1, and this experiment). The apparent  $K_m$  values obtained are only tentative, as the AHB and AL used in these studies are racemic mixtures.

Table 4.3.2. Comparison of  $K_m$  values for AL and AHB for IR from various systems

Species	$K_m, M$		Source
	AL	AHB	
<u>S. typhimurium</u>	$1.9 \times 10^{-4}$	$3.4 \times 10^{-4}$	Armstrong and Wagner (1961)
<u>E. coli</u>	$6.5 \times 10^{-4}$	$7.3 \times 10^{-4}$	Radhakrishnan <u>et al.</u> (1960)
<u>N. crassa</u>	$3.2 \times 10^{-4}$	$1.6 \times 10^{-4}$	Kiritani <u>et al.</u> (1966)
<u>P. radiatus</u>	$5.5 \times 10^{-3}$	$1.2 \times 10^{-4}$	Satyannarayana and Radhakrishnan (1965)

Fig. 4.3.2. Substrate saturation of IR of S. pombe. Crude extract was prepared as before (section 4.2.1a) and activity was assayed in the standard assay mixture with increasing concentrations of substrate. With AHB, 50  $\mu$ ls CE were assayed at each substrate concentration, and 100  $\mu$ ls CE was used with AL. Insets show Lineweaver-Burk transformations for determination of  $K_m$  values.

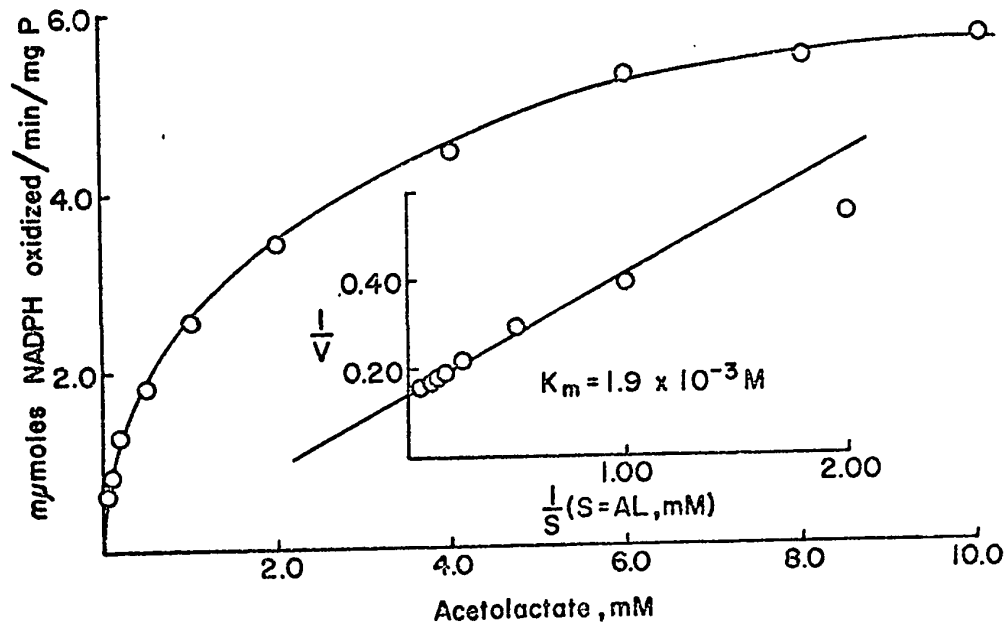
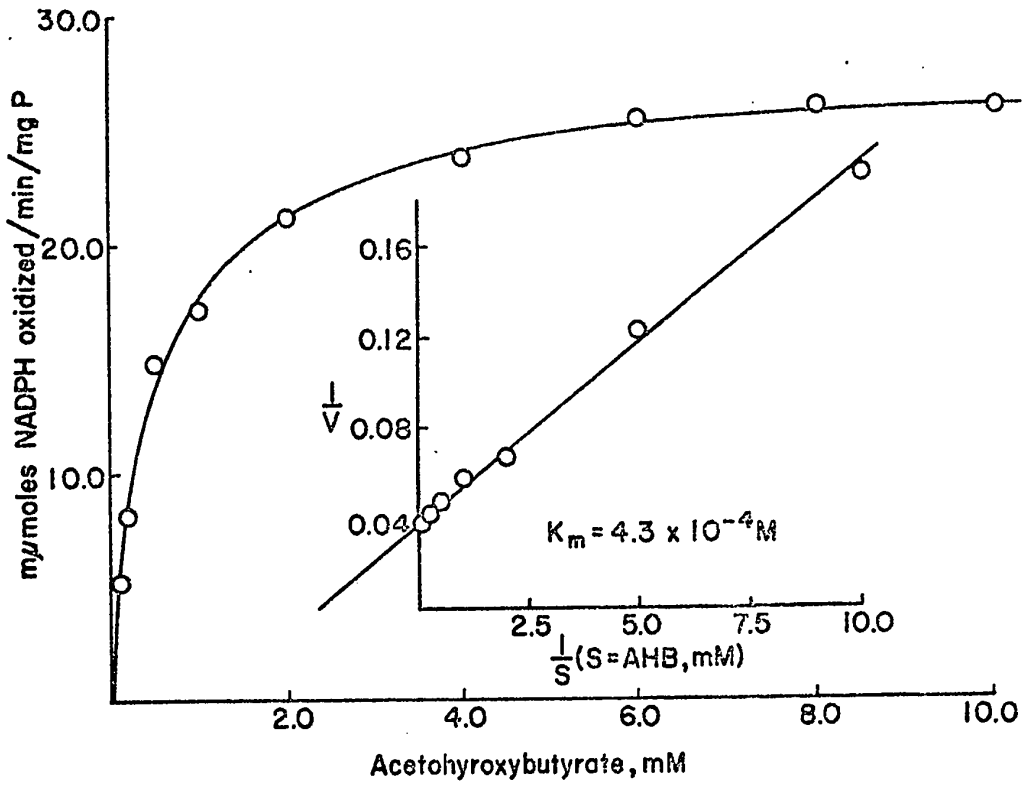


Fig. 4.3.2.

#### 4.3.4. Discussion of IR data

No attempts were made to purify the IR from S. pombe or examine other kinetic aspects of the enzyme, for it was our interest only to characterize it sufficiently in order to study its behaviour in repression experiments (to be presented in a later section).

The enzyme from S. pombe exhibits properties similar to the IR of other systems which have been investigated: same pH optimum, requirement for  $Mg^{2+}$ , 4-5 fold greater activity with AHB than with AL, and relatively high affinities for its substrates (of the order of  $10^{-4}M$ ). No data on the characteristics of the IR from S. cerevisiae are available other than that it shows NADPH and  $Mg^{2+}$  requirements (Watanabe et al., 1959; Strassman et al., 1960). These workers assay IR activity in crude cell homogenates at pH 8.0, but no rigorous pH study is reported. Magee and Hereford (1969) assay IR activity in bakers' yeast at pH 7.7, while Bussey and Umbarger (1969) and Kakar and Wagner (1964) used pH 7.5.

No attempts were made to determine the activities of several reductases which are often found in association with the IR isolated from E. coli (Umbarger et al. 1960) and N. crassa (Radhakrishnan et al., 1960). It was shown for S. typhimurium (Armstrong & Wagner, 1961) that purified IR showed one band for IR activity and 3 separate bands possessing reductase activity after starch gel electrophoresis. These workers concluded that the reductase activities probably represented altered forms of IR.

#### 4.4. DIHYDROXYACID DEHYDRATASE (DH)

Cell-free extracts of S. pombe have been shown to contain dihydroxyacid dehydratase (DH) activity (3.2.4), which catalyzes the conversion of dihydroxyisovalerate and dihydroxymethylvalerate to ketoisovalerate and ketomethylvalerate, respectively. The experiments described in this section were performed in order to characterize further the DH of S. pombe with respect to optimum pH for activity and its kinetic behaviour towards its two substrates.

##### 4.4.1. Requirements for activity

A crude cell-free preparation was prepared from cells of S. pombe as previously described (4.2.1.a), using 0.1 M potassium phosphate buffer, pH 7.5, to extract the enzyme. Enzyme activity was assayed in the assay mixture as described in section 3.2.4; 50  $\mu$ l of the crude extract were incubated for 15 min at pH 8.0. The results are given in table 4.4.1; relative activity is presented because the protein concentration of the extract was not determined. The DH in the crude extract is fairly resolved for  $Mg^{2+}$ , for activities with both substrates could be at least doubled by inclusion of  $Mg^{2+}$  in the assay mixture. The enzyme was not tested for any other cofactors, but  $Mn^{2+}$  could be substituted for  $Mg^{2+}$  when enzyme activity was assayed in Tris buffers. The DH from E. coli and N. crassa (Myers and Adelberg, 1954) and bakers' yeast (Wixom et al., 1960) all require  $Mg^{2+}$  for optimal activity.

Table 4.4.1. Requirements of the DH from S. pombe

Conditions	µmoles ketoacid formed per ml enzyme per min
Complete with DHIV	0.334
complete with DHMV	0.212
- DHIV	0.002
- DHMV	0.000
DHIV - Mg <sup>2+</sup>	0.118
DHMV - Mg <sup>2+</sup>	0.105

4.4.2. Effect of pH on DH activity from S. pombe

The effect of pH on DH activity with each substrate is illustrated in fig. 4.4.1. With DHIV as substrate, activity was optimal between pH 7.4 - 7.6, while with DHMV, a rather broad pH optimum around pH 7.6 - 8.0 was obtained. In subsequent experiments, all DH assays were performed in potassium phosphate buffer at pH 7.6.

4.4.3. Effect of substrate concentration

DH activity in a crude extract of S. pombe was assayed in increasing concentrations of each of its substrates, and the results are given in fig. 4.4.2. The kinetics of substrate saturation of the DH are strictly Michaelian for both substrates. The corresponding double reciprocal

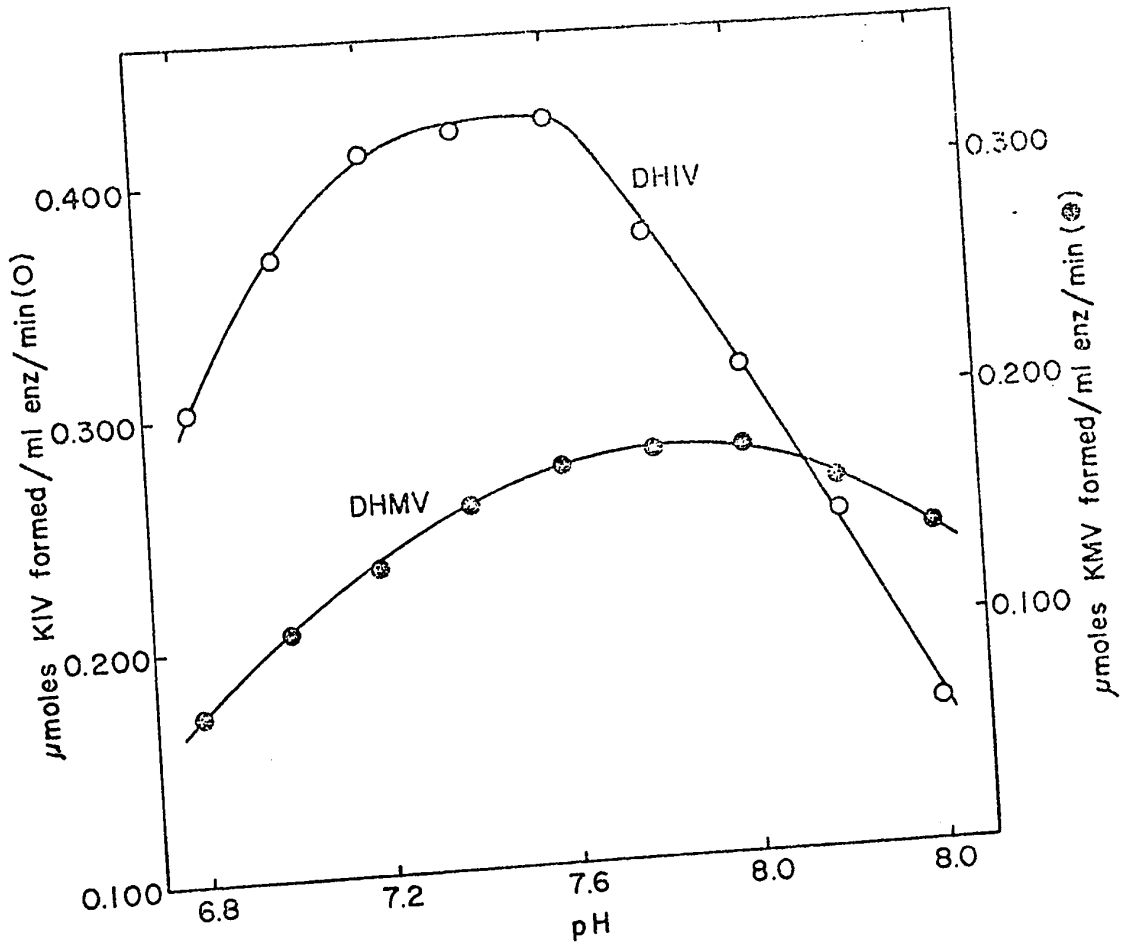


Fig. 4.4.1. Effect of pH on *S. pombe* DH activity. Crude extract was prepared as before (section 4.4.1) and assayed in the standard reaction mixture containing 200 micro-moles/ml Tris-HCl buffer adjusted to the indicated pH values. 20  $\mu$ ls CE assayed with DHIV or DHMV for 20 min.

► Fig. 4.4.2. Substrate saturation kinetics of DH. Proteins of S. pombe were extracted into 0.1M potassium phosphate buffer, pH 7.4, containing 20% glycerol and 1 mM DTT. Standard reaction mixtures containing DHIV in concentrations 1.0-50 mM, and DHMV at 0.1-50 mM, were inoculated with 50  $\mu$ ls CE (1.2 mg protein) and incubated for 15 min. The products, KIV and KMV, were determined as described (section 3.2.4). Insets show Lineweaver-Burk transformations of the results.

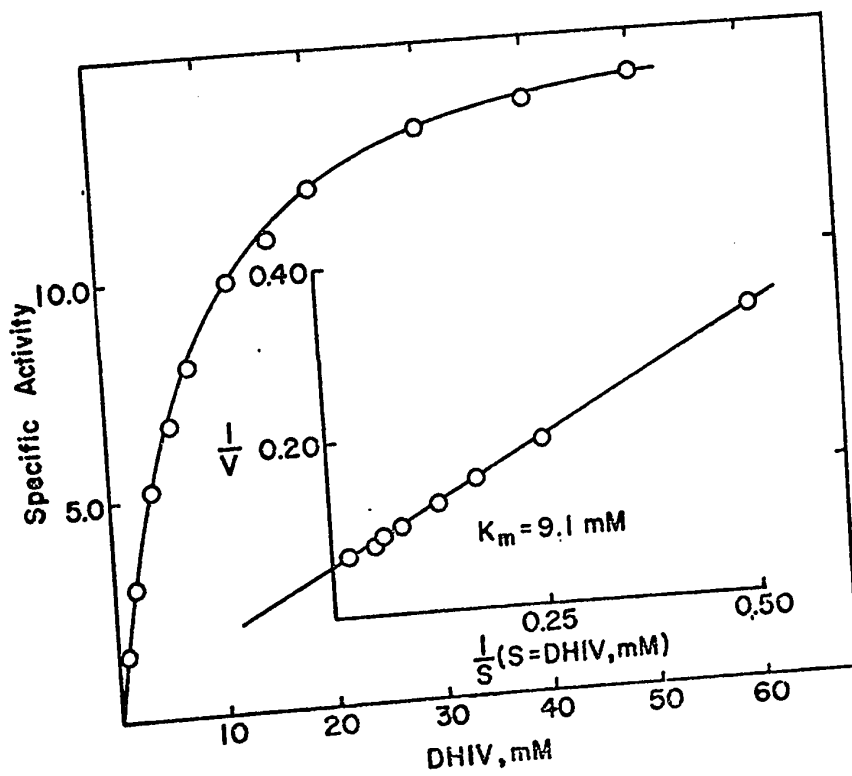
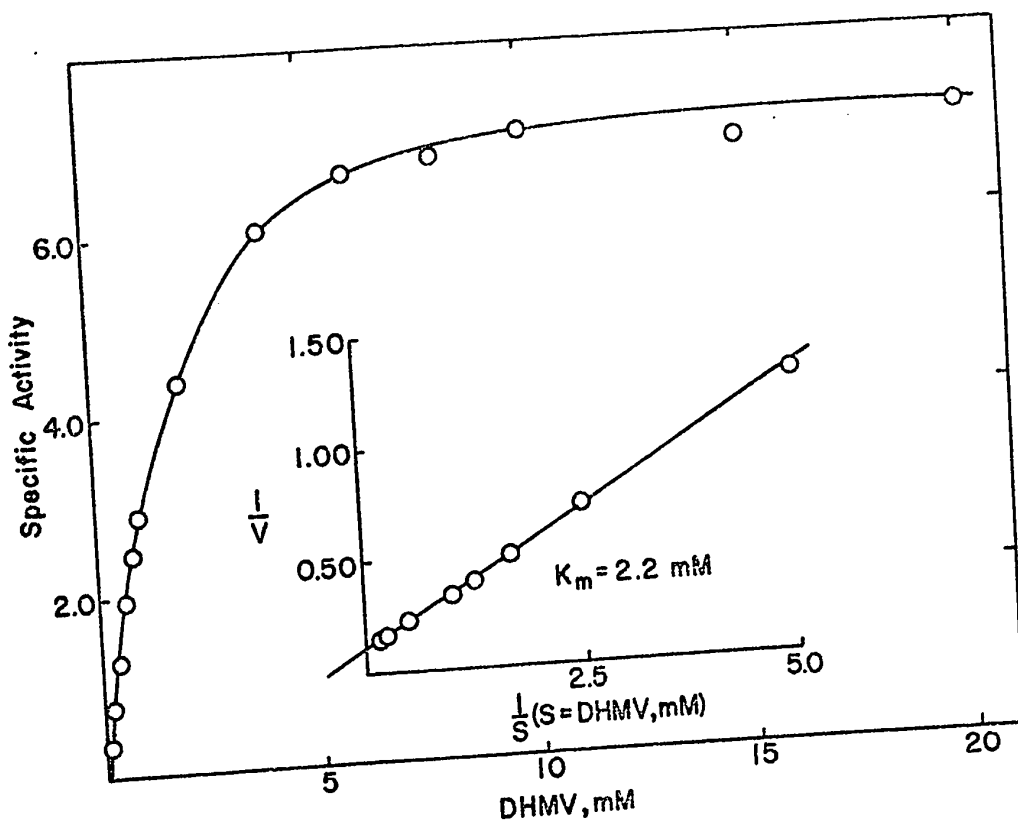


Fig. 4.4.2.

plots are linear, and yield apparent  $K_m$  values of 9.1 mM for DHIV and 2.2 mM for DHMV. These  $K_m$  values are rough estimates only, for the DHIV and DHMV used were racemic mixtures. Nevertheless, the DH appears to have a higher affinity for DHMV as substrate than for DHIV. With DHIV, the enzyme is saturated at greater than 50 mM substrate, whereas with DHMV, the enzyme is saturated by 10 mM substrate.

The observation that, for the S. pombe DH, DHMV is a better substrate for the enzyme is interesting, because in E. coli (Myers, 1961), N. crassa (Kiritani et al., 1966) and in S. cerevisiae (Wixon et al., 1960), DHIV is the substrate which is most favourably utilized. The enzyme isolated from Phaseolus radiatus (Satyanarayana and Radhakrishnan, 1964), however, has a higher affinity for DHMV than for DHIV.

#### 4.4.4. Discussion of DH observations

It is evident that S. pombe possesses the enzymatic ability to dehydrate the dihydroxy acid precursors to the keto acid precursors of isoleucine and valine. With the demonstration of transaminase B activity in extracts of S. pombe (3.2.5), it is reasonable to conclude that isoleucine and valine are synthesized in S. pombe by the pathway which has been demonstrated in most other microorganisms (Umbarger and Davis, 1962).

The DH of S. pombe is similar in its properties to the enzymes of other microorganisms and plants (table 4.4.2) except for the apparent greater affinity for DHMV as substrate, which is probably not a significant difference.

No further studies were performed on the DH from S. pombe, such as purification, tests for additional cofactors (e.g. pyridoxal phosphate).

Table 4.4.2. Comparison of DH from various species

Organism	pH.opt.	Metal ion requirement	$K_m, M$		Source
			DHIV	DHEV	
<u>N. crassa</u>	7.7-8.3	Mg <sup>2+</sup>	5.8x10 <sup>-4</sup>	1.2x10 <sup>-3</sup>	Kiritani <u>et al.</u> (1966)
<u>E. coli</u>	7.8-7.9	Fe <sup>2+</sup> , Mg <sup>2+</sup> or Mn <sup>2+</sup>	8x10 <sup>-5</sup>	1.7x10 <sup>-4</sup>	Myers (1961)
<u>S. cerevisiae</u>	7.4	Mg <sup>2+</sup> or Mn <sup>2+</sup>	1.7x10 <sup>-3</sup>	—	Wilcox <u>et al.</u> (1960)
<u>P. radiatus</u>	ca.8.0	Mg <sup>2+</sup> , Mn <sup>2+</sup> or Fe <sup>2+</sup>	2.4x10 <sup>-3</sup>	9x10 <sup>-4</sup>	Satyannarayana and Radhakrishnan (1964)
<u>S. pombe</u>	7.6	Mg <sup>2+</sup> or Mn <sup>2+</sup>	9.1x10 <sup>-3</sup>	2.2x10 <sup>-3</sup>	

#### 4.5. $\alpha$ -ISOPROPYLMALATE SYNTHETASE (IPMS)

IPMS catalyzes the first step in leucine biosynthesis, namely the acetylation of  $\alpha$ -ketoisovalerate by acetyl CoA to give  $\alpha$ -isopropylmalate. It was shown in a previous section (3.2.7) that cell-free extracts of S. pombe were able to catalyze this reaction in vitro. As IPMS is the first enzyme of the leucine pathway, it was desirable to characterize the enzyme from S. pombe with respect to its activity as a function of pH and to determine whether it is subject to feedback inhibition by leucine.

##### 4.5.1. Effect of pH on IPMS activity and feedback properties

The pH profile of IPMS from S. pombe (fig. 4.5.1) shows a rather broad peak of optimal activity in the pH range 7.5-8.5; activity drops off sharply on either side of this plateau.

The effect of pH on inhibition of IPMS activity by L-Leucine is strikingly different. Maximum inhibition occurs in the acid range between pH 6.5 and 7.0 (54% inhibition at pH 6.5), and falls off to a plateau around pH 7.7; inhibition by leucine at 5 mM is around 10-15% in the pH range 7.6-9.0. The curve (open triangles) showing % inhibition by leucine (fig. 4.5.1) resembles a titration curve with an inflection point near pH 7.2. Kohlhaw et al. (1969) observed a similar inhibition pattern with the IPMS of S. typhimurium; they conclude that histidine may be involved in the binding site (s) for leucine, since the observed range of the pK value of the imidazole group in proteins is 5.6-7.0.

L-Isoleucine and L-valine (at 5 mM) inhibited IPMS activity from S. pombe 20.7% and 12.6%, respectively, at pH 7.3. In contrast, 1 mM

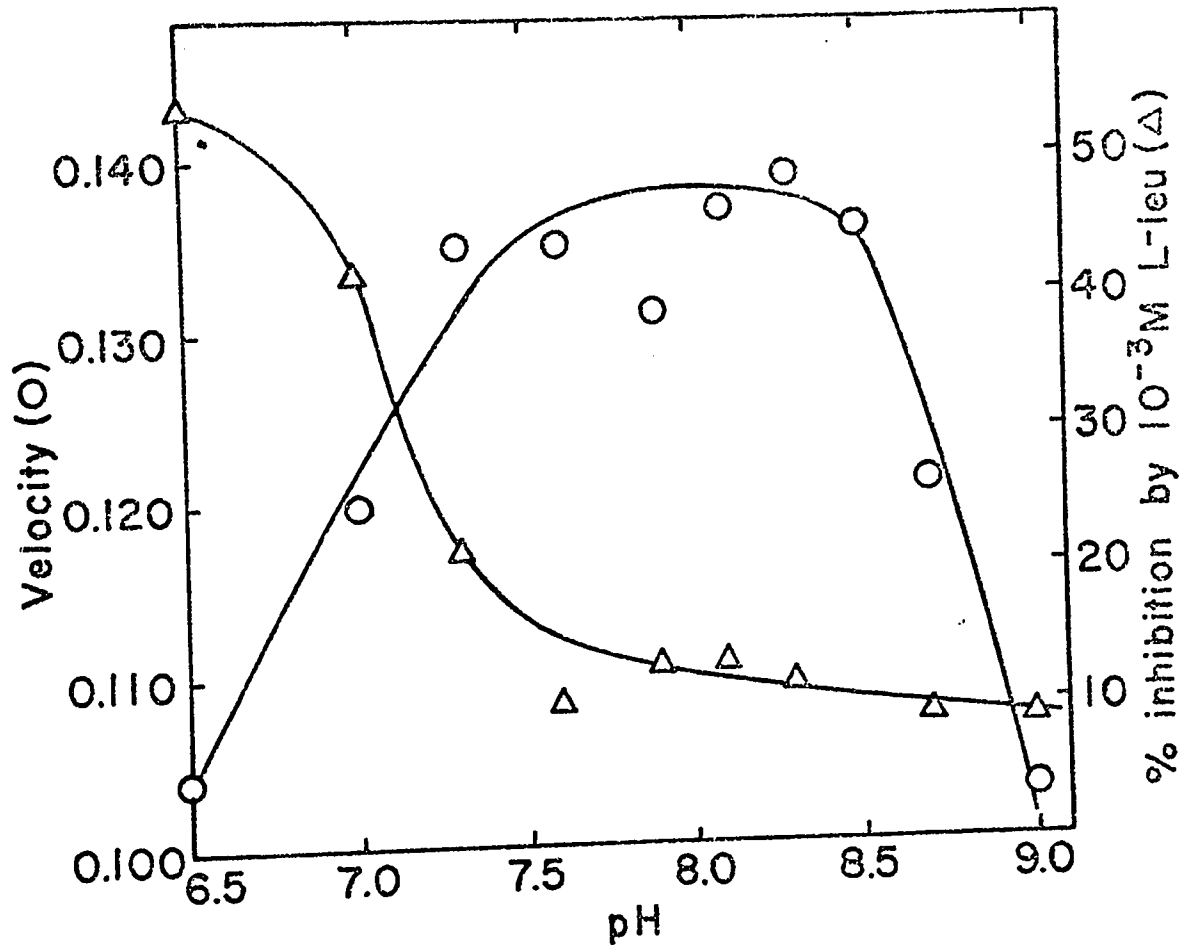


Fig. 4.5.1. Effect of pH on activity and leucine sensitivity of *S. pombe* IHMS. Crude extract was prepared using 0.1 M tris-HCl buffer, pH 8.0, containing 20% glycerol, 1 mM EDT and 0.1 mM L-leucine. One ml of the CE was filtered through Sephadex G-25, and the eluate was used for assay. Standard reaction mixtures were prepared with Tris-HCl buffers ranging in pH from 6.5 - 9.0; a blank without KIV was run at each pH to correct for endogenous AcCoA cleavage. 10  $\mu$ ls of G-25 eluate (0.11 mg protein) were incubated for 10 min in each reaction mixture. Leucine sensitivity was measured in the presence of 1 mM L-leucine. Activity is expressed as  $\mu$ moles SH formed / ml enzyme / min.

leucine produced 21.5% inhibition at the same pH; leucine is about 5 times as effective as isoleucine.

#### 4.5.2. Discussion

From these very preliminary studies, it is evident that the IPMS of S. pombe appears to be a typical regulatory enzyme. It is subject to retroinhibition by L-leucine, which bears little steric similarity to ketoisovalerate. The marked difference between the pH optimum for activity of the enzyme and the pH for optimal inhibition by leucine is typical of regulatory enzymes.

The IPMS from S. pombe is similar, with respect to these properties, to the enzymes isolated from S. typhimurium (Kohlhaw et al., 1969) and S. cerevisiae (Satyanarayana et al., 1968; Ulm and Kohlhaw, 1971). The enzymes from N. crassa (Webster and Gross, 1965) and P. aeruginosa (Rabin et al., 1968) have lower pH optima at about 7.5 and 7.0, respectively. All IPMS's studied are subject to retroinhibition by leucine.

IPMS is repressible by leucine present in excess. The situation in S. cerevisiae, however, warrants some comment. When bakers' yeast was grown in minimal medium supplemented with leucine, the levels of IPMS were consistently higher than when the cells were grown in unsupplemented medium (Satyanarayana et al., 1968a). Further, it was found that only threonine, of all the amino acids tested, caused repression of IPMS; leucine enhanced the effect of threonine, while isoleucine and valine abolished it. In view of these findings, would be of great interest to study the effect of amino acid supplements to HM on the levels of IPMS in S. pombe. A complete study of the leucine pathway, must, however, await the

availability of commercial quantities of  $\alpha$ -isopropylmalate, the substrate required for the assay of the two intermediate enzymes of the leucine pathway.

4.6. REGULATION OF THE SYNTHESIS OF THE ISOLEUCINE-VALINE-FORMING ENZYMES  
IN S. POMBE

In E. coli, the levels of the enzymes involved in isoleucine-valine biosynthesis are controlled by multivalent repression (Freundlich et al., 1962): these enzymes are repressed only when excess isoleucine, valine and leucine are all present. In this way, a deficiency of one of the endproducts results in derepression of the enzymes for the pathway.

Radhakrishnan and Adelberg (1965) showed that in E. coli K-12, the structural genes for isoleucine-valine enzymes are tightly clustered and constitute two (possibly three) operons. In bakers' yeast, however, the genes coding for the isoleucine-valine enzymes are scattered on different chromosomes (von Borstel, 1969). In S. pombe, of the three branched chain amino acids synthesized via the ile-val-leu pathways, only 3 markers of the leucine chain have been mapped: leu 2 is situated on Chromosome I, and leu 1 and leu 3 are linked on Chromosome II, but are not adjacent (Flores da Cunha, 1970). In N. crassa, the four cistrons specifying the leucine biosynthetic enzymes are located on three different linkage groups, and the leu 3 and leu 4 genes which are linked are 10-15 map units apart (Kashmiri and Gross, 1970). An exception to unlinked genes is the ura-2 gene in S. cerevisiae (Lue and Kaplan, 1971): this gene appears to be a polycistronic operon coding for aspartate transcarbamylase, carbamyl phosphate synthetase, and possibly a regulatory polypeptide. But, generally, it appears that in eucaryotes, structural genes for the enzymes of a pathway are not organized into functional units (operons) as in bacteria, at least as far as the isoleucine, valine and leucine pathways are concerned. One would expect, then, that the

regulation of the synthesis of these enzymes in eucaryotes would be different, if not less efficient, from the mechanisms operating in pro-caryotes. However, it has been shown independently by Bussey and Um-barger (1969) and by Magee and Hereford (1969), that in bakers' yeast the isoleucine-valine enzymes are non-coordinately multivalently repressed.

As this was the first time that multivalent repression was demon-strated in eucaryotes, it was of interest to examine the patterns of re-pression and derepression of the isoleucine-valine enzymes in S. pombe, in order to gain more information on the ubiquity of multivalent repres-sion in eucaryotes.

#### 4.6.1. Media and growth conditions

In these experiments, the two basic media used were YPG (complete medium) and HM (minimal medium), prepared as described in section 3.1.1.2. In addition, supplemented HM was used: HM + ilv is minimal supplemented with isoleucine, leucine and valine, at 5 mM each; HM + ile, HM + val and HM + leu are minimal supplemented with the appropriate amino acid at a concentration of 5 mM.

Cells of S. pombe (strain 972h<sup>-</sup>) were precultured in YPG, harvested, washed once with water and resuspended in water to a cell density of 2.0-2.5 x 10<sup>8</sup> cells/ml. This suspension served to inoculate the cul-tures to be used in the experiments. The cells were grown as previously described (3.1.1.3), and harvested when the cultures reached Klett<sub>660</sub> readings of 200-250 units.

The cells were smashed as described (4.1.1.a), and the enzymes were extracted into 0.1 M potassium phosphate buffer, pH 7.4, supplemented

with 20% glycerol, 1 mM DTT, 5 mM Mg SO<sub>4</sub>, 2 mM TPP and 20 µg/ml FAD. As soon as the cell-free extracts were obtained, they were filtered through 0.9 x 15 cm columns of Sephadex G-25 pre-equilibrated with the buffer used in the enzyme extraction. Protein was eluted from the columns using the same buffer, and the enzyme activities of the extracts were assayed immediately. The G-25 eluate was used as such for assays of AHAS, DH and TrB activities; it was diluted 1:1 with buffer solution for the TD and IR assays.

Each experiment consisted of measuring the specific activity of each enzyme (in triplicate) in two separate identical cultures. In many cases, the experiment was repeated 2-4 times. The specific activities for each enzyme from each identical culture used were finally averaged for a mean specific activity of the enzyme in cells grown under that particular condition.

#### 4.6.2. Growth of *S. pombe* in the various media used

It soon became obvious that the growth of *S. pombe* was rather substantially affected by the different media used in these experiments.

*S. pombe* 972h<sup>-</sup> was grown from precultures in 25 ml cultures of the various media exactly as described in the previous section (4.6.1). Sidearm flasks were used, which allowed the growth of the culture (i.e. turbidity) to be monitored at various time intervals. Readings were usually taken at two-hour intervals in a Klett-Summerson colorimeter fitted with a no. 66 filter. The mean generation times were determined from a semi-log plot of the results. Table 4.6.1 lists the mean generation times of *S. pombe* in various unsupplemented and supplemented media;

where the mean generation time for a specific medium was determined more than twice, the corresponding standard deviation from the mean is presented.

Cells of S. pombe grew slightly faster in YPG than in HM, as expected. Isoleucine and leucine cause a statistically significant (at the 1% level) increase in mean generation time when they are present as a supplement in HM. When Klett readings of a culture of cells growing in HM + isoleucine or HM + leucine one plotted against time, it can be seen that there is a

Table 4.6.1. Generation times of S. pombe in various media

Medium	Generation time (hrs)
HM	3.3 ± 0.3
HM + isoleucine	4.1 ± 0.2
HM + leucine	4.3 ± 0.2
HM + ile + val + leu	4.1 ± 0.8
HM + valine	3.7
HM + ile + val	3.7 ± 0.1
HM + yeast extract + bacto-peptone	2.9

prolonged lag phase (fig. 4.6.1); it appears that isoleucine and leucine affect growth in the early stages of the cycle rather than in later stages. Cells inoculated into HM + valine grow at a rate intermediate to that of HM and HM supplemented with isoleucine or leucine. Valine

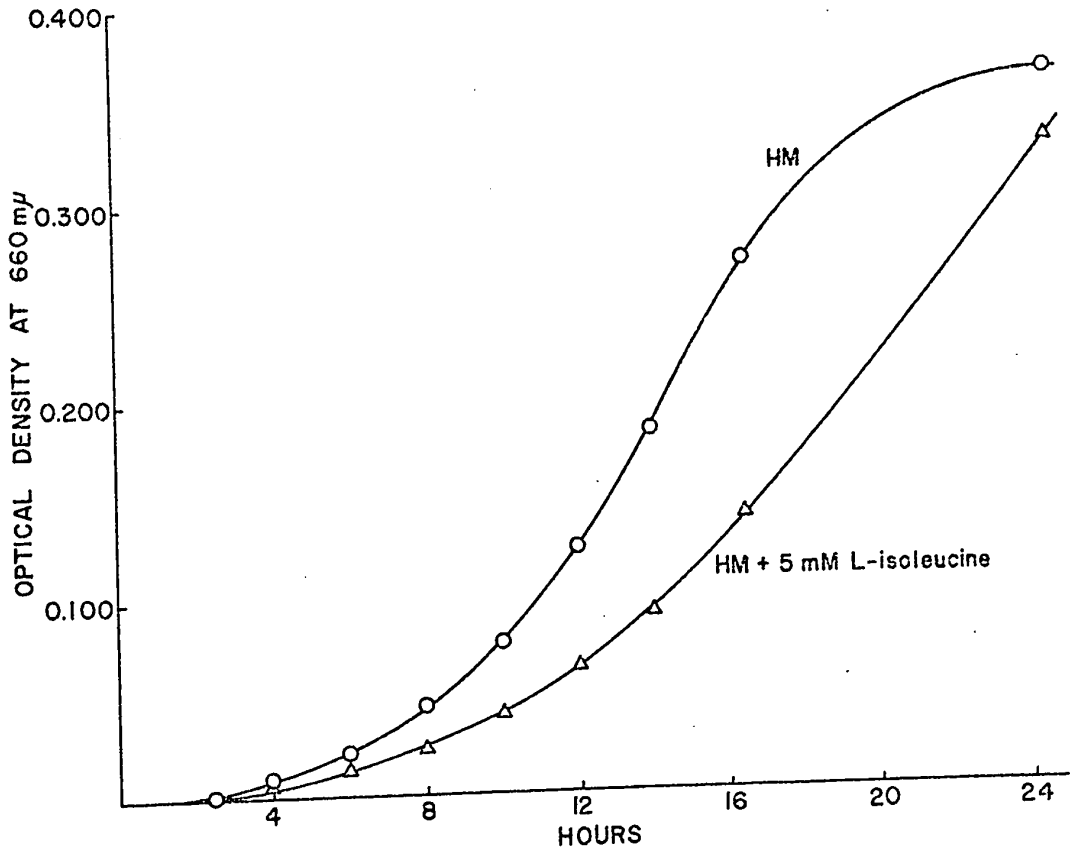


Fig. 4.6.1. Growth of *S. pombe* in Halvorson minimal medium (HM) and HM supplemented with 5 mM L-isoleucine. Cells were pre-cultured in YPG, washed once with water and resuspended to  $2 \times 10^8$  cells/ml. Inoculum consisted of 0.5 ml of this cell suspension in 50 mls medium. Turbidity was measured in Klett-Summerson colorimeter with a red no. 66 filter.

was able at least to partially overcome growth inhibition by isoleucine; it had no such effect with leucine. Growth in HM supplemented with isoleucine, valine and leucine was similar to that in HM supplemented with each amino acid singly. The difference in mean generation time between HM and HM + ilv cultures was not statistically significant at the 5% level. However, in all the experiments performed where HM + ilv was used, it required 1-2 hrs more incubation at 30 C to reach 200 Klett units than it did for HM only, all other parameters being equal.

The increase in mean generation time observed when cells were grown in HM + isoleucine was not sufficient to allow the isolation of isoleucine-resistant colonies of S. pombe (even when the isoleucine concentration in the medium was increased to 0.2 M).

#### 4.6.3. Levels of isoleucine-valine-forming enzymes

Table 4.6.2. shows the specific activities ( $\pm$  standard error of the mean) of the isoleucine-valine biosynthetic enzymes in wild-type strain 972 h<sup>-</sup> of S. pombe grown on various media. The specific activities of each enzyme were compared for pairs of media using the Student's t test to determine the significance of differences; the pairs tested were the following: HM vs YPG; HM vs HM + ilv; HM vs HM + ile; and YPG vs HM + ilv. All differences were statistically significant at the 1% level

(where the tests were feasible) except the following:

TD levels in YPG and HM  
AHAS levels in YPG and HM + ilv  
DH levels in YPG, HM and HM + ilv  
IR levels in YPG and HM + ilv  
TrB levels in all media tested

Several general conclusions may be drawn from the data of table 4.6.2. The isoleucine-valine (ilva) enzymes are present in generally higher levels in cells grown in HM than in those grown in YPG. AHAS and IR show the most marked changes, AHAS exhibiting the characteristic two-fold derepression of fungi (Magee and Hereford, 1969). AHAS, IR (and possibly TrB) are repressed in cells grown in HM supplemented with isoleucine, valine and leucine (ilv) to levels close to those found in cells grown in YPG, with the synthetase again showing the most marked response. DH is unaffected, while surprisingly, TD appears to be derepressed about 22% over levels in HM-grown cells. The ilva enzymes in cells grown in HM supplemented with isoleucine, valine or leucine singly are further derepressed over levels in minimal-grown cells; isoleucine elicits the most pronounced response, with AHAS being further derepressed two-fold over minimal medium levels. The important exception to note here is the fact that while valine causes substantial derepression of TD, DH and IR over HM levels, it represses the AHAS level (as would be expected) to near that of YPG-grown cells.

The TD does not show any marked, let alone significant, fluctuations except when media containing isoleucine or valine (perhaps also leucine) are used. TrB seems to be similarly resistant to any changes in level

Table 4.6.2. Levels of ilva enzymes in S. pombe

Growth conditions	Specific activity <sup>1</sup>					
	TD	AHAS	IR	DH	TrB	
YFG	0.201 ± 0.009 <sup>2</sup>	0.122 ± 0.003	17.16 ± 0.84	0.008 ± 0.000	0.020 ± 0.000	
HM	0.228 ± 0.012	0.245 ± 0.013	25.23 ± 0.37	0.008 ± 0.001	0.022 ± 0.001	
HM + ilv	0.279 ± 0.009	0.139 ± 0.020	18.34 ± 0.81	0.008 ± 0.001	0.020 ± 0.001	
HM + ile	0.370 ± 0.011	0.473 ± 0.023	35.41 ± 3.16	0.015 ± 0.000	0.031 <sup>3</sup>	
HM + val <sup>3</sup>	0.306	0.171	30.31	0.012	0.024	
HM + leu <sup>3</sup>	0.282	0.315	31.74	0.009	0.023	

1 TD: μmoles α-KB/min/mgP  
 AHAS: μmoles AL/min/mgP  
 IR: μmoles NADPH oxidized/min/mgP  
 DH: μmoles KIV/min/mgP  
 TrB: μmoles KIV/min/mgP

2 Specific activities ± S.E.M.

3 all determinations in duplicate only



from one medium to another, except again when isoleucine is present as a supplement. AHAS shows the most clear-cut pattern of repression and derepression. When cells were grown in HM supplemented with fructose as carbon source (instead of glucose) and 0.2M DL- $\alpha$ -aminobutyrate, the generation was increased to about 7 hrs; however, the levels of the isoleucine-valine enzymes did not show any marked derepression.

Only AHAS and IR appear to be subject to multivalent repression by isoleucine, valine and leucine included in the minimal medium. Except for TD, the remaining ilva enzymes appear to be resistant to any changes in level as a result of repression or derepression (at least under the conditions outlined here). The role of pantothenic acid (which is derived ultimately from  $\alpha$ -ketoisovaleric acid) was not investigated, but it is present in the basic HM in optimal amounts for the growth of S. pombe, and is thus not a limiting factor for growth.

Levels of IPMS were not investigated in these studies, because the details of its assay were not worked out until later.

#### 4.6.4. Discussion of repression experiments

The pattern of multivalent repression such as is found in enterobacteria (Freundlich et al., 1962) and in bakers' yeast (Magee and Hereford, 1969; Bussey and Umberger, 1969) does not appear to be present in S. pombe, at least as far as can be determined from studies with the wild-type strain. All three branched-chain amino acids are required for repression of AHAS and IR to levels near those obtained in cells grown in YPG; DH and TrB levels remain unchanged under the conditions of repression and derepression used. These latter two enzymes may be present

in the cell at a constitutive level, no matter what type of medium the cells are grown in; that is, they are present at a given, partially-de-repressed level which is independent of fluctuations in the intracellular pools of the branched-chain amino acids. It is of interest that the level of TD increases significantly when all three endproducts are present in the medium.

The similar behaviour of AHAS and IR in repression and derepression is of interest. It may be that IR in S. pombe is inducible by the products of AHAS, namely AL and AHB (which are also the substrates of IR); in other words, the level of IR could be mediated by the level of AHAS in the cell and its extent of activity. The substrate induction of IR was actually demonstrated directly in E. coli and A. aerogenes (Arfin et al., 1969), but such a demonstration in yeast is complicated by the impermeability of yeast to the acid intermediates of the ilv pathway (Magee and de Robichon-Szulmajster, 1968a). Control by substrate induction has been demonstrated by Gross (1965) for the leucine pathway in Neurospora, and by Lacroute (1968) for the pyrimidine pathway in S. cerevisiae. The results of Marinus and Loutit (1969) could be similarly interpreted in terms of substrate induction: in P. aeruginosa, AHAS and IR only are subject to multivalent repression, and these same two enzymes are derepressed simultaneously and appear to be coordinately synthesized. These authors speculate that the behaviour of these enzymes may be evidence for an operon.

Since the three branched-chain amino acids depend on four common enzymes for their synthesis, it may be that a deficiency of two of these amino acids results when growing cells are exposed to an excess supply of the third amino acid; an imbalance in the appropriate supply of these

three amino acids required for protein synthesis may, under these conditions, relieve the repression control over the ilva enzymes and their synthesis is increased to derepressed levels. Under these growth conditions, cells of S. pombe were found to contain higher levels of the ilva enzymes than those found in minimal-grown cells. The levels of DH and TrB were higher, although not enough determinations were done to test the statistical significance of these increases. AHAS was, of course, repressed by valine, but curiously the TD level of cells grown in the presence of isoleucine was markedly higher.

The observation of increased levels of TD in the presence of its endproduct inhibitor, isoleucine, is not unique. It was observed that the level of IPMS in cells of bakers' yeast grown in minimal medium supplemented with L-leucine was higher than that in cells grown in unsupplemented medium (Satyanarayana et al., 1968a). These authors suggest that the effect of leucine may not be a direct one, because isoleucine was as effective, and valine almost as effective, in derepressing this enzyme as was leucine. In P. aeruginosa (Marinus and Loutit, 1969), a wild type strain grown on minimal medium supplemented with isoleucine had a slightly higher specific activity of TD (10.3 units) as compared with the same cells grown in unsupplemented medium (8.3 units); they did not consider this difference to be significant. Horvath et al. (1967) observed that in Mycobacterium pellegrino addition of valine (at 0.2 mM) to minimal medium caused a 6-fold increase in AHAS content of these cells; this increased level of AHAS was attributed to an isoleucine deficiency caused by feedback inhibition by valine of AHAS, and subsequent increased synthesis of this enzyme due to derepression, thus resulting in making up the isoleucine requirement. This interpretation was supported by the fact that

exogenous isoleucine added with the valine prevented the increase in AHAS level.

The simplest interpretation of the increased level of TD activity in cells grown in the presence of isoleucine (and to some extent valine) is that the TD of these cells is stabilized by the high levels of isoleucine which are present both in the intracellular pool and in the external medium. The isoleucine in the cells themselves could help to preserve TD during the preparation of the crude extract, when the proteins in the homogenate could very well be exposed to local changes in temperature in the smashing bottle, despite the external cooling with CO<sub>2</sub>. Activity is preserved during the G-25 filtration by the presence of PLP, as the endogenous isoleucine of the extract is removed. In an earlier experiment where threonine replaced isoleucine in HM supplemented with valine and leucine, the level of TD in cells grown in this medium was intermediate between the TD level of HM-grown cells and YPG-grown cells. In this connection, it is interesting to note the analogous effect of UTP on the stabilization of feedback sensitivity of ATCase from bakers' yeast (Kaplan et al., 1969). In a uracil-requiring mutant growing under conditions of limiting uracil, derepression of ATCase was accompanied by a progressive loss of sensitivity to UTP; excess uracil added to the medium reversed this effect. Intracellular UTP appeared to be the stabilizing agent, and it was subsequently shown that UTP added to the buffer in which the cells were smashed could stabilize the feedback site during extraction, and enhance somewhat the specific activity of the enzyme. Another interesting and relevant example is the up to 10-fold increase in rat liver tryptophan pyrrolase observed in animals injected with excess tryptophan; this was first reported as an example

of enzyme induction in a mammalian system by Knox (1951). It was later shown, however, that the radioactivity of pre-labeled enzyme was not lost when tryptophan was administered, as opposed to its rapid loss in untreated animals (Schimke et al., 1965); this result suggested that tryptophan administration resulted in stabilization of the enzyme in vivo, thereby accounting for accumulation of enzyme by preventing its normal and rapid degradation while synthesis continued at normal rates.

The derepressed levels of ilva enzymes in cells grown in the presence of one of the branched-chain amino acids may also be due in part to the slowed growth response of S. pombe in the presence of isoleucine or leucine, as described in section 4.6.2. It was shown that leucine, as well as isoleucine, inhibits TD activity (fig. 4.1.14), and that isoleucine, as well as valine, inhibits AHAS (section 4.2.18). Generally, when the growth of a microorganism is retarded purposely, e.g. by an analog of a metabolite, the biosynthetic enzymes involved with that metabolite are derepressed (Demain, 1971).

Deviations from the observed pattern of multivalent repression of the ilva enzymes in the enterobacteria are noted in other organisms. For example, in P. aeruginosa, AHAS and IR are repressed by isoleucine + valine + leucine, and derepressed by isoleucine alone, both enzyme responses taking place in a coordinate manner. However, in both unsupplemented and supplemented media, the levels of TD and DH were unaffected (Marinus and Loutit, 1969). In addition, AHAS and IR eluted together after gel-filtration on Sephadex G-200. Thus, in this microorganism, the synthesis of two of the five enzymes is controlled by multivalent repression, and three are not. Barritt (1971), using extracts from wild type and isoleucine-valine auxotrophs of R. spheroides, was unable to

show any repression or derepression of the level of TD; the microorganism was grown both aerobically and under photosynthetic conditions. He concludes that the biosynthesis of isoleucine and valine is regulated predominantly by feedback inhibition mechanisms in this organism.

A study of repression and derepression cannot be complete unless these phenomena can be studied in isoleucine-valine auxotrophs. Unfortunately, despite repeated attempts, no such auxotrophs of S. pombe were isolated. Another aspect not considered in these studies is the role of isoleucyl-, valyl-, and leucyl-tRNA's in the repression mechanism. In the multivalent repression of the ilva enzymes, Eidlic and Neidhart (1965) showed that isoleucine, valine and leucine had to be activated by their respective tRNA synthetases for repression to occur. Freundlich (1967) demonstrated that repression of the isoleucine-valine enzymes in E. coli W required that valine not only be activated, but also charged to valyl tRNA. In S. cerevisiae, the activity of isoleucyl-tRNA synthetase is essential for multivalent repression of the isoleucine-valine enzymes (McLaughlin et al., 1969); this suggests that it is a product of the reaction catalyzed by the isoleucyl-tRNA synthetase, and not free isoleucine, which regulates enzyme synthesis.

## 5. GENERAL DISCUSSION

### 5.1. BIOSYNTHESIS OF ISOLEUCINE, VALINE AND LEUCINE IN S. POMBE

Through use of standard assay procedures, it has been possible to demonstrate the presence, in cell-free extracts of S. pombe, of all the enzymes known from studies of other species to be involved in the biosynthesis of isoleucine and valine; namely: threonine deaminase, aceto-hydroxy acid synthetase, aceto-hydroxy acid isomeroreductase, dihydroxy acid dehydratase, and transaminase B. In addition, the first enzyme of the leucine pathway,  $\alpha$ -isopropylmalate synthetase, was studied. AHAS, IR, DH and TrB were shown to react with the appropriate intermediate from both the isoleucine and valine pathways, and it may thus be concluded that, as in other systems tested (Umbarger and Davis, 1962), the isoleucine and valine pathways share these four enzymes.

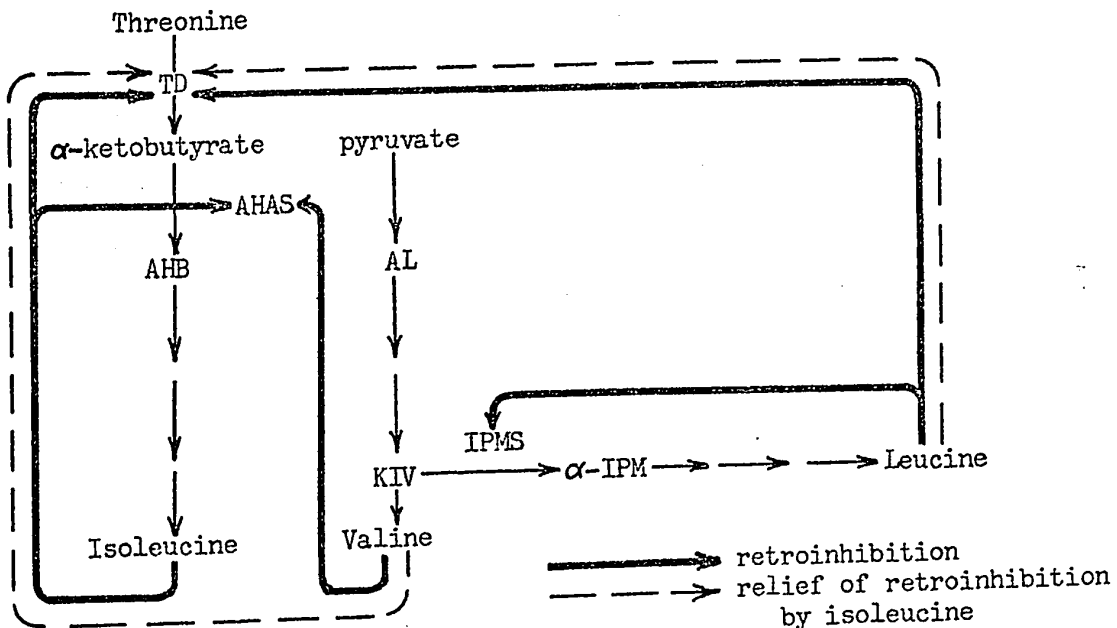
The possibility that the isoleucine and valine pathways each have a complete set of enzymes specific for that pathway (isozymes) under distinct regulatory and genetic control cannot be disregarded. Each enzyme would have to be purified and checked for a single protein band in ultracentrifugation and electrophoretic studies. Activities towards each substrate should be constant at all steps of purification. Finally, if one enzyme is responsible for both reactions at each step in the pathways, it should be possible to show kinetic competition between the two substrates. Such evidence obtained with other species (e.g. E. coli and N. crassa, Myers and Adelberg, 1954; Myers, 1961) strongly suggest a single set of enzymes common to both pathways. Moreover, genetic evidence shows that mutants blocked at any one of the steps after threonine deamination

require both isoleucine and valine or the appropriate precursors (Umbarger and Davis, 1962).

Our studies indicate that S. pombe possesses the capability of synthesizing the branched-chain amino acids by means of the pathways which have been elucidated for other prototrophic microorganisms.

### 5.2. REGULATORY ASPECTS OF BRANCHED-CHAIN AMINO ACID SYNTHESIS

Regulation of the branched-chain amino acids appears to be controlled in S. pombe primarily through mechanisms of feedback inhibition. It was shown that TD, AHAS and IPMS were each inhibited by their respective end-product, namely isoleucine, valine and leucine respectively. In addition, there are inter-pathway effects of these amino acids: thus, valine relieves isoleucine inhibition of TD, TD is inhibited by leucine, AHAS is inhibited by isoleucine as well as valine. These endproduct inhibition patterns are summarized in the following illustration:



The pattern of multivalent repression (Freundlich et al., 1962) shown to exist in bacterial systems (Umbarger, 1969) and bakers' yeast (Magee and Hereford, 1969; Bussey and Umbarger, 1969), could not be demonstrated in S. pombe; rather, only AHAS and IR showed clear evidence of multivalent repression. These same two enzymes showed the highest level of derepression as well, under appropriate conditions. AHAS was shown to be repressible by valine alone.

On the basis of the present evidence, then, one must tentatively conclude that S. pombe regulates its synthesis of these amino acids through interplay of its feedback inhibition mechanisms and its rather limited repression patterns.

The physiological significance of the extreme sensitivity of TD to isoleucine is immediately apparent - it serves as a rapid and sensitive control on the activity of the isoleucine pathway, thereby limiting the size of the isoleucine pools in the cell. Isoleucine also affects the activity of AHAS to some extent, as does valine, the natural endproduct inhibitor of that enzyme.

The physiological roles of valine activation of TD and the reversal of isoleucine inhibition by valine and leucine are not so apparent; one can only speculate on the roles of these amino acids in the regulation of the isoleucine-valine-leucine pathways. It is conceivable that valine and leucine act as signals or indicators of the requirement of the branched-chain amino acids for protein synthesis, to the "machinery" in the cell responsible for their synthesis (the ilvasome (?), a complex of isoleucine-valine enzymes which can, in vitro, synthesize these amino acids from pyruvate and  $\alpha$ -ketobutyrate and cofactors, as shown for Neurospora by Wagner, 1964). More specifically, valine and leucine may in

some way be indicators of the pool sizes of valine and leucine with respect to the isoleucine pool. From the kinetic studies, the isoleucine pathway is the most sensitive to endproduct control, and in view of this fact, the endogenous conversion pool of isoleucine may very well be small in relation to the valine and leucine pools which may be larger because of the less stringent control on their size.

In the absence of any supporting evidence, it is not unreasonable to assume that, to a first approximation, the three branched-chain amino acids may be required in approximately equal amounts for protein synthesis. It is interesting to note the amino acid content of hydrolysates of bakers' yeast protein, as given by Long (1961) (expressed as % of dry weight):

isoleucine	2.86
leucine	3.01
valine	2.32

Contrast these values with, for instance, the glutamic acid content of yeast protein at 5% of dry weight, or that of tryptophan at 0.66% of dry weight. These data support the assumption we have made above.

It seems possible that valine concentration (pool size) is a one-way signal controlling not only its own synthesis but that of isoleucine as well, much as the purines of bakers' yeast have been shown to be a one-way control of the pyrimidine pathway (Satyanarayana and Kaplan, 1971). If so, then even when the isoleucine pathway is shut down due to retroinhibition of TD by the isoleucine pool, growth and onset of protein synthesis could rely on increased isoleucine production if valine disinhibits this pathway. This frankly speculative hypothesis has at least three testable consequences: 1, it predicts that the isoleucine

pool size is significantly smaller than the valine pool; 2, it suggests that prior to the onset of the maximal rate of protein synthesis, there is a transient increase in the size of the valine pool; 3, it also predicts that there is a balanced and approximately equivalent synthesis of isoleucine and valine. With respect to no. 1 above, it may be of significance to note that there is very little evidence obtained with growing E. coli cells that the sensitivity of AHAS to valine is significant in vivo (Roberts et al. (1955), as described in more detail in section 4.2.22.2). These authors demonstrated that the flow of carbon over the valine pathway was virtually the same whether the growth medium contained excess valine or not. This would tend to support the notion of a relatively large intracellular valine pool.

### 5.3. GENERAL CONSIDERATIONS

It has been pointed out by Gross (1969) that the relatively small variations in the levels of enzymes involved in the synthesis of essential metabolites in fungi probably occur because the level of production of the biosynthetic enzymes is set relatively high under normal growth conditions. In this respect, the same author points out that the Neurospora wild type strain, when grown on minimal medium, possesses more isopropylmalate synthetase than it needs to satisfy the demand for leucine in protein synthesis; it was shown that feedback-insensitive mutants which produce near or less-than-normal amounts of IPMS, excrete large quantities of  $\alpha$ -ketoisocaproate and leucine during growth.

The relatively small difference in the levels of the ilva enzymes observed in S. pombe grown under varying conditions certainly bear out

Gross' observations. Slightly larger differences between repressed and derepressed levels of enzymes are found in S. cerevisiae (Magee and Hereford, 1969; Bussey and Umberger, 1969). As pointed out in the Introduction, although S. pombe has been reasonably well characterized genetically, precious little has been done correlating genetic evidence with biosynthetic metabolism, save for aspartate transcarbamylase (Megnet, 1958), phosphoribosylpyrophosphate: glutamine amidotransferase (Nagy, 1970), histidine and tryptophan (as cited by Leupold, 1970). No information is available on the levels of those enzymes investigated under conditions of repression and derepression. It is thus impossible to say whether the repression results obtained with the ilva enzymes are typical for S. pombe or not.

The notion of interpathway links served by the effect of valine and leucine on isoleucine inhibition of TD, is supported by the various findings of other workers. For instance, it was reported that in histidine-requiring mutants of N. crassa grown on limiting histidine, the last two enzymes of the tryptophan pathway were derepressed; excess histidine added to the medium prevented this derepression (Carsiotis and Lacy, 1965). Other examples have been cited by Jensen (1969): prephenate dehydratase involved in phenylalanine synthesis is not only inhibited by phenylalanine but is also activated by either leucine or methionine; leucine, methionine, phenylalanine, tryptophan and alanine all activate aspartokinase in B. polymyxa. From these and other observations, Jensen suggested that a regulatory superstructure of interactions exerted between metabolic pathways acts to reinforce and modify the basic allosteric controls operating within specific pathways.

Evidence for such interacting signals in metabolically interconnected pathways is available for yeast. At least two purine ribonucleotides exert significant effects on the first enzymes of the pyrimidine pathway in bakers' yeast. ATP significantly reduced the feedback inhibition of aspartate transcarbamylase by UTP (Kaplan et al., 1967), and XMP strongly activated carbamyl phosphate synthetase (Lue, 1970). The first enzyme of the purine pathway in yeast, phosphoribosylpyrophosphate amidotransferase, was shown to be sensitive to feedback inhibition by various purine ribonucleotides but not to the final products of the pyrimidine pathway (Satyanarayana and Kaplan, 1971). These in vitro results corroborate the in vivo studies of Burns (1964) on a yeast mutant deficient for adenine, histidine and uracil, where accumulation of high pool levels of purine nucleotides overcome the internal inhibition of the pyrimidine pathway; no reciprocal effect of pyrimidines on purine synthesis was observed. Thus the purines seem to play a role in assuring the balanced synthesis of the intermediates required for nucleic acid synthesis.

It should be apparent that a cell should have "global" control over the production levels of all the amino acids, purines and pyrimidines it requires for growth, both in the interests of cell economy and in maintaining a proper balance of flow of intermediates through its many metabolic pathways. Indeed, Jensen (1969) further suggests that the control mechanisms involving the key enzymes in metabolic pathways are primitive characteristics of those enzymes, and that modifications superimposed over these by secondary mutations in the course of evolution would tend to enhance the survival value of the basic patterns and therefore promote their conservation.

Finally, very little is known about the natural biology and ecology of the fungal species of genetic and regulatory interest to researchers, namely N. crassa, S. cerevisiae, Aspergillus and S. pombe. Without such knowledge, it is difficult to rationalize, in teleonomic terms, the evolutionary basis for gene relationships on the chromosomes and the myriad regulatory mechanisms employed. Clearly, these phenomena must be studied from the viewpoint of the cell facing and adapting to competition for survival under natural conditions.

#### 5.4. AVENUES FOR FUTURE RESEARCH

Having established the basic isoleucine-valine-leucine biosynthetic pathways in S. pombe, and the regulation of the enzymes involved, it is now possible to develop aspects of the system in more detail. A few general lines for investigation follow.

The most immediate effort should be directed toward an intensive search for isoleucine, leucine, and isoleucine-valine auxotrophs; standard techniques can be utilized, e.g. mutagenesis with nitrosoguanidine or ethyl-methanesulfonate, and enrichment with nystatin or 2-deoxyglucose. The availability of classes of such mutants would allow complementation and mapping studies to determine whether or not the genes coding for the ilva enzymes are contiguous as in an operon, or, as is more likely, are unlinked and residing on different chromosomes. Isoleucine-valine auxotrophs would allow a more complete study of repression mechanisms in S. pombe; limitation of growth could be achieved by limiting one branched-chain amino acid, and one might observe coordinate derepression of all the ilva enzymes.

TD and AHAS should be purified to homogeneity. Then, direct binding studies of threonine, isoleucine and valine could be carried out to determine the number of sites involved, and whether or not they are all distinct from one another. The same would apply with respect to valine and pyruvate. With pure TD, accurate molecular weight determinations could be made, the subunit structure of the enzyme could be determined and the role of PLP in the stabilization and activation of TD could be elucidated. By comparison with the TD purified from bakers' yeast (Ahmed and Magee, 1971), it would then be possible to investigate models

for eucaryotic TD's, similar to that devised for the enzyme from S. typhimurium (Hatfield and Burns, 1970).

In addition to the isolation of isoleucine-valine auxotrophs, a search must be made for mutants with feedback-insensitive TD. A promising lead in this direction is our recent observation that S. pombe appears to be more sensitive to certain analogs when the carbon and energy source included in the minimal medium is fructose instead of glucose. For example, the doubling time in HM + fructose (HMF) is 3.4 hrs (as for HM with glucose) but the inclusion of 0.1 M L- $\alpha$ -aminobutyrate in HMF increases the doubling time to 7.5 hrs, compared to only 5.8 hrs in HM + glucose + 0.1 M AB. Thiaisoleucine and azaleucine are good growth inhibitors of S. pombe growing in HMF. Results similar to these were first reported for P. aeruginosa by Calhoun and Jensen (1971). With a genetically desensitized mutant in hand, one could investigate the role of TD in the repression of the isoleucine pathway, in analogy with the findings of Kovach et al. (1969). These authors showed that in S. typhimurium, the normal kinetic pattern of repression of the histidine pathway enzymes was altered and the repressibility disappeared when the regulatory site of the first enzyme of the pathway was altered so as to render it insensitive to retroinhibition. Similar results were very recently reported for the isoleucine pathway of bakers' yeast by Bollon and Magee (1971).

Further studies of the repression process should involve the tRNA synthetases. Duda et al. (1968) showed that the first allosteric enzyme of the aromatic amino acid biosynthetic pathway strongly binds phenylalanyl-tRNA; they proposed that a complex of the charged tRNA with the allosteric enzyme may be the repressor for this system. When TD from

S. typhimurium is reconstituted from its constituent subunits in vitro, an inactive species of the enzyme is formed which specifically and reversibly binds leucyl-tRNA (Hatfield and Burns, 1970). A similar study is under way on the purified TD of bakers' yeast (Bollon and Magee, 1971). It should be added that tRNA synthetases have been associated with repression mechanisms for several years (Neidhart, 1966); it was shown by McLaughlin et al. (1969) that in bakers' yeast, charged isoleucyl-tRNA is essential for the multivalent repression of TD and AHAS.

Another interesting approach would be the demonstration, in both bakers' and fission yeast, of an isoleucine-valine-synthesizing particle or "ilvasome", as characterized from Neurospora by Wagner and Bergquist (1963). An ilvasome is a system (or complex) which sediments in the 39,000 x g particulate fraction of wild type Neurospora homogenates, and which can synthesize valine and isoleucine from pyruvate and  $\alpha$ -ketobutyrate contained in a reaction mixture along with the necessary cofactors and NADPH-generating system. There is evidence that this isoleucine-valine synthesizing particle is associated with the mitochondria (Cassady and Wagner, 1969). In S. pombe, methods have been developed for fractionating cellular components (Duffus, 1969), and a crude preparation of mitochondria could be suitably used to test for the occurrence of ilvasome activity, using the methods described by Wagner and Bergquist (1963) or by Kiritani et al. (1965 a, b).

The comparison of the regulation of the leucine pathway in S. pombe with that of S. cerevisiae should prove extremely interesting. Only 3 classes of leucine auxotrophs have been isolated for S. pombe: leu 2 is on chromosome I, while leu 1 and leu 3 map on chromosome II and are not adjacent (Flores da Cunha, 1970). In contrast, there are 10 different

loci in S. cerevisiae which affect the first three leucine enzymes: leu 6 specifies IPMS, leu 1, leu 4, leu 5, leu 7, leu 8 and leu 10 all affect  $\alpha$ -isopropylmalate isomerase, and leu 2, leu 3 and leu 9 specify  $\beta$ -isopropylmalate dehydrogenase (Satyanarayana et al., (1968). In Salmonella (Burns et al., 1966) and in Neurospora (Gross, 1965), four structural genes have been shown to specify the first three leucine enzymes. In addition, IPMS in S. cerevisiae is repressed not by leucine alone as in Salmonella (Burns et al., 1966) and Neurospora (Gross, 1965), but by leucine plus threonine (Satyanarayana et al., 1968a). Thus, a search for more leucine auxotrophs of S. pombe should be undertaken in order to determine whether the multiplicity of genes for the leucine pathway is common in yeasts or is a characteristic peculiar to S. cerevisiae.

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