The isoleucine-valine biosynthesis pathway in Staphylococcus aureus

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THE ISOLEUCINE-VALINE BIOSYNTHESIS PATHWAY IN 

STAPHYLOCOCCUS AUREUS 

by 

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# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>LITERATURE REVIEW</td>
<td>3</td>
</tr>
<tr>
<td>MATERIALS AND METHODS</td>
<td>22</td>
</tr>
<tr>
<td>Media and Chemicals</td>
<td>22</td>
</tr>
<tr>
<td>Bacterial Strains</td>
<td>23</td>
</tr>
<tr>
<td>Growth Studies</td>
<td>23</td>
</tr>
<tr>
<td>Enzyme Assays</td>
<td>26</td>
</tr>
<tr>
<td>RESULTS</td>
<td>30</td>
</tr>
<tr>
<td>Growth Curves</td>
<td>30</td>
</tr>
<tr>
<td>General Properties of the Enzymes</td>
<td>30</td>
</tr>
<tr>
<td>Preparation of Extracts</td>
<td>43</td>
</tr>
<tr>
<td>Inhibition of the Enzymes <em>In Vitro</em></td>
<td>44</td>
</tr>
<tr>
<td>Repression and Derepression Studies</td>
<td>61</td>
</tr>
<tr>
<td>DISCUSSION</td>
<td>70</td>
</tr>
<tr>
<td>SUMMARY</td>
<td>79</td>
</tr>
<tr>
<td>ACKNOWLEDGEMENTS</td>
<td>81</td>
</tr>
<tr>
<td>LITERATURE CITED</td>
<td>82</td>
</tr>
</tbody>
</table>
INTRODUCTION

Investigations of the processes by which organisms synthesize or degrade cellular components have produced metabolic maps that depict the series of enzymatic steps involved in the reactions. While all the reactions which are necessary to permit growth and maintenance of cells are by no means known, it is very evident that these processes require a highly integrated, enzyme-mediated interplay of anabolism and catabolism.

Some of these metabolic processes concern the synthesis of amino acids, which are the basic units of enzymes and other proteinaceous cellular components. Since the mechanisms of metabolism depend upon enzymes as the functional factors, the regulation of the synthesis and activity of enzymes is of primary importance to the cell.

The higher plants and animals are multicellular organisms consisting of many differentiated, specialized and interacting cells. The complexity of these organisms renders it difficult to recognize the mechanisms that regulate cellular functions. The use of microorganisms, however, has the advantages of a greatly decreased level of interaction between cells, rapid growth, and yet a broad range of nutritional requirements.

This study is concerned with the biosynthesis of isoleucine and valine in Staphylococcus aureus. This organism was chosen not only because it is a pathogen of man and therefore of clinical importance, but also because previous studies of its biosynthetic properties have been mainly concerned with these functions involved with pathogenicity or with biochemical genetics. It is hoped that studies of the type reported here on S. aureus
will aid in understanding the relationships of this organism to other organisms, and in understanding cellular functions and their regulation.
LITERATURE REVIEW

Our knowledge of amino acid biosynthesis has been accumulated in several overlapping phases. The initial phase concerned the isolation and identification of amino acids in protein hydrolysates. This led to nutritional studies to determine which amino acids are required by mono- and multi-cellular organisms, providing the basis for the concept that certain amino acids could be synthesized by a particular organism while others could not. Since isotopes could be easily traced and isolated, their application to biological studies provided a convenient means of determining biosynthetic sequences. And since microorganisms displayed great variations in nutritional requirements, they were a logical selection for use in investigations of the biosynthesis of amino acids.

One of the results of these investigations has been the development of metabolic maps depicting the series of reactions by which amino acids (and other cellular components) are formed. Such a sequence for the biosynthesis of isoleucine and valine, as considered to exist in microorganisms, is presented in Fig. 1. As illustrated, the over-all biosynthetic pathway involves, except for the first step in isoleucine biosynthesis, reactions that are catalyzed by bifunctional enzymes, i.e., each enzyme that catalyzes a step in the valine pathway catalyzes the corresponding step in the isoleucine pathway. In addition, an intermediate, alpha-ketoisovalerate (KV) in the valine pathway is involved in the biosynthesis of leucine and pantothenate.

Studies on this biosynthetic sequence were considerably advanced when Beadle and Tatum (1941) introduced the use of auxotrophic mutants of
Fig. 1. Pathway of biosynthesis of isoleucine and valine (De Robichon-Szulmajster and Magee, 1968; Allaudeen and Ramakrishnan, 1968). Enzyme 1 = threonine deaminase; enzyme 2 = acetohydroxy acid synthetase; enzyme 3 = reductoisomerase; enzyme 4 = dihydroxy acid dehydrase; enzyme 5 = transaminase
Neurospora crassa. One of these mutants, strain 16117, was found to require both isoleucine and valine. Subsequent studies by Bonner, Tatum and Beadle (1943) demonstrated that mutant 16117 could utilize alpha-ketoisovalerate (KV*) and alpha-keto-beta-methylvalerate (KI) in place of isoleucine and valine. However, when Bonner (1946) attempted to repeat the growth experiments with chemically synthesized keto acids, he found that the mutant could utilize synthetic KV in a medium supplemented with isoleucine, but could not utilize the synthetic KI in a medium containing valine. He therefore proposed that the genetic block in the isoleucine pathway resulted in an accumulation of an intermediate which then interfered with the valine biosynthetic pathway.

These early studies were supplemented by results obtained with mutant and wild-type strains of Escherichia coli. Tatum (1946) found that the growth of E. coli strain K-12 was inhibited by valine, and that the inhibition could be reversed by isoleucine. In addition, certain mutants of strain K-12 required only isoleucine for growth. However, Teas (1948) observed that these mutants could also utilize alpha-ketobutyrate (KB) or synthetically prepared KI (Bonner, 1946).

Since the wild-type strains of E. coli and N. crassa could grow in media lacking isoleucine and valine, the isolation of mutants requiring either one or both of the amino acids suggested that the isolates were genetically blocked at some step in the formation of these amino acids.

*The chemical names given to the intermediates in the isoleucine-valine biosynthetic pathway are not the same in all publications. Therefore, to avoid confusion, the chemical names accepted at present have been substituted for any different names given in the references.
Therefore, mutants were grown in media containing limiting amounts of isoleucine and valine to determine whether compounds that might be intermediates in the biosynthesis of isoleucine and valine would accumulate in the media. These procedures were used to isolate and identify KV and KI (the immediate precursors of isoleucine and valine) from culture filtrates of a mutant of strain K-12 of *E. coli* (Umbarger and Magasanik, 1951). Similar methods were used to isolate two dihydroxy acids from culture filtrates of strain 16117 of *N. crassa*. Adelberg and Tatum (1950) and Adelberg et al. (1951) identified the acids as alpha, beta-dihydroxyisovalerate (DHV, the dihydroxy acid precursor of valine), and alpha, beta-dihydroxy-beta-methylvalerate (DHI, the dihydroxy acid precursor of isoleucine).

The accumulation of the dihydroxy acids by mutant 16117 of *N. crassa* indicated that the mutant must be blocked prior to the formation of the keto acids. It was considered chemically possible that the keto acids could be precursors of isoleucine and valine, while a reaction that would allow the dihydroxy acids to be the immediate precursors was considered to be improbable. Therefore, Umbarger and Adelberg (1951) repeated the growth experiments with mutant 16117 in a medium containing enzymatically synthesized keto acids, and found that the mutant could utilize them in place of isoleucine and valine. A comparison of Bonner's synthetic KI with several other keto acids demonstrated that it was actually KB. Because they also found accumulations of DHV and DHI in culture filtrates of the mutant, they proposed that, while the biosynthesis of isoleucine and valine occurred in separate pathways, the final steps consist of the conversion of DHI (DHV) to KI (KV) to isoleucine (valine).

An early step in the biosynthesis of isoleucine was indicated by Teas
(1948) and by Umbarger and Adelberg (1951). Both reports demonstrated that certain isoleucine-requiring mutants of *E. coli* could utilize KB for growth. Indirect evidence that KB was involved in the pathway was provided by isotope studies. Adelberg (1954) reported that $^{14}$C$^{1}$-1,2-threonine was converted to $^{14}$C$^{1}$-1,2-DHI by mutant 16117 of *N. crassa* and suggested that the biosynthesis of isoleucine involved deamination of threonine to form KB. Strassman *et al.* (1954) found carbon atoms from labeled acetate in isoleucine, and they suggested that a condensation of acetaldehyde with KB would form alpha-aceto-alpha-hydroxybutyrate (AHB), which would provide the carbon skeleton of isoleucine.

Similar studies on the valine pathway were conducted by Strassman *et al.* (1953). They found that $^{14}$C$^{2}$-2,3-valine was formed from $^{14}$C$^{2}$-2-lactate, suggesting a condensation reaction involving two lactate molecules. Additional isotopic studies by Abelson (1954) confirmed the role of KB in the synthesis of isoleucine and also indicated that pyruvate was involved in the synthesis of KV and valine. The results of Strassman *et al.* (1953) could be explained since it was known that pyruvate could be formed from lactate.

These studies, indicating which compounds were possible intermediates in the biosynthesis of isoleucine and valine, were accomplished primarily by the use of isotopes and by identifying the compounds accumulated or utilized by mutants that required one or both of the amino acids. These data indicated a very close relationship between the isoleucine and valine biosynthetic pathways. However, the hypothesis (Bonner, 1946) that the accumulation of an intermediate in the isoleucine pathway inhibited an analogous step in the valine pathway prevailed until studies on
Individual enzymes were reported.

The transamination of the keto acid precursors of isoleucine and valine, which is the final step in the pathway, was the first reaction to be intensely studied at the enzyme level. Rudman and Meister (1953) purified two transaminases from extracts of *E. coli* strain W. Transaminase A catalyzed reversible reactions between alpha-ketoglutaric acid and either phenylalanine, tyrosine, tryptophan or aspartic acid. Transaminase B was apparently involved in the isoleucine-valine (*ilv*) pathway since it catalyzed a reversible transamination between glutamate and the keto acid precursors of isoleucine, valine and leucine. In addition, each of the three amino acids could be used as the amino donor to the keto acid precursors of the other two amino acids.

In the same study, these authors also investigated mutants of *E. coli* strain W that had an absolute requirement for isoleucine and a partial requirement for valine. Extracts of these mutants lacked transaminase B activity. However, another enzyme, which could use alanine or alpha-aminobutyrate in a weak reaction with KV but not with KI, was purified from these extracts, which explained the absolute requirement for isoleucine and the partial requirement for valine. The enzyme was also present in extracts of wild-type cells. The reaction was described as a non-specific transamination (although KI was not an acceptor), and the authors concluded that a single enzyme, transaminase B, was primarily responsible for the transamination reaction in the *ilv* pathway. Similar observations were made on extracts of *Salmonella typhimurium* strain LT-2 (Wagner and Bergquist, 1960), indicating that Bonner's hypothesis was not correct since one enzyme could perform the transamination reaction for both
pathways, and the loss of this enzyme might block both pathways.

These results indicated the presence of at least three distinct transaminases in *E. coli* and *S. typhimurium*. However, assays of crude extracts of *N. crassa* revealed at least four different transaminases, two of which required pyridoxal phosphate for activity (Fincham and Boulter, 1956). Seecof and Wagner (1959a, 1959b) also found four transaminases in extracts of *N. crassa*. They purified a "phenylpyruvate transaminase" which formed isoleucine and valine from their keto acid precursors. They found, in contrast to the results obtained with transaminase B of *E. coli*, that at least ten amino acids could be used as amino donors in reversible reactions, and that the highest rate of transamination occurred with phenylalanine as the amino donor to KI. They concluded that a single enzyme, which displayed enhanced activity in the presence of pyridoxal phosphate, catalyzed the transamination of KI and KV, and that the transaminases in *E. coli* and *N. crassa* differ in their patterns of specificity.

The initial reaction in the biosynthesis of isoleucine was thought to be the deamination of threonine to form KB. An enzyme that could catalyze this reaction was purified from extracts of *N. crassa* (Yanofsky and Reissig, 1953). The "dehydrase" exhibited increased activity in the presence of pyridoxal phosphate and, while the enzyme demonstrated a higher rate of activity with threonine, it could also deaminate serine. Sayre and Greenberg (1956) reported similar results with a purified enzyme from extracts of sheep liver. They found that threonine deaminase (TD) activity was retained only when the concentration of phosphate buffer in the assay tubes was 0.05 M or greater, and that pyridoxal phosphate was required for maximal activity. Ultracentrifugation indicated that a single
protein was present in the purified preparation, but since the enzyme also weakly deaminated serine, they concluded that contamination with a serine deaminase had occurred, or that the TD could also react with serine.

However, Umbarger and Brown (1956, 1957) found that threonine deamination can be accomplished by more than one enzyme. They demonstrated the presence of two deaminases in crude extracts of strains W and K-12 of E. coli. One of the enzymes is produced under anaerobic conditions and may be a degradative enzyme. The other deaminase, considered to be the biosynthetic enzyme, is produced only under aerobic conditions, is inhibited by isoleucine, and is absent in isoleucine-requiring mutants which can utilize KB for growth. The biosynthetic enzyme lost activity during storage under varied conditions and was unstable during ammonium sulfate precipitation, dialysis and incubation in the absence of substrate. However, both enzymes required pyridoxal phosphate and were active against threonine and serine.

Isoleucine inhibits the activity of threonine deaminases of E. coli (Umbarger and Brown, 1958a), Saccharomyces cerevisiae (Kakar and Wagner, 1964), S. typhimurium (Freundlich and Umbarger, 1963a; Maeba and Sanwal, 1966), Mycobactarium tuberculosis (Allaudeen and Ramakrishnan, 1968) Bacillus subtilis (Hayashibe and Uemura, 1961), Rhodopseudomonas capsulata (Hughes et al., 1964) and Rhodopseudomonas spheroides (Datta, 1966). In contrast, TD activity is weakly inhibited by isoleucine only at low substrate concentrations in Rhodospirillum rubrum (Ning and Gest, 1966). However, the organism was grown under anaerobic conditions and the enzyme examined may have been the degradative TD. Leucine also inhibits TD activity in E. coli, but not as effectively as isoleucine, while valine
was reported to have no effect (Umbarger and Brown, 1958a). Similar results were obtained with extracts of *S. cerevisiae* (Kakar and Wagner, 1964). However, Changeux (1963) reported that valine reversed isoleucine inhibition of TD activity in *E. coli* strain K-12, but, at concentrations above 0.01 M, valine was also inhibitory.

Reports concerning stability of the biosynthetic TD in extracts of different organisms varied, but in all instances, there was a decrease in specific activity when extracts were prepared or assayed in solutions of low ionic strength. The TD of *S. cerevisiae* is very sensitive to dilution and is stabilized when stored at low temperature in 0.1 M phosphate buffer (De Robichon-Szulmajster and Magee, 1968). Harding (1969) reported that stability of the enzyme in extracts of *E. coli* is greatly decreased at low enzyme concentration and is optimal at high (5 mM) substrate and phosphate buffer (0.5 M or higher) concentration.

A comparison of the properties of threonine deaminases of different organisms shows certain similarities. Enzyme activity is inhibited by isoleucine (except possibly in *R. rubrum*), is lost or greatly diminished in solutions of low ionic strength, is enhanced by pyridoxal phosphate and is observed with threonine or serine as substrates.

Certain organisms convert acidic products to neutral products during glucose catabolism by a condensation reaction in which pyruvate and acetaldehyde form acetolactate (AL). Decarboxylation of the AL yields acetylmethylcarbinol (AMC) (Gale, 1947). Knowledge of this reaction led Umbarger *et al.* (1957) to investigate the possibility that such a condensation might be involved in the formation of AL in the ilv pathway. They noted that a mutant of *E. coli* strain W accumulated AL when grown in a
medium containing limiting amounts of valine. Extracts of the mutant pro-
duced AL from pyruvate at approximately 10 times the rate of extracts of
the parent strain. They also reported that valine inhibited the reaction.
They proposed that AL is an intermediate in the valine pathway and that
valine controls its own biosynthesis by a feedback inhibition of the
enzyme that forms AL.

Umbarger and Brown (1958b) also investigated the accumulation of AL
by a mutant of E. coli, and found that the formation of the enzyme was
repressed by valine, and valine was also an inhibitor of the reaction.
The synthesis of AL by extracts of mutant and wild-type strains was opti-
mal at pH 8.0 in a phosphate-buffered system containing magnesium ions and
thiamine pyrophosphate (TPP).

These data strongly indicated that a condensation reaction similar
to the one involved in glucose catabolism might also form AL in the valine
pathway. This suggested that a single enzyme (or enzyme system) performs
the condensation for valine biosynthesis and glucose catabolism, or that
two enzymes are capable of the condensation reaction. Halpern and Umbarger
(1959) investigated both possibilities in whole cells and crude extracts of
Enterobacter aerogenes and obtained evidence that two enzymes are involved.
One enzyme is optimally active at pH 7.5 and has properties similar to
those found in E. coli. The other enzyme displays optimal activity at pH
6.0, is only slightly stimulated by TPP, does not require magnesium ions,
and valine does not affect either the formation or activity of the enzyme.
However, extracts of cells grown at pH 5.8 had no AL-forming activity at
pH 8.0, indicating that the system functioning at pH 6.0 provides AL for
valine biosynthesis in cells grown at the lower pH. The condensation
reaction was further investigated by Leavitt and Umbarger (1961, 1962) in several strains of *E. coli*. Their results indicate that, in crude extracts, AL and AHB are formed by the same enzyme, since the formation of either intermediate is inhibited to the same degree by valine, is stimulated by TPP and magnesium ions, has the same pH optimum, and shows the same loss in activity when the extracts are diluted or stored under varied conditions. In addition, the growth of strain K-12 is inhibited by valine but the growth of strain W is not. The authors demonstrated that the AL- and AHB-forming system is more highly sensitive to valine in strain K-12 than it is in strain W.

The AL-forming system of *S. typhimurium* strain LT-2 was reported by Armstrong *et al.* (1963) to be different from the same system found in *E. coli*. They stated that the reaction had a pH optimum of 6.0 to 6.5 and was not inhibited by valine. However, Bauerle *et al.* (1964) demonstrated that the enzyme (the acetohydroxy acid synthetase, AAS) in strain LT-2 has a pH optimum of 8.0, and, at that pH, is inhibited by valine. They also found that the enzyme is unstable and requires, in addition to TPP and magnesium ions, an unidentified factor that could be supplied by boiled extracts of Salmonella or yeast. Størmer and Umbarger (1964) identified the factor as flavine adenine dinucleotide (FAD).

These data indicate that one enzyme, AAS, catalyzes the formation of both AL and AHB, but the evidence is not conclusive. Partial purification of the enzyme from extracts of *N. crassa* was accomplished, but the preparation had a pH optimum between 6.0 and 7.0, and, while activity was stimulated by TPP and magnesium ions, formation of the enzyme was not affected by valine. In addition, crude extracts formed both AL and AMC, indicating
the presence of a decarboxylase (Radhakrishnan and Snell, 1960). Therefore, the enzyme may be similar to the pH 6.0 enzyme of E. aerogenes.

Attempts to purify the AAS of E. coli and E. aerogenes were not successful due to the instability of the enzyme. In addition, only mutants of N. crassa were shown to accumulate both AL and AHB or their decarboxylated products (Wagner et al., 1959). The latter may be due to the activities of two different AL-forming enzymes in E. coli (Radhakrishnan and Snell, 1960) and in E. aerogenes, one of which has a pH optimum of 6.0 while the other has a pH optimum of 8.0. These organisms may have to lose the activities of both enzymes in order to show accumulations of AL and AHB.

Inhibition of the activity of the AAS by valine has been reported in E. aerogenes (Halpern and Umbarger, 1959), E. coli (Leavitt and Umbarger, 1961, 1962), S. typhimurium (Bauerle et al., 1964), S. cerevisiae (Magee and De Robichon-Szulmajster, 1968a, 1968b), N. crassa (Radhakrishnan and Snell, 1960), Pseudomonas aeruginosa (Varga and Horvath, 1967a), and M. tuberculosis (Allaudeen and Ramakrishnan, 1968). In all of these organisms, except perhaps N. crassa, the enzyme was reported to be unstable, generally losing 30 to 50 per cent activity within 24 hours. However, the presence of FAD prevents heat inactivation of the AAS in P. aeruginosa and may also decrease the loss of activity during attempts to purify the enzyme (Varga and Horvath, 1967a, 1967b). But, in addition to P. aeruginosa, the effects of FAD have apparently been investigated only in S. typhimurium (Størmer and Umbarger, 1964), a streptomycin-dependent mutant of E. coli (Desai and Polglase, 1965), and M. tuberculosis (Allaudeen and Ramakrishnan, 1968), with only the latter organism demonstrating no effect when the cofactor was added to assays of AAS activity. These reports did
not include investigations of the effects of FAD on AHB formation.

Certain similarities in the properties of AAS activities in the organisms listed above include: activity is enhanced by TPP and magnesium ions (and, in some instances, FAD), is optimal in phosphate-buffered systems, and is inhibited by valine.

In the search for compounds that might be intermediates in the ilv pathway, a role for AL was suggested when it was shown to be converted to DHV by an extract of a mutant of *E. coli* (Umbarger *et al.*, 1957), and to KV (in a reaction that indicated involvement of reduced triphosphopyridine nucleotide, TPNH) by an extract of *S. cerevisiae* (Strassman *et al.*, 1958). The related compound, AHB, had been suggested as an intermediate in the biosynthesis of isoleucine (Strassman *et al.*, 1954).

Confirmation that AL and AHB are intermediates was obtained by demonstrating the conversion of both compounds to the dihydroxy acids in extracts of mutant 16117 of *N. crassa* (Wagner *et al.*, 1958). However, as the authors pointed out, the reaction is a two-step process in which there is a repositioning of a methyl (or ethyl) group, and a reduction. If the rearrangement occurs first, alpha-keto-beta-hydroxyisovalerate (HKV) and alpha-keto-beta-hydroxy-beta-methylvalerate (HKI) would be intermediates in the synthesis of DHV and DHI, respectively. These keto-hydroxy acids (HKV and HKI) were synthesized and, in extracts of strain 16117, were converted to the dihydroxy acids. The enzyme (the keto-hydroxy acid reductase) that catalyzes the reaction was active in crude and purified extracts of the wild-type strain, mutant 16117, and mutant 304 (which accumulates AL and AHB) of *N. crassa*. These data suggest that two enzymes are involved in the conversion of AL and AHB to the dihydroxy acids. However,
attempts to isolate HKV and HKI were unsuccessful, even when TPNH was omitted from the assays. These observations indicate that the compounds exist only as enzyme-bound intermediates.

Crude extracts of several strains of E. coli and E. aerogenes were also capable of converting AL and AHB to the dihydroxy acids (Umbarger et al., 1960). The authors demonstrated a correlation between TPNH oxidation and the appearance of the dihydroxy acids, which provided the basis for a spectrophotometric assay for oxidation of TPNH in the presence of AL or AHB. While reduction of HKV and HKI was also demonstrated, they suggested that a single enzyme performed the rearrangement and reduction of AL and AHB.

Similar results were reported by Radhakrishnan et al. (1960) in crude extracts of E. coli and N. crassa. Using extracts of both organisms, however, these authors accomplished purification of the reductase as well as a reductoisomerase (RI), which converted AL and AHB to the dihydroxy acids. Extensive experimentation with the purified enzymes indicated that the reductase might not be involved in the ilv pathway, or that it might be an altered form of the RI. The latter was based on an observation that a purified RI with no initial reductase activity gradually gained reductase activity as it lost RI activity. Nearly identical results were obtained with a purified reductase and RI of S. typhimurium (Armstrong and Wagner, 1961a, 1961b).

However, the question concerning the involvement of one or two enzymes in the rearrangement and reduction steps has not been completely resolved. Some data indicate the participation of one enzyme which may exist in more than one form (possibly due to an alteration which does not
exist in vivo. But Allaudeen and Ramakrishnan (1968) found still a third enzyme, the acetohydroxy acid isomerase, in crude extracts of M. tuberculosis. The authors noted that the activity of the RI was enhanced by the presence of L-ascorbic acid. Therefore, TPNH was omitted in order to determine if ascorbic acid affected the isomerase step. From assays containing ascorbic acid and either AL or AHB, either HKV or HKI was isolated and then converted to the corresponding dihydroxy acid. However, because the enzymes were not purified, these results only demonstrate that HKV and HKI can be formed, and that they can act as intermediates in the ilv pathway. In addition, the data do not exclude the possibility that one enzyme can effect isomerization and reduction. Support for this possibility was obtained by Arfin and Umbarger (1969) who extensively purified a reducto-isomerase from extracts of S. typhimurium. The properties of the enzyme include the ability to perform the reverse isomerization of HKV to AL, reduce HKV to DHV, and form DHV from AL. No effect was noted when ascorbic acid was added to the assays.

An additional difficulty in determining the properties of the RI and reductase is that some of the supporting and direct data were obtained in crude extracts by the spectrophotometric assay of Umbarger et al. (1960). The validity of the spectrophotometric assay of activity in crude extracts was questioned by Wagner et al. (1964) who demonstrated three distinct enzymes in N. crassa which oxidize TPNH in the presence of AL or AHB. Only one enzyme has the properties of the RI; the other two enzymes apparently are non-specific oxidases since they can perform the reaction with various other substrates.

Therefore, the accumulated data indicate that one enzyme, the
reductoisomerase, accomplishes the over-all conversion of AL and AHB to the dihydroxy acids. The enzyme requires magnesium ions and TPNH, and generally loses approximately 50 per cent activity in 24 hours in crude extracts. A reductase and an isomerase may exist as separate, distinct enzymes, but present evidence strongly suggest that at least one reductase is an altered form of the RI.

The remaining step in the ilv pathway is the conversion of the dihydroxy acids (DHV and DHI) to the keto acids (KV and KI). Myers and Adelberg (1954) first demonstrated this conversion in crude extracts of E. coli and N. crassa. The enzyme, dihydroxy acid dehydrase, was shown to require only magnesium ions for maximal activity in the presence of DHV or DHI. The enzyme is stabilized by cysteine, and activity is not inhibited by isoleucine or valine. These properties are also found for the dehydrase in crude extracts of S. cerevisiae (Wixom et al., 1960). There is apparently only one report on the properties of a purified dihydroxy acid dehydrase, which was presented by Myers (1961). The enzyme, which was purified from extracts of strain K-12 of E. coli, utilizes both DHV and DHI as substrates, indicating that a single enzyme is responsible for both reactions in the ilv pathway. Activity is optimal in a tris-HCl buffer at pH 7.8, and is maximal in the presence of ferrous ions and a reducing agent (cysteine or glutathione).

The intermediates in the ilv pathway have been identified and some of the properties of the enzymes which catalyze the reactions have been determined. Except for the deamination of threonine to form KB, each step in the biosynthesis of both isoleucine and valine is apparently catalyzed by a single enzyme, giving these enzymes bifunctional capabilities.
Regulation of the formation and activities of these enzymes poses a serious problem for living cells, since not only does the pathway involve the synthesis of isoleucine and valine, but the keto acid precursor of valine, KV, is an intermediate in the biosynthesis of leucine and pantothenate (Abelson, 1954; Ramakrishnan and Adelberg, 1964). This suggests that the regulatory mechanisms for the ilv pathway might be more complex than the mechanisms which would influence a pathway with only one end-product.

Regulation of the ilv pathway is at least partially exerted by the end-products of the pathway; e.g., isoleucine inhibits the activity, and to a slight extent represses the formation, of the threonine deaminase of E. coli (Umbarger and Brown, 1958a), and valine inhibits the activity and represses the formation of the acetohydroxy acid synthetase of the same organism (Umbarger and Brown, 1958b). Repression of the formation of the AAS is strongly indicated as a major factor in the inhibition of growth of strain K-12 of E. coli, since the enzyme is also involved in the biosynthesis of isoleucine.

Inhibition of the growth of strain K-12 was reported by Cohen (1958) to be the result of the formation of "false protein". Addition of valine to an exponentially growing culture does not result in complete inhibition of growth, but does cause a decrease to a linear rate of growth. Under these conditions, Cohen concluded that valine is substituted for isoleucine in protein molecules, with the result that many of the enzymes are inactive. However, Temple et al. (1965) not only demonstrated that active enzymes are formed, but that the formation of inducible enzymes could be accomplished during valine inhibition. They
concluded that valine acts by inhibiting the over-all rate of protein synthesis by preventing the biosynthesis of isoleucine.

These results indicate that valine has an important role in regulating the \textit{ilv} pathway in the valine-sensitive strain of \textit{E. coli}. However, many bacteria are not inhibited by valine. Freundlich, Burns and Umbarger (1962) and Freundlich and Umbarger (1963b) demonstrated that in strain \textit{W} of \textit{E. coli} (which is not sensitive to valine) isoleucine, valine, leucine and pantothenate are necessary for maximal repression of the enzymes catalyzing the reactions of the \textit{ilv} pathway. The authors termed this "multivalent repression".

An additional aspect in regulation of the pathway was noted by Wagner and Bergquist (1963). They observed that the mitochondrial fraction of extracts of \textit{N. crassa} could synthesize isoleucine and valine when supplied with KB, pyruvate and all of the cofactors necessary for each step. The supernatant fraction of the extracts also possessed all of the enzymes of the \textit{ilv} pathway. However, extracts of certain isoleucine-valine requiring mutants of \textit{N. crassa}, while demonstrating all of the enzymes in the supernatant fraction, could not perform the over-all synthesis with the mitochondrial fraction. Therefore, they suggested that the defect in these mutants is not due to an inability to produce active enzymes, but to an inability to organize them properly \textit{in vivo}. Cronenwett and Wagner (1965) found that membrane fractions of extracts of \textit{S. typhimurium} could synthesize isoleucine, but not valine, under similar conditions.

These results indicate the complexity of the regulatory mechanisms operating in the \textit{ilv} pathway. Enzyme inhibition and repression may be
accompanied by a necessity to organize the enzymes in a particular manner in order for the biosynthesis of isoleucine and valine to occur in vivo.

Roth and Sanderson (1966) determined the orientation of the structural genes that code for the enzymes of the ilv pathway in strain LT-2 of *S. typhimurium*, and also suggested that the sequence is similar in strain K-12 of *E. coli*. Substituting the names of the enzymes for the structural genes, the sequence is; transaminase, dehydrase, TD, RI, AAS. In addition, Ramakrishnan and Adelberg (1964) reported that the TD, dehydrase and transaminase are regulated by multivalent repression in strain K-12, but that the AAS and RI are not. They suggested that the TD, dehydrase and transaminase are in one operon, and that the AAS and RI are regulated by a separate operator gene.

However, Smith and Pattee (1967) reported that the sequence of genes within the ilv pathway of strain 655 of *Staphylococcus aureus* is identical to the sequence in which the enzymatic reactions occur. This suggests that the mechanism by which the ilv pathway in *S. aureus* is regulated would be different from the method of regulation in strain K-12, because the structural genes in *S. aureus* could not be arranged in operons in the same sequence as they are in strain K-12 of *E. coli*. Therefore, this study was undertaken in an attempt to determine the manner in which the ilv pathway is regulated in *S. aureus*. 
MATERIALS AND METHODS

Media and Chemicals

The media employed in this study included brain heart infusion (BHI, Difco), trypticase soy broth (TSB, Baltimore Biological Laboratories), and the synthetic medium of Kloos and Pattee (1965). The derepression synthetic (DS) broth lacked isoleucine, valine and leucine, and the complete synthetic (CS) broth contained 30 μg of isoleucine, 80 μg of valine and 90 μg of leucine per ml. Unless otherwise stated, all compounds employed throughout this study were the L-isomers.

Alpha-ketobutyric acid (KB), the ethyl ester of alpha-acetolactic acid, alpha-ketoisovaleric acid (KV), and acetylmethylcarbinol (AMC) were obtained from Calbiochem. D, L-alpha, beta-dihydroxyisovaleric acid (D, L-DHV) was obtained from Reef Laboratories, Lafayette, Indiana, and L-alpha, beta-dihydroxyisovaleric acid (L-DHV) was kindly supplied by Dr. Frank Armstrong, North Carolina State University. All other reagents are commercially available.

The free alpha-acetolactic acid (AL) was prepared from its ethyl ester by hydrolysis. The ester was slowly added with constant stirring to five ml of freshly-prepared 2 N NaOH in a beaker surrounded by an ice bath. Aliquots of the ester were dissolved in the solution until the pH was 7.0, after which the solution was diluted with H₂O to achieve a final concentration of 100 μM of AL per ml.

Alpha-naphthol was purified by sublimation and placed in a foil-wrapped flask in a desiccator over CaCl₂ and NaOH pellets. When stored in this manner, very little deterioration of the reagent occurred within
six months. The 2, 4-dinitrophenylhydrazine was reprecipitated from hot ethyl acetate and stored in a similar manner.

Bacterial Strains

The majority of the experiments were performed with strain 655 of Staphylococcus aureus. In addition, strains 152 and U9 of S. aureus were used in growth studies. These strains have been described previously (Pattee and Baldwin, 1961). Strain LT-2 of Salmonella typhimurium was used as a control in certain enzyme assays.

Primary stock cultures of these strains were maintained at 4 C on BHI agar slants in screw-cap tubes and were transferred at three-month intervals. Secondary stock cultures were prepared by inoculating cotton-stoppered BHI slants which were incubated at 37 C for 12 hours and then stored at 4 C. The secondary stock cultures were replaced each week.

Growth Studies

Growth curves

All media used in the following procedures were prewarmed at 37 C unless otherwise indicated. Tubes containing 10 ml of DS broth were inoculated with cells of the appropriate strain of S. aureus and shaken in a water bath shaker (Warner-Chilcott Laboratories, Richmond, California, Model 2156) at a maximum setting of "10" at 37 C for 12 hours. The contents of the tubes were combined, and 2-ml aliquots were added to 250-ml side-arm flasks containing 50 ml of DS or CS broth. Valine, leucine or isoleucine, at final concentrations of 50, 100 or 500 µg per ml, were added to the flasks containing DS broth. These additions were made at the time of inoculation, or after the cultures had entered the log
phase of growth. The flasks were shaken at 37°C on a New Brunswick Platform Shaker at the minimal shaker setting of "one". Optical density (O.D.) readings were taken at appropriate intervals on a Bausch and Lomb Spectronic 20 spectrophotometer at 535 mp.

Repression and derepression studies

To characterize the enzymes of the isoleucine-valine biosynthetic pathway in strain 655, the cells were inoculated into tubes of TSB and incubated at 37°C in a Warner-Chillcott water bath shaker at a shaker setting of "10" for six hours. The TSB cultures were combined and used to inoculate Fernbach flasks containing from 500 to 1600 ml of DS broth. The initial O.D. of these flasks was approximately 0.15. The flasks were shaken at 37°C on a New Brunswick Platform Shaker at the minimal shaker setting of "one" until an O.D. of 1.15 to 1.3 was obtained (approximately 5 hours). The cells were centrifuged at 10,000 rpm for 10 minutes in a Sorvall RC-2B centrifuge at 0-10°C, resuspended in cold 1 M phosphate buffer at pH 8.5 (hereafter referred to as phosphate buffer), centrifuged as above, weighed, and resuspended in phosphate buffer at a ratio of 10 ml of buffer per gm wet weight of cells. All cell suspensions were frozen at -15°C until lysed, or were lysed immediately. Similar procedures were employed to obtain cell suspensions of strain 655 grown in CS broth, DS broth containing 200 µg of valine per ml, CS broth containing 240 µg of valine per ml, and CS broth containing 50 µg of calcium pantothenate per ml.

Cells were also grown in DS broth containing inhibitory concentrations of isoleucine or leucine. Tubes containing 10 ml of TSB were inoculated and incubated as described above. Two Fernbach flasks,
containing 1600 ml of DS broth per flask, were each inoculated with 30 ml of the combined TSB cultures. The flasks were shaken as previously described until the O.D. reached 1.1 to 1.2. The cells were then harvested aseptically in a prewarmed (37 C) GSA rotor with the centrifuge chamber operating at room temperature. These cells, resuspended in 10 ml of DS broth, are referred to as the cell suspension in the following procedures.

Four Fernbach flasks, each containing 1600 ml of DS broth, were each inoculated with 4.2 ml of the cell suspension (initial O.D. of approximately 0.55) and shaken at 37 C as previously described. One cell sample, which was later used to prepare the "0 hour" or control extract, was removed after one hour of incubation. Isoleucine, at a concentration of 50 μg per ml of DS broth, was then added to each flask, and cell samples were removed each hour for four hours. The cells from each sample were collected by centrifugation in the cold (5 C) at 10,000 rpm for 10 minutes. The cells were weighed, resuspended in phosphate buffer at a ratio of 10 ml of buffer per gm wet weight of cells, and frozen at -15 C. These procedures were repeated using leucine at a concentration of 200 μg per ml of DS broth.

**Preparation of cell-free extracts**

Three procedures were employed to lyse the cells. These included lysis with the staphylolytic enzyme (Burke and Pattee, 1967), and use of the Bronwill Tissue Homogenizer and the Bronwill Biosonik II. In the latter two procedures, the cell suspension was mixed with approximately two times its volume of 0.11-0.12 mm glass beads and cooled to 0-5 C. The cells were lysed by agitation for four minutes in the CO₂-cooled
chamber of the tissue homogenizer, or by sonication with the Biosonik II, using the standard probe at maximum power. For sonication, the cell-glass bead suspension was placed in a glass beaker surrounded by an ice bath and sonicated at 20-second intervals for a total of six minutes of sonication. The procedure required approximately 25 minutes since one-minute intervals were needed between periods of sonication to prevent heating of the probe and cell-glass bead suspension.

The extract of the cells was separated from the beads by centrifugation at 3,000 rpm for five minutes. The supernatant fluid was decanted and then centrifuged at 20,000 rpm for 15 minutes to remove whole cells and cellular debris. When the cells were lysed with the staphylolytic enzyme, only the second centrifugation was necessary. All centrifugations were accomplished at 0-10°C unless otherwise indicated. The protein concentration in the extracts was determined by the Biuret method, using 3X recrystallized lysozyme as the standard.

Enzyme Assays

**Threonine deaminase**

The standard threonine deaminase (TD) assay procedure was a modification of the procedure of Umbarger and Brown (1957). The reaction mixture contained 500 μM of phosphate buffer at pH 8.5, 20 μg of pyridoxal phosphate, 40 μM of threonine and crude extract in a total volume of one ml in a Corex centrifuge tube. After incubation at 37°C for 30 minutes, the reaction was terminated by addition of one ml of 20 per cent trichloroacetic acid. The tubes were centrifuged at 1500 x g for 10 minutes to remove precipitated material.
The supernatant fluid was assayed for the presence of KB by a modification of the procedures of Friedemann and Haugen (1943) and Sayre and Greenberg (1956). To 0.5 ml of the supernatant fluid was added 0.5 ml of 0.1 per cent 2, 4-dinitrophenylhydrazine in 2 N HCl. The tubes were allowed to stand at room temperature for 5 minutes; one ml of 95 per cent ethyl alcohol was then added, followed by the addition of 2.5 ml of 2.5 N NaOH. The O.D. of the tubes was determined at 520 μm after 10 minutes.

**Acetohydroxy acid synthetase**

The acetohydroxy acid synthetase (AAS) assay procedure was a modification of the procedures of Kakar and Wagner (1964) and Størmer and Umbarger (1964). The standard assay solution contained 500 μM of phosphate buffer at pH 6.5 (which put the pH of the assay at 6.7 since the extract was prepared in 1 M phosphate buffer at pH 8.5), 200 μM of Na pyruvate, 10 μM of MgCl₂, 80 μg of thiamine pyrophosphate (TPP), 4 μg of flavine adenine dinucleotide (FAD), and crude extract in a total volume of one ml in a Corex centrifuge tube. The tubes were incubated at 37 C for 30 minutes and the reaction was stopped by the addition of one ml of 20 per cent trichloroacetic acid. The tubes were then incubated at 45 C for 30 minutes to ensure complete decarboxylation of AL to AMC (Radhakrishnan and Snell, 1960).

The concentration of AMC in the tubes was determined by a modification of the procedure of Westerfeld (1945). Each tube received the following: 2.6 ml of prewarmed (45 C) H₂O; 0.4 ml of 2.5 N NaOH to neutralize the solution; one ml of prewarmed (45 C) 0.5 per cent creatine; one ml of a freshly-prepared alpha-naphthol solution (0.1 gm/2 ml of
2.5 N NaOH). The contents of each tube were uniformly mixed after each addition. The tubes were incubated at 30 C for 45 minutes, centrifuged at 1500 x g for 10 minutes, and the supernatant fluids were decanted into spectrophotometer tubes. The O.D. of each sample was determined at 530 μm after a total of 60 minutes had elapsed since addition of the alpha-naphthol.

Reduc-to-isomerase

The assay for reductoisomerase (RI) activity followed the procedure of Umbarger et al. (1960). Alpha-acetolactate reduction was correlated spectrophotometrically to oxidation of TPNH in a reaction mixture containing 300 μM of phosphate buffer at pH 7.5, 10 μM of MgCl₂, 50 μM of neutralized AL, 0.5 μM of TPNH, and crude extract in a total volume of 3 ml. The reagents, except TPNH, were added to a Quartz cuvette and incubated at 37 C for 10 minutes. Prewarmed (37 C) TPNH was added to the cuvette, the contents were rapidly mixed, and the oxidation of TPNH was recorded at 37 C on a Beckman DB recording Spectrophotometer at 340 μm. A control tube, lacking substrate, was included in each assay to record non-specific oxidation of TPNH.

Dihydroxy acid dehydrase

The activity of the dehydrase in the cell-free extracts was determined by the procedure of Myers and Adelberg (1954). The Corex centrifuge tubes contained 100 μM of phosphate buffer at pH 8.0, 10 μM of MgSO₄, 15 μM of L-DHV, and crude extract in a total volume of 0.5 ml. The tubes were incubated at 37 C for 30 minutes and the reaction was terminated by the addition of 0.5 ml of 20 per cent trichloroacetic acid. The concentration of KV in the assay tubes was determined by the procedures
Lactic dehydrogenase

The standard assay for lactic dehydrogenase (LD) activity was a modification of the procedure of Nachlas et al. (1960). The assay tubes contained 700 µM of phosphate buffer at pH 9.0, 0.9 mg of DPN, 60 µM of Na lactate, and 0.1 ml of the crude extract in a total volume of one ml. The stock solutions of the substrate and DPN were prepared in 1 M phosphate buffer at pH 9.0. The reagents, except the enzyme, were added to Corex centrifuge tubes and incubated at 37 C for 10 minutes. After adding prewarmed (37 C) crude extract, each tube was incubated at 37 C for one minute. The reaction was stopped by the addition of one ml of 20 per cent trichloroacetic acid. The concentration of pyruvic acid in the assay tubes was determined by the procedures described for the TD assay.
RESULTS

Growth Curves

A primary concern in this study was to determine the growth responses of strain 655 of *Staphylococcus aureus* to exogenous isoleucine, valine or leucine. As illustrated in Fig. 2, 50 μg per ml of isoleucine or leucine added to DS broth at the time of inoculation were inhibitory, whereas 50 μg of valine per ml of DS broth did not decrease the growth rate. Isoleucine or leucine, at final concentrations of 100 μg per ml, exerted an inhibitory effect on exponentially growing cultures. This inhibition, also, was reversed by the addition of 100 μg of valine per ml four hours after the addition of isoleucine or leucine (Fig. 3).

By repeating these experiments with strains U9 and 152 of *S. aureus*, it was determined that this growth response of strain 655 to exogenous isoleucine, valine and leucine was not unique to this strain. However, as identical results were obtained with all three strains, only the results obtained with strain 655 are presented.

General Properties of the Enzymes

Threonine deaminase

Preliminary assays of threonine deaminase (TD) activity in crude extracts demonstrated that the enzyme was very unstable. Lysis of the cells or assay for TD activity in systems containing varied concentrations of tris-HCl buffer, triethanolamine-HCl (TEA) buffer, or 0.1 or 0.5 M phosphate buffer, all of which lacked or contained combinations of 25 μg of isoleucine per ml, 0.05 M mercaptoethanol, 0.1 M KCl or 20 μg of pyridoxal phosphate (PP) per ml, resulted in a 50 to 100 per cent loss
Fig. 2. Growth responses of strain 655 in CS broth (○), DS broth (●), and DS broth containing 50 μg per ml of isoleucine (△), leucine (▲), or valine (■). Conditions for growth are described in the text.
Fig. 3. Growth responses of strain 655 in DS broth. After 7.5 hr, 100 µg per ml of valine (■), leucine (○, ●) or isoleucine (△, △) were added. After 11.5 hr, 100 µg per ml of valine (○, △) were added.
in activity. However, an extract prepared in 0.5 M phosphate buffer containing 0.1 M KCl and 25 µg of isoleucine per ml gave the most reproducible, although low, specific activity. Since isoleucine is an inhibitor of TD activity in many organisms, portions of the extract were dialyzed for six hours against phosphate buffers at concentrations of 0.1, 0.4, 0.8 and 1 M. Subsequent assays demonstrated that the specific activity was enhanced in the extracts dialyzed against 0.8 and 1 M buffer. When an extract was prepared by lysing cells suspended in 1 M phosphate buffer, assays conducted immediately and after 10 hours demonstrated no differences in specific activities.

A dilution effect was also noted in the TD assays. The specific activity of the TD decreased approximately 60 per cent in assay tubes containing a 1:20 dilution of the crude extract as compared to assay tubes containing a 1:10 dilution. Increasing the concentration of substrate, PP, or phosphate buffer, or addition to the assay tubes of the supernatant fluid or the precipitate from boiled extract, or of bovine serum albumin dissolved in 1 M phosphate buffer did not diminish the loss in activity. Similar results have been reported for the TD of \textit{E. coli} (Harding, 1969), and of \textit{S. cerevisiae} (De Robichon-Szulmajster and Magee, 1968).

The requirement for 1 M phosphate buffer to maintain TD activity interfered with attempts to purify the enzyme by ammonium sulfate precipitation, as crystals of ammonium phosphate precipitated, and the enzyme was dispersed in the 40, 50 and 60 per cent ammonium sulfate fractions. Acetone precipitation of the enzyme caused a complete inactivation which was not reversed by extensive dialysis against 1 M
phosphate buffer.

Subsequent studies demonstrated that the TD has a pH optimum of 8.5 (Fig. 4) and is stable in 1 M phosphate buffer when stored at -15 C for as long as seven days. The enzyme is saturated at a substrate concentration of 40 µM of threonine. Pyridoxal phosphate is required for maximal specific activity; assay tubes lacking this cofactor exhibited a 25 to 30 per cent decrease in activity (Table 1).

Table 1. Requirements for the enzymatic conversion of threonine to KB. In addition to the components listed in the table, each assay tube contained 40 µM of threonine and crude extract of strain 655 of S. aureus in a total volume of 1.0 ml. The pH of the phosphate buffer was 8.5. The assay was conducted as described in Materials and Methods.

<table>
<thead>
<tr>
<th>Additions</th>
<th>Phosphate buffer (pM)</th>
<th>Pyridoxal phosphate (pg)</th>
<th>Specific activity (pM/mg protein/hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
<td>2.48</td>
</tr>
<tr>
<td>0</td>
<td>20</td>
<td></td>
<td>5.32</td>
</tr>
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<td>19.67</td>
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<tr>
<td>500</td>
<td>10</td>
<td></td>
<td>18.30</td>
</tr>
<tr>
<td>500</td>
<td>0</td>
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<td>14.36</td>
</tr>
</tbody>
</table>
Determination of the pH optimum of the threonine deaminase (Δ), acetohydroxy acid synthetase (○) and lactic dehydrogenase (●) in 1 M phosphate buffer. The specific activities (μM/mg protein/hr) at the pH optimum for each enzyme were: TD - 24.4, AAS - 2.89, LD - 28.3
Acetohydroxy acid synthetase

Initial attempts to obtain reproducible results with the acetohydroxy acid synthetase (AAS) assay procedure of Kakar and Wagner (1964), as modified by StÜrmer and Umbarger (1964), were unsuccessful. The preliminary assays indicated that at least part of the difficulty was due to the procedure (Westerfeld, 1945) involved in determining the concentration of acetylmethylcarbinol (AMC) in the supernatant fluid. Therefore, assays were conducted using the cofactors, known concentrations of AMC, and bovine serum albumin.

It was discovered that one cause of the variations in the assays was the use of commercial alpha-naphthol. This variable was eliminated by using alpha-naphthol that had been purified by sublimination under reduced pressure. In addition, it was noted that temperature played an important role, since temperatures below 25 C delayed development of color while temperatures above 45 C caused a change in color from the usual shade of red to a dark purple. This variable was minimized by first adding a constant amount (2.6 ml) of prewarmed (45 C) deionized H2O to each tube, and, after adding the remaining reagents, placing the tubes in a 30 C incubator for 45 minutes. Precipitated protein was removed by centrifugation. Assays conducted in this manner (with known concentrations of AMC) gave highly reproducible results.

With a satisfactory assay procedure established to determine the concentration of AMC in the tubes, a series of experiments were conducted to determine the properties of the AAS. The enzyme was unstable when stored at 5 C or -15 C in any of the buffers that were employed in the TD assays or in the presence of 1 M phosphate buffer containing up to 800 µg
of TPP per ml. However, the loss of activity, which amounted to 25 to 40 per cent within 24 hours after preparation of the extract, was minimal in 0.5 or 1 M phosphate buffer. The instability of the enzyme required, therefore, that the AAS assay be conducted immediately after preparation of the extract.

When dilutions were performed in 1 M phosphate buffer, all tubes corresponded very well with the tube containing the lowest dilution. However, when the extract was diluted prior to assay in 0.1 M phosphate buffer, 0.1, 0.2 or 0.5 M tris or TEA buffers lacking or containing 0.05 M mercaptoethanol, 25 µg of isoleucine per ml, or 80 µg of TPP per ml, an immediate and irreversible 25 to 90 per cent loss in specific activity occurred.

As demonstrated in Fig. 4, the specific activity of the AAS is maximal at a pH of 6.7 to 6.8. The enzyme is saturated at a substrate concentration of 200 µM of pyruvate and, as indicated in Table 2, requires TPP, FAD and MgCl₂ for maximum activity. It is also noted in Table 2 that a decrease in the concentration of phosphate buffer in the assay tubes from 500 to 0 µM resulted in a loss of approximately 16 per cent of the specific activity.

**Reductoisomerase**

Activity of the reductoisomerase (RI) was difficult to determine accurately since the highest concentration of TPNH that could be employed in the spectrophotometric assay was 0.7 µM (yielding an initial O.D. of approximately 1.0), and the oxidation of TPNH was nearly complete within two minutes after adding the cofactor to the cuvette. The pH optimum for the assay was approximately 8.0, although repeated attempts, in a pH
Table 2. Requirements for the enzymatic conversion of Na pyruvate to alpha-acetolactate. In addition to the components listed in the table, each assay tube contained 200 μM of Na pyruvate and crude extract of *S. aureus* strain 655 in a total volume of 1.0 ml. The assay was conducted at pH 6.7 as described in Materials and Methods.

<table>
<thead>
<tr>
<th>Additions</th>
<th>Specific activity (μM/mg protein/hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TPP (μg)</td>
<td>FAD (μg)</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
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<tr>
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<td>80</td>
<td>4</td>
</tr>
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<td>80</td>
<td>0</td>
</tr>
</tbody>
</table>

The results of Wagner et al. (1964) demonstrated that, in * Neurospora crassa*, the spectrophotometric determination of RI activity is not a valid assay since there are at least two other enzymes present in crude extracts that oxidize TPNH in the presence of alpha-acetolactate and several other substrates. Therefore, extracts of strain 655 of *S. aureus* and strain LT-2 of *Salmonella typhimurium* were assayed for oxidation of
TPNH using Na pyruvate, alpha-ketobutyrate (KB), or alpha-acteolactate (AL) or its ethyl ester as substrates. The procedures for the growth of strain LT-2 and the preparation of extracts were identical to the procedures described for strain 655.

As indicated in Table 3, strain LT-2 oxidizes TPNH at a much greater rate than does strain 655. The results with strain LT-2 compare favorably with those obtained by Armstrong and Wagner (1961a) using the same strain. In addition, strain 655 oxidizes TPNH in the presence of KB at approximately the same rate as in the presence of AL. These results indicate that oxidation of TPNH by extracts of strain 655 is not the result of activity of a substrate-specific enzyme, but may be the result of a nonspecific oxidase(s).

Dihydroxy acid dehydrase

The sample of the L-isomer of alpha, beta-dihydroxyisovaleric acid (L-DHV) supplied by Dr. Armstrong was sufficient to allow only a very limited characterization of the dehydrase. Assays conducted with the L-isomer demonstrated no loss in specific activity when extracts were prepared in 1 M phosphate buffer and stored at −15°C for up to one week. The specific activity was in the range of 10 to 12 μM of keto acid formed per mg of protein per hour when extracts were prepared from cells grown in DS broth. An assay conducted without MgSO₄ indicated a loss of 32 percent of specific activity.

Assays of dehydrase activity employing D, L-DHV as substrate resulted in nearly complete inhibition of the enzyme. A loss of approximately 30 percent of the activity occurred in an assay mixture containing 15 μM of L-DHV and 22.4 μM of D, L-DHV. Extracts of S. typhimurium strain LT-2,
Table 3. Oxidation of TPNH by extracts of strain 655 of *S. aureus* and strain LT-2 of *S. typhimurium*. In addition to the substrates listed in the table, each assay tube contained 0.5 μM of TPNH, 10 μM of MgCl₂, 300 μM of phosphate buffer at the pH indicated, and crude extract in a total volume of 3 ml. The assay was conducted as described in Materials and Methods.

<table>
<thead>
<tr>
<th>Extract</th>
<th>Substrate</th>
<th>pH</th>
<th>Specific activity (μM/mg protein/hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I. Strain LT-2</td>
<td>AL</td>
<td>8.0</td>
<td>1.200</td>
</tr>
<tr>
<td></td>
<td>AL</td>
<td>7.5</td>
<td>2.000</td>
</tr>
<tr>
<td></td>
<td>AL (ethyl ester)</td>
<td>7.5</td>
<td>0.820</td>
</tr>
<tr>
<td></td>
<td>KB</td>
<td>7.5</td>
<td>0.076</td>
</tr>
<tr>
<td>II. Strain 655</td>
<td>AL</td>
<td>7.5</td>
<td>0.382</td>
</tr>
<tr>
<td></td>
<td>AL</td>
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<td></td>
<td>AL</td>
<td>8.5</td>
<td>0.369</td>
</tr>
<tr>
<td></td>
<td>KB</td>
<td>8.0</td>
<td>0.380</td>
</tr>
<tr>
<td></td>
<td>pyruvate</td>
<td>8.0</td>
<td>0.290</td>
</tr>
<tr>
<td></td>
<td>AL (ethyl ester)</td>
<td>8.0</td>
<td>0.260</td>
</tr>
</tbody>
</table>

*a* The concentration of all substrates was 50 μM.

however, utilized both the L- and D, L-forms.

D, L-DHV was synthesized by the procedures of Sjolander *et al.* (1954), but attempts to obtain the L-isomer by their quinine salt procedure were unsuccessful. Use of the quinine salt procedure to isolate the L-isomer from D, L-DHV obtained from Reef Laboratories was also unsuccessful. A satisfactory grade of anhydrous quinine was not
available, which may have contributed to the lack of success in these procedures.

**Lactic dehydrogenase**

The activity of the lactic dehydrogenase (LD) was employed as a control to correlate the activities of the isoleucine-valine (ilv) enzymes to an enzyme unrelated to the ilv pathway. The initial assays of LD activity were conducted using the spectrophotometric method of Nachlas *et al.* (1960), which correlates the reduction of DPN to the production of pyruvate from lactate. However, the procedure of Friedemann and Haugen (1943), which was used to determine the concentration of the keto acid end-product (KB) in the TD assay, could be used to determine the concentration of the keto acid end-product (pyruvate) in the LD assay. This procedure would minimize the amount of time required to perform both assays since they could be conducted simultaneously. Therefore, the activity of the LD in several crude extracts was determined by both procedures, and, as nearly identical results were obtained, the latter procedure was then routinely employed.

The requirement for 1 M phosphate buffer to maintain TD activity precluded the use of other buffers in the preparation of extracts. However, phosphate buffers have a very limited buffering capacity at pH values above 8.5. While the pH optimum for LD activity in the presence of 1 M phosphate buffer was determined to be 9.0 (Fig. 4), assays were conducted with TEA buffers in a pH range of 8.8 to 9.6, and with phosphate and TEA buffers in concentrations of 0.1 to 1 M (Table 4). The results indicate that: a) increasing the concentration of TEA buffer from 0.2 to 1 M slightly decreases specific activity; b) the pH optimum in the presence
Table 4. The effects of concentration and type of buffer on the enzymatic conversion of Na lactate to pyruvate by crude extracts of strain 655 of S. aureus. In addition to the components listed in the table, each assay tube contained 60 μM of substrate and crude extract in a total volume of 1.0 ml. The assay was conducted as described in Materials and Methods.

<table>
<thead>
<tr>
<th>Buffer</th>
<th>Phosphate (μM)</th>
<th>TEA[^a] (μM)</th>
<th>pH</th>
<th>DPN (mg)</th>
<th>Specific activity (μM/mg protein/hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>800</td>
<td>---</td>
<td>9.0</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>700</td>
<td>---</td>
<td>9.0</td>
<td>0.90</td>
<td>21.10</td>
<td></td>
</tr>
<tr>
<td>70</td>
<td>---</td>
<td>9.0</td>
<td>0.90</td>
<td>18.33</td>
<td></td>
</tr>
<tr>
<td>140</td>
<td>---</td>
<td>9.0</td>
<td>0.90</td>
<td>19.34</td>
<td></td>
</tr>
<tr>
<td>350</td>
<td>---</td>
<td>9.0</td>
<td>0.90</td>
<td>20.44</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>---</td>
<td>8.5</td>
<td>0.90</td>
<td>17.94</td>
<td></td>
</tr>
<tr>
<td>750</td>
<td>---</td>
<td>9.0</td>
<td>0.45</td>
<td>13.65</td>
<td></td>
</tr>
<tr>
<td>---</td>
<td>140</td>
<td>9.0</td>
<td>0.90</td>
<td>20.67</td>
<td></td>
</tr>
<tr>
<td>---</td>
<td>350</td>
<td>9.0</td>
<td>0.90</td>
<td>20.30</td>
<td></td>
</tr>
<tr>
<td>---</td>
<td>700</td>
<td>9.0</td>
<td>0.90</td>
<td>20.10</td>
<td></td>
</tr>
<tr>
<td>---</td>
<td>140</td>
<td>8.8</td>
<td>0.90</td>
<td>20.00</td>
<td></td>
</tr>
<tr>
<td>---</td>
<td>140</td>
<td>9.2</td>
<td>0.90</td>
<td>20.54</td>
<td></td>
</tr>
<tr>
<td>---</td>
<td>140</td>
<td>9.6</td>
<td>0.90</td>
<td>20.20</td>
<td></td>
</tr>
</tbody>
</table>

[^a]Triethanolamine-HCl buffer.

of TEA buffer is approximately 9.0; c) activity is slightly enhanced in the presence of 1 M phosphate buffer. Therefore, in order to prevent
possible pH changes in the assays, stock solutions of substrate and DPN were prepared in 1 M phosphate buffer at pH 9.0.

Optimal activity was obtained at a substrate concentration of 60 μM of Na lactate and a DPN concentration of 0.9 mg. Increasing the concentration of substrate or cofactor did not significantly increase activity, while assays conducted without DPN demonstrated no activity. No loss in LD activity was noted in extracts stored at -15 C for as long as one week.

**Preparation of Extracts**

Cell-free extracts, obtained by lysing the cells with the staphyloytic enzyme, lacked TD activity since the staphyloytic enzyme is optimally active in 0.1 M TEA buffer and is inhibited by 1 M phosphate buffer (Burke and Pattee, 1967). Cells suspended in 0.1 M TEA and lysed with the staphyloytic enzyme failed to yield an active TD even when the preparation was diluted with an equal volume of 2 M phosphate buffer immediately after lysis.

Sonication proved to be the most convenient method for lysing cells of *S. aureus*. The Bronwill Tissue Homogenizer required less time, but it was difficult to control temperature during lysis, and cell suspensions with volumes of less than 15 ml could not be used with this instrument. As sonication did not present these disadvantages, it was routinely used to prepare the cell-free extracts.

Since it was not possible to lyse all cell suspensions immediately after harvesting the cells, the specific activities of the enzymes were determined in crude extracts prepared from fresh and frozen cells. Cells
grown in DS broth were resuspended in cold (5 C) phosphate buffer and divided into three equal volumes. One of the cell suspensions was lysed and assayed immediately, and the other two suspensions were frozen at -15 C. A second suspension was lysed and assayed after being frozen for six days, and the third suspension was lysed and assayed after being frozen for ten days. No differences were noted in the specific activities of the TD, AAS or LD enzymes in any of the extracts.

Inhibition of the Enzymes In Vitro

The stock solutions of valine, leucine, isoleucine, KV and calcium pantothenate employed in the in vitro inhibition studies were prepared in 1 M phosphate buffer at the pH optimum for the particular enzyme. This prevented a decrease in the concentration of the buffer in the assays. The stock solutions were kept frozen at -15 C in sealed screw-cap tubes until used, and were not sterilized in order to prevent possible changes in concentration.

Threonine deaminase

Inhibition of TD activity occurred in the presence of either isoleucine, valine or leucine. The enzyme was most sensitive to inhibition exerted by isoleucine, with a concentration of 0.5 μM yielding approximately 50 per cent loss in specific activity in extracts of cells grown in DS broth (Fig. 5). In contrast, the same extract yielded a 50 per cent loss in specific activity in the presence of approximately 21 μM of leucine (Fig. 6), and 100 μM of valine resulted in only a 22 per cent loss in specific activity (Fig. 7). However, valine reversed the inhibition exerted by isoleucine (Fig. 8) and by leucine (Table 5). The level of
Fig. 5. Inhibition exerted by isoleucine on TD activity in crude extracts obtained from cells grown in DS (●) and CS (○) broth. Control tubes lacking isoleucine had specific activities of 21.8 μM and 2.84 μM per mg of protein per hr, respectively.
% SPECIFIC ACTIVITY

ISOLEUCINE CONCENTRATION (µM)
Fig. 6. Effects of leucine on TD activity in crude extracts obtained from cells grown in DS (●) and CS (○) broth. Control tubes lacking leucine had specific activities of 21.8 μM and 2.84 μM per mg of protein per hr, respectively.
Fig. 7. Inhibition exerted by valine on TD activity in crude extracts obtained from cells grown in DS (●) and CS (○) broth. Control tubes lacking valine had specific activities of 21.8 and 2.84 μM per mg of protein per hr, respectively.
Fig. 8. Inhibition of TD activity exerted by 1 μM of isoleucine and reversal of the inhibition by an increasing concentration of valine. The specific activity of the TD in control assays lacking isoleucine or valine was 21.4 μM per mg of protein per hr.
Table 5. The effects of increasing valine concentration on the inhibition, exerted by leucine, of TD activity

<table>
<thead>
<tr>
<th>Leucine concentration (μM)</th>
<th>Valine concentration (μM)</th>
<th>Specific activity (μM/mg/hr)</th>
<th>Per cent loss in specific activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>21.51</td>
<td>0.0</td>
</tr>
<tr>
<td>5</td>
<td>0</td>
<td>16.78</td>
<td>22.0</td>
</tr>
<tr>
<td>5</td>
<td>10</td>
<td>18.20</td>
<td>15.4</td>
</tr>
<tr>
<td>10</td>
<td>0</td>
<td>14.42</td>
<td>33.0</td>
</tr>
<tr>
<td>10</td>
<td>10</td>
<td>16.33</td>
<td>24.1</td>
</tr>
<tr>
<td>10</td>
<td>20</td>
<td>17.98</td>
<td>16.4</td>
</tr>
</tbody>
</table>

Inhibition exerted by isoleucine was also reversed by increasing the substrate concentration (Table 6). No loss in activity resulted from the addition of 1 to 10 μM of calcium pantothenate.

In order to determine whether growth of the cells in the presence of isoleucine, valine and leucine would alter the in vitro responses of the TD, AAS and LD enzymes to the three amino acids, a crude extract of cells grown in CS broth was prepared. As illustrated in Fig. 5, the in vitro response of the TD to isoleucine differed very little, whereas the inhibition exerted by leucine (Fig. 6) and valine (Fig. 7) decreased. It was also noted that the specific activity of the TD in crude extracts of cells grown in DS broth (21.8 μM/mg protein/hr) was nearly eight times greater than the specific activity of the TD in the crude extracts of cells grown in CS broth (2.84 μM/mg protein/hr).
Table 6. The effects of increasing substrate concentration on the inhibition, exerted by isoleucine, on TD activity

<table>
<thead>
<tr>
<th>Threonine concentration (µM)</th>
<th>Isoleucine concentration (µM)</th>
<th>Specific activity (µM/mg/hr)</th>
<th>Per cent loss in specific activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>40</td>
<td>0</td>
<td>18.25</td>
<td>0.0</td>
</tr>
<tr>
<td>80</td>
<td>0</td>
<td>18.45</td>
<td>0.0</td>
</tr>
<tr>
<td>120</td>
<td>0</td>
<td>18.60</td>
<td>0.0</td>
</tr>
<tr>
<td>40</td>
<td>5</td>
<td>2.66</td>
<td>85.4</td>
</tr>
<tr>
<td>80</td>
<td>5</td>
<td>7.62</td>
<td>58.3</td>
</tr>
<tr>
<td>120</td>
<td>5</td>
<td>13.64</td>
<td>25.3</td>
</tr>
<tr>
<td>40</td>
<td>10</td>
<td>2.48</td>
<td>86.4</td>
</tr>
<tr>
<td>80</td>
<td>10</td>
<td>7.09</td>
<td>61.1</td>
</tr>
<tr>
<td>120</td>
<td>10</td>
<td>10.45</td>
<td>42.7</td>
</tr>
<tr>
<td>40</td>
<td>20</td>
<td>2.66</td>
<td>85.4</td>
</tr>
<tr>
<td>80</td>
<td>20</td>
<td>7.09</td>
<td>61.1</td>
</tr>
<tr>
<td>120</td>
<td>20</td>
<td>10.19</td>
<td>44.2</td>
</tr>
</tbody>
</table>

**Acetohydroxy acid synthetase**

All assays of acetohydroxy acid synthetase (AAS) activity were performed immediately after preparation of the extracts due to the instability of the enzyme. Inhibition of AAS activity occurred in the presence of isoleucine, valine, leucine and alpha-ketoisovalerate (KV). The enzyme was most sensitive to inhibition by KV, demonstrating an average loss of 50 per cent in specific activity at a concentration of 20 µM in
extracts of cells grown in DS broth (Fig. 9). The same extracts lost an average of 17.5 per cent in specific activity in the presence of 20 \( \mu M \) of valine (Fig. 9), 15 per cent in specific activity in the presence of 20 \( \mu M \) of isoleucine, and 11 per cent in the presence of 20 \( \mu M \) of leucine (Fig. 10). It was not possible to increase the concentration of isoleucine or leucine above 50 \( \mu M \) in the assays because of the limited solubility of these amino acids in 1 M phosphate buffer. No loss in AAS activity resulted from the addition of 1 to 10 \( \mu M \) of calcium pantothenate or of 40 \( \mu M \) of threonine.

AAS activity was inhibited by isoleucine and valine to the same extent in extracts of cells grown in DS or CS broth. However, the extract of cells grown in CS broth yielded a higher degree of inhibition of AAS activity by KV (Table 7). In addition, increasing the substrate concentration did not affect the inhibition exerted by valine or leucine, but did decrease the level of inhibition exerted by KV (Table 8).

Several attempts to determine the degree of inhibition exerted by combinations of KV, isoleucine, valine or leucine on AAS activity yielded results that were quite variable, but in no instance did the presence of one compound reverse the inhibition exerted by another. In addition, the combined effects of any two or three of the compounds were not additive, e.g.; the per cent inhibition exerted by a combination of 10 \( \mu M \) of isoleucine and 10 \( \mu M \) of KV was lower than the sum of the per cent inhibitions exerted by the compounds separately at the same concentration.

**Lactic dehydrogenase**

The specific activity of the lactic dehydrogenase was not diminished by isoleucine, valine, leucine, threonine, KV or calcium pantothenate at
Fig. 9 Effects of valine (●) and KV (○) on AAS activity in extracts of cells grown in DS broth. Each point in the curves is the average of 2 to 4 assays.
Fig. 10. Effects of isoleucine (○) and leucine (□) on AAS activity in extracts of cells grown in DS broth. Each point in the curves is the average of 3 to 4 assays.
Table 7. A comparison of the level of inhibition exerted by isoleucine, valine and alpha-ketoisovalerate (KV) on AAS activity in extracts of cells grown in CS broth and in DS broth. Specific activity is defined as \( \mu \text{M} \) of product formed per mg of protein per hr.

<table>
<thead>
<tr>
<th>Additions</th>
<th>Extract from repressed cells(^a)</th>
<th></th>
<th>Extract from derepressed cells(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Specific activity (( \mu \text{M} ))</td>
<td>Per cent loss in specific activity</td>
<td>Specific activity (( \mu \text{M} ))</td>
</tr>
<tr>
<td>None</td>
<td>3.30</td>
<td>0.0</td>
<td>3.10</td>
</tr>
<tr>
<td>Isoleucine (30 ( \mu \text{M} ))</td>
<td>2.55</td>
<td>22.7</td>
<td>2.48</td>
</tr>
<tr>
<td>Isoleucine (50 ( \mu \text{M} ))</td>
<td>2.34</td>
<td>29.1</td>
<td>2.08</td>
</tr>
<tr>
<td>Valine (25 ( \mu \text{M} ))</td>
<td>2.65</td>
<td>20.0</td>
<td>2.46</td>
</tr>
<tr>
<td>Valine (50 ( \mu \text{M} ))</td>
<td>2.38</td>
<td>27.9</td>
<td>2.22</td>
</tr>
<tr>
<td>KV (10 ( \mu \text{M} ))</td>
<td>1.69</td>
<td>48.8</td>
<td>2.34</td>
</tr>
</tbody>
</table>

\(^a\)Extract of cells grown in CS broth.

\(^b\)Extract of cells grown in DS broth.
Table 7. (Continued)

<table>
<thead>
<tr>
<th>Additions</th>
<th>Extract from repressed cells&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Extract from derepressed cells&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Specific activity (µM)</td>
<td>Per cent loss in specific activity</td>
</tr>
<tr>
<td>KV</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(20 µM)</td>
<td>1.11</td>
<td>66.3</td>
</tr>
<tr>
<td>KV</td>
<td>0.81</td>
<td>75.5</td>
</tr>
</tbody>
</table>

<sup>a</sup> Table continues on the next page.
Table 8. The effects of increasing substrate concentration on the inhibition of AAS activity by isoleucine, valine or KV. The specific activity is defined as μM of product formed per mg of protein per hr.

<table>
<thead>
<tr>
<th>Pyruvate concentration (μM)</th>
<th>Isoleucine (μM)</th>
<th>Valine (μM)</th>
<th>KV (μM)</th>
<th>Specific activity</th>
<th>Per cent loss in specific activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>200</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>2.56</td>
<td>0.0</td>
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<td>--</td>
<td>2.62</td>
<td>0.0</td>
</tr>
<tr>
<td>600</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>2.65</td>
<td>0.0</td>
</tr>
<tr>
<td>200</td>
<td>30</td>
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<td>--</td>
<td>1.99</td>
<td>22.3</td>
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<td>30</td>
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<td>--</td>
<td>1.95</td>
<td>25.8</td>
</tr>
<tr>
<td>600</td>
<td>30</td>
<td>--</td>
<td>--</td>
<td>1.96</td>
<td>24.7</td>
</tr>
<tr>
<td>200</td>
<td>--</td>
<td>75</td>
<td>--</td>
<td>1.66</td>
<td>35.2</td>
</tr>
<tr>
<td>400</td>
<td>--</td>
<td>75</td>
<td>--</td>
<td>1.67</td>
<td>36.1</td>
</tr>
<tr>
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<td>--</td>
<td>75</td>
<td>--</td>
<td>1.71</td>
<td>35.4</td>
</tr>
<tr>
<td>200</td>
<td>--</td>
<td>--</td>
<td>25</td>
<td>1.19</td>
<td>53.5</td>
</tr>
<tr>
<td>Pyruvate concentration (μM)</td>
<td>Isoleucine (μM)</td>
<td>Valine (μM)</td>
<td>KV (μM)</td>
<td>Specific activity</td>
<td>Per cent loss in specific activity</td>
</tr>
<tr>
<td>---------------------------</td>
<td>----------------</td>
<td>------------</td>
<td>---------</td>
<td>------------------</td>
<td>----------------------------------</td>
</tr>
<tr>
<td>400</td>
<td>--</td>
<td>--</td>
<td>25</td>
<td>1.46</td>
<td>44.4</td>
</tr>
<tr>
<td>600</td>
<td>--</td>
<td>--</td>
<td>25</td>
<td>1.85</td>
<td>30.2</td>
</tr>
</tbody>
</table>
concentrations similar to those employed for the inhibition studies with TD and AAS enzymes.

Repression and Derepression Studies

Since isoleucine and leucine demonstrated inhibitory effects on the growth of strain 655, and valine reversed this inhibition (Fig. 2 and 3), a comparison was made of the specific activities of the TD, AAS and LD enzymes in extracts of cells grown in DS and CS broth. As demonstrated in Table 9, growth in the presence of isoleucine, valine and leucine (CS broth) resulted in a severely decreased level of TD activity, but little change in AAS or LD activities.

However, in order to follow more closely the effects of isoleucine, valine and leucine on the levels of the activities of these enzymes, an additional experiment was performed. As illustrated in Fig. 11, two flasks (3200 ml) of CS broth were inoculated with cells grown in TSB. The two flasks (and all other flasks in this experiment) were shaken at 37 C on a New Brunswick Platform Shaker, and the cells were harvested aseptically by centrifugation when the O.D. of the flasks reached 1.2 (4.5 hours of incubation). An aliquot of the resuspended cells was used as the control or "0 hour repressed cells". The remaining cells were used to inoculate six flasks (9,000 ml) of DS broth. These flasks were incubated for four hours, and a cell sample was removed each hour. The fourth-hour sample was harvested aseptically, with 10 ml of the resuspended cells used as the "derepressed cells". Four flasks (6,000 ml) of CS broth were inoculated with the remaining cells. These flasks were incubated for four hours and a cell sample was removed each hour. The
Table 9. A comparison of enzyme activities in extracts of cells grown in synthetic media supplemented as indicated in the table. Growth conditions and assay procedures are described in Materials and Methods.

<table>
<thead>
<tr>
<th>Synthetic media</th>
<th>Threonine deaminase</th>
<th>Acetohydroxy acid synthetase</th>
<th>Lactic dehydrogenase</th>
</tr>
</thead>
<tbody>
<tr>
<td>DS broth</td>
<td>19.67</td>
<td>3.17</td>
<td>27.80</td>
</tr>
<tr>
<td>CS broth</td>
<td>2.93</td>
<td>3.25</td>
<td>28.60</td>
</tr>
<tr>
<td>CS broth with 50 μg per ml of pantothenate</td>
<td>4.46</td>
<td>2.65</td>
<td>29.40</td>
</tr>
<tr>
<td>DS broth with 200 μg per ml of valine</td>
<td>9.29</td>
<td>3.89</td>
<td>38.66</td>
</tr>
<tr>
<td>CS broth with 240 μg per ml of valine</td>
<td>13.94</td>
<td>2.72</td>
<td>28.91</td>
</tr>
</tbody>
</table>

0.0D. of each flask, and the results of the assays conducted on extracts of the cell samples are presented in Table 10. As indicated, the activity of the TD increased with time in cells grown in DS broth, and decreased with time in cells grown in CS broth.

There were only slight variations in the activity of the AAS in all extracts, but these variations indicate that the AAS was repressed slightly with time in cells grown in DS broth, and was derepressed to a very limited degree with time in cells grown in CS broth. The LD activity remained relatively constant throughout these procedures.
Fig. 11. Summary of procedures used for the growth of strain 655 of *S. aureus* in CS broth with sequential transfer of cells to DS broth and then to CS broth
Table 10. The specific activities of the TD, AAS and LD enzymes in extracts of cells grown in CS broth with sequential transfer to DS broth and then to CS broth. The O.D. of each flask was determined each time a cell sample was removed.

<table>
<thead>
<tr>
<th>Medium</th>
<th>Time</th>
<th>Ave. O.D.</th>
<th>Specific activity (µM/mg/hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Threonine deaminase</td>
</tr>
<tr>
<td>I. DS broth</td>
<td>0 hr&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.20</td>
<td>2.84</td>
</tr>
<tr>
<td></td>
<td>1 hr</td>
<td>0.63</td>
<td>6.46</td>
</tr>
<tr>
<td></td>
<td>2 hr</td>
<td>0.75</td>
<td>8.18</td>
</tr>
<tr>
<td></td>
<td>3 hr</td>
<td>0.86</td>
<td>9.84</td>
</tr>
<tr>
<td></td>
<td>4 hr&lt;sup&gt;d&lt;/sup&gt;</td>
<td>1.10</td>
<td>12.48</td>
</tr>
</tbody>
</table>

<sup>a</sup> Hours after inoculation of the flasks.

<sup>b</sup> Average optical density of the flasks.

<sup>c</sup> Cells used to inoculate the DS broth. The cells had been grown in CS broth and represent "repressed cells".

<sup>d</sup> A sample of these cells were used to inoculate the CS broth and represent the "derepressed cells".

Table 10. (Continued)

<table>
<thead>
<tr>
<th>Medium</th>
<th>Time</th>
<th>Ave. O.D.</th>
<th>Threonine deaminase</th>
<th>Acetohydroxy acid synthetase</th>
<th>Lactic dehydrogenase</th>
</tr>
</thead>
<tbody>
<tr>
<td>II. CS broth</td>
<td>1 hr</td>
<td>0.65</td>
<td>11.54</td>
<td>2.92</td>
<td>27.84</td>
</tr>
<tr>
<td></td>
<td>2 hr</td>
<td>0.85</td>
<td>8.08</td>
<td>3.22</td>
<td>27.64</td>
</tr>
<tr>
<td></td>
<td>3 hr</td>
<td>1.10</td>
<td>4.00</td>
<td>3.16</td>
<td>26.52</td>
</tr>
<tr>
<td></td>
<td>4 hr</td>
<td>1.26</td>
<td>2.14</td>
<td>3.32</td>
<td>29.90</td>
</tr>
</tbody>
</table>
Valine reversed the inhibition exerted by isoleucine and leucine on the growth of strain 655 (Fig. 3), and also reversed the inhibition exerted by isoleucine on the activity of the TD in vitro (Fig. 8). To determine whether an excess of valine in the growth medium would affect the synthesis of the enzymes, extracts were prepared of cells grown in CS broth containing 240 μg of valine per ml, and in DS broth containing 200 μg of valine per ml. In addition, the effects of pantothenate (which is synthesized from KV) on the levels of the enzymes were determined by conducting assays on an extract of cells which had been grown in CS broth containing 50 μg of calcium pantothenate per ml. As shown in Table 9, synthesis of the AAS was repressed in cells grown in CS broth containing pantothenate or an excess of valine, and was maximally derepressed in cells grown in DS broth containing an excess of valine. However, synthesis of the TD was repressed by approximately 50 per cent in cells grown in DS broth containing an excess of valine, was severely repressed in cells grown in CS broth containing pantothenate, and was only slightly repressed in cells grown in CS broth containing excess valine. LD activity remained constant except in the extract of cells grown in DS broth containing an excess of valine, where LD activity was significantly increased.

The specific activities of the enzymes in extracts of cells grown in DS broth containing inhibitory concentrations of either isoleucine (50 μg per ml) or leucine (200 μg per ml) were also determined. The inhibition required that the flasks be inoculated with sufficient cells to obtain a high initial O.D. (approximately 0.57), and the flasks were incubated for one hour before adding the amino acid. The O.D. of each flask was
determined as each cell sample was removed. As indicated in Table 11, the synthesis of both the TD and the AAS was repressed significantly with time in cells grown in the presence of either isoleucine or leucine. However, full repression of either enzyme was not obtained in any of the extracts. The LD activity remained relatively constant with time in all extracts. A fourth-hour sample was not obtained from the flasks containing leucine because the O.D. indicated that the cells might have entered the stationary phase.
Table 11. The specific activities of the TD, AAS and LD enzymes in extracts of cells grown in DS broth containing isoleucine or leucine. The O.D. of each flask was determined each time a cell sample was removed.

<table>
<thead>
<tr>
<th>Medium</th>
<th>Time</th>
<th>Ave. O.D.</th>
<th>Threonine deaminase</th>
<th>Acetohydroxy acid synthetase</th>
<th>Lactic dehydrogenase</th>
</tr>
</thead>
<tbody>
<tr>
<td>I. DS broth with</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0 hr</td>
<td>0.72</td>
<td>15.47</td>
<td>3.94</td>
<td>30.87</td>
</tr>
<tr>
<td></td>
<td>1 hr</td>
<td>0.82</td>
<td>12.59</td>
<td>3.18</td>
<td>27.41</td>
</tr>
<tr>
<td></td>
<td>2 hr</td>
<td>0.90</td>
<td>11.33</td>
<td>2.82</td>
<td>27.00</td>
</tr>
<tr>
<td></td>
<td>3 hr</td>
<td>0.96</td>
<td>11.02</td>
<td>2.83</td>
<td>23.94</td>
</tr>
<tr>
<td></td>
<td>4 hr</td>
<td>1.02</td>
<td>10.40</td>
<td>2.61</td>
<td>32.40</td>
</tr>
</tbody>
</table>

*aHours after addition of isoleucine or leucine.

*bThe average of the optical densities of the flasks.
<table>
<thead>
<tr>
<th>Medium</th>
<th>Time</th>
<th>Ave.</th>
<th>Threonine deaminase (µM/mg/hr)</th>
<th>Acetohydroxy acid synthetase (µM/mg/hr)</th>
<th>Lactic dehydrogenase (µM/mg/hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>II. DS broth with 200 µg per ml of leucine</td>
<td>0 hr</td>
<td>0.69</td>
<td>15.12</td>
<td>3.84</td>
<td>31.20</td>
</tr>
<tr>
<td></td>
<td>1 hr</td>
<td>0.86</td>
<td>11.00</td>
<td>2.74</td>
<td>28.14</td>
</tr>
<tr>
<td></td>
<td>2 hr</td>
<td>1.02</td>
<td>7.74</td>
<td>2.48</td>
<td>24.00</td>
</tr>
<tr>
<td></td>
<td>3 hr</td>
<td>1.15</td>
<td>7.00</td>
<td>2.35</td>
<td>28.35</td>
</tr>
</tbody>
</table>
The growth of strain W of *Escherichia coli* is not affected by isoleucine, valine or leucine. However, strain K-12 of *E. coli* differs in that growth is inhibited by valine, and isoleucine reverses this inhibition. As demonstrated in this study, three strains of *Staphylococcus aureus* respond in still a different manner to exogenous isoleucine, valine and leucine (Fig. 2 and 3). These data demonstrate that:  
a) each strain is able to grow in synthetic broth lacking isoleucine, valine and leucine (DS broth);  
b) addition of either isoleucine or leucine to DS broth inhibits growth;  
c) valine reverses the inhibition exerted by isoleucine or leucine;  
d) addition of valine does not decrease the growth rate.  

These data suggest that regulation of the ilv pathway in *S. aureus* may include a feedback inhibition exerted by isoleucine and leucine. Therefore, studies were conducted to determine the in vitro effects of isoleucine, valine and leucine on the enzymes of the ilv pathway.

As previously stated, attempts to characterize the reductoisomerase (RI) of strain 655 were unsuccessful because of an apparent non-specific oxidation of TPNH in the presence of several substrates. In addition, the limited supply of L-DHV allowed only a preliminary investigation of the properties of the dihydroxy acid dehydrase. However, the results demonstrate that the dehydrase is specific for the L-isomer of DHV, as the activity of the dehydrase is completely inhibited by D, L-DHV. The D, L-DHV was utilized as substrate by extracts of *Salmonella typhimurium*, indicating that the racemic mixture is biologically active in this system.
The assays of threonine deaminase (TD) activity indicate that the properties of the TD of strain 655 do not differ significantly from the properties of the analogous enzyme of other organisms. The activity of the TD is inhibited by isoleucine and leucine, while valine, which exerts slight inhibition only at relatively high concentrations, reverses the inhibition exerted by isoleucine and leucine.

In contrast, numerous differences have been reported for the properties of the acetohydroxy acid synthetase (AAS) of various organisms. An example is the acetolactate-forming system of Enterobacter aerogenes, which exhibits pH optima of 6.0 and 7.5. The pH 7.5 enzyme is inhibited by valine and is considered to be active in the biosynthesis of this amino acid. The pH 6.0 enzyme is not affected by valine and is considered to be involved in glucose catabolism (Halpern and Umbarger, 1959). In contrast to E. aerogenes, Neurospora crassa possesses only one AAS, which has a broad pH optimum of 6.0 to 7.0 and is not inhibited by valine (Radhakrishnan and Snell, 1960). The AAS of Mycobacterium tuberculosis has a pH optimum of 6.9, and does not have a requirement for FAD, but valine does inhibit activity (Allaudeen and Ramakrishnan, 1968).

The AAS of strain 655 has a pH optimum of 6.7 (Fig. 3), which is intermediate between the pH optima of 6.0 and 7.5 found for the two acetolactate-forming systems of E. aerogenes. Indirect evidence that the AAS is involved in valine biosynthesis is provided by two observations. First, the acetolactate (AL) formed in the assays is not converted to acetylmethylcarbinol (AMC) without acid treatment. This suggests that the acetolactate-decarboxylase, which is active in producing AMC spontaneously during glucose catabolism (Radhakrishnan and Snell,
1960), is not active in strain 655 under the assay conditions employed in this study. Secondly, isoleucine, valine, leucine and alpha-ketoisovalerate (KV) inhibit the activity of the AAS (Fig. 9 and 10), and it is doubtful that an enzyme which is not involved in the ilv pathway would be inhibited by these compounds.

In E. coli, the AAS of strain K-12 has been found to be more sensitive to valine than is the AAS of the valine-resistant strain W. Leavitt and Umbarger (1962) suggested that the increased sensitivity of the AAS of strain K-12 is the cause of the inhibition of growth of this strain in the presence of valine. However, the inhibition is not complete, but results in a decrease from an exponential to a linear rate of growth. Cohen (1958) attributed the linear rate of growth to the synthesis of false protein formed by the incorporation of valine in place of isoleucine. Temple et al. (1965) obtained contradictory evidence when they demonstrated that the activities of certain enzymes did not decrease when strain K-12 was grown in the presence of valine. They suggested that the linear rate of growth may be an artifact created by the method of depicting a decreasing exponential growth.

A similar decrease in the rate of growth of strain 655 occurs in the presence of isoleucine and, to a lesser extent, leucine (Fig. 3). However, as indicated in Table 11, the activities of the AAS and TD enzymes decreased in extracts of cells grown in DS broth containing isoleucine or leucine, but the activity of the lactic dehydrogenase (LD) remained nearly constant. These data indicate that either isoleucine or leucine represses formation of the AAS and TD, but the two amino acids do not substitute for valine in the LD. Therefore, it is unlikely that the
decrease in the rate of growth of strain 655 in the presence of isoleucine or leucine is the result of the formation of false protein.

Freundlich et al. (1962) and Freundlich and Umbarger (1963b) reported that maximum repression of the enzymes of the ilv pathway in strain W of E. coli and strain LT-2 of S. typhimurium occurs when each of the end-products, isoleucine, valine, leucine and pantothenate, is present in excessive concentrations. This pattern of control by end-products of the enzymes involved in a biosynthetic pathway has been termed "multivalent repression".

Several experiments were conducted to determine whether multivalent repression is exerted on the TD and AAS of strain 655. The activities of the TD, AAS and LD in these procedures were compared to the activities obtained in an extract of cells grown in DS broth. The results of these experiments (Table 9) indicate that formation of the TD and the AAS is not affected to the same degree, nor in the same manner, in cells grown in various synthetic media. TD synthesis was maximally repressed (85 per cent loss in specific activity) in cells grown in CS broth, or CS broth containing pantothenate (77 per cent loss in activity), and was moderately repressed in cells grown in CS or DS broth containing an excess of valine. The latter two observations correlate with the data obtained from assays of TD activity in vitro, as valine, by itself, was slightly inhibitory, but also decreased the level of inhibition exerted by leucine and isoleucine. In addition, growth of the cells in DS broth containing isoleucine or leucine did not completely repress the TD (Table 11). Therefore, while the presence of pantothenate had little effect, these data indicate that repression of the TD is regulated by a mechanism
similar to multivalent repression, but requiring that all three amino acids be present in a particular ratio.

In contrast, AAS formation was not affected in cells grown in CS broth containing pantothenate, decreased with time in cells grown in DS broth containing isoleucine or leucine, was enhanced in cells grown in DS broth with an excess of valine, but was decreased in cells grown in CS broth containing an excess of valine. The latter two observations indicate that valine (at this relatively low concentration as compared to the concentration needed for inhibition of the AAS in vitro) may stimulate AAS synthesis in vivo, but, in conjunction with isoleucine and leucine, represses the AAS when present at a concentration three times greater than is used in CS broth. In addition, the increase in AAS synthesis in cells grown in DS broth containing an excess of valine may explain why the rate of growth of strain 655 is optimal in DS broth containing valine (Fig. 2).

These results indicate that AAS synthesis is maximally repressed by pantothenate or a combination of the three amino acids and pantothenate, but not to a degree (16 per cent loss in specific activity) which is consistent with the concept that a mechanism similar to multivalent repression would be an effective regulator of AAS formation.

In order to further examine the patterns of repression and derepression of the TD and the AAS, strain 655 was grown in CS broth with sequential transfer to DS broth and then to CS broth (Fig. 11). The data in Table 10 show that TD synthesis was repressed in cells grown in CS broth, was gradually derepressed in cells grown in DS broth, and was subsequently repressed with time in cells grown in CS broth. It is noted
that repression of TD formation occurred more rapidly and completely within three hours of cellular growth in CS broth than occurred in three hours of growth in DS broth containing an excess of isoleucine or leucine. These data indicate that all three amino acids repress the synthesis of the TD more effectively than does isoleucine or leucine alone, which supports the concept that multivalent repression is effective in regulating TD formation.

However, AAS synthesis decreased in cells obtained after three and four hours of growth in DS broth, and increased slightly in cells transferred to CS broth. The decrease in AAS formation may aid in explaining why the rate of growth of strain 655 is slower in DS broth than it is in CS broth (Fig. 2), but it is difficult to explain how the AAS is regulated. It is clear that AAS formation is not greatly affected by the presence of any one, or all three, of the amino acids, again indicating that multivalent repression is not an effective mechanism for regulating the AAS. It is possible that the concentrations of the three amino acids employed in CS broth are sufficient to limit synthesis of KV, which exerted the most effective inhibition of AAS activity in vitro. The decrease in AAS activity observed in extracts of cells grown in DS broth may be the result of repression or inhibition of the AAS by KV, which would be synthesized during growth in DS broth. Hence, the synthesis or activity of the AAS may be primarily regulated by KV. Alternatively, AAS synthesis may be regulated by a multivalent repression involving the combined effects of isoleucine, valine, leucine, pantothenate and KV, or some combination of these compounds.

Indirect support for the concept that KV is the primary regulator
of the AAS is suggested by the observation that KV more effectively inhibited AAS activity in extracts of cells grown in CS broth than in extracts of cells grown in DS broth (Table 7). This observation indicates that the AAS is more sensitive to KV in extracts of cells grown under conditions in which KV synthesis should be minimal. Therefore, the decrease in AAS activity in extracts of cells grown under conditions in which KV is synthesized may result from the presence of the intermediate. However, further experimentation is necessary to determine the effects of KV on the growth of strain 655 and on the activity of the AAS in extracts of cells grown in the presence of KV.

It was also noted that valine and leucine were less effective as inhibitors of TD activity in the extract of cells grown in CS broth than in the extract of cells grown in DS broth (Fig. 7 and 8). This observation suggests that growth in the presence of isoleucine, valine and leucine decreases the in vitro sensitivity of the TD to inhibition exerted by leucine and valine. This decrease in sensitivity may result from a change in conformation of the TD caused by binding of leucine and valine (and, perhaps, isoleucine) during growth of the cells under conditions that repress the TD. The change in conformation may result in a decreased affinity of the TD for binding leucine and valine.

However, as the TD is repressed under these conditions of growth, the concentration of extract employed in these assays was ten-fold greater than the concentration employed in assays of TD activity in extracts of cells grown under derepressed conditions. Therefore, leucine and valine may not be as effective in inhibiting TD activity in assays with increased protein concentration but decreased TD activity.
Multivalent repression of the enzymes of the $ilv$ pathway is exhibited in strain LT-2 of $S. typhimurium$ (Freundlich et al., 1962), and in strain W of $E. coli$ (Umbarger and Freundlich, 1965). The linear arrangement of the structural genes of the $ilv$ pathway has been reported to be similar in strain LT-2 of $S. typhimurium$ and in strain K-12 of $E. coli$. Employing the names of the enzymes to identify the genes which code for the enzymes, the arrangement in these organisms has been established as transaminase, dehydrase, TD, RI, AAS (Roth and Sanderson, 1966). This arrangement does not occur in strain 655 of $S. aureus$. Smith and Pattee (1967) reported that the linear arrangement in this organism is identical to the sequence in which the enzymatic reactions occur (Fig. 1).

Ramakrishnan and Adelberg (1964) demonstrated that multivalent repression is effective only in regulating the TD, dehydrase and transaminase in strain K-12 of $E. coli$, which, in correlation with the linear arrangement of the structural genes, would constitute one operon. They suggested that the genes for the AAS and the RI are outside the operon in which the other three genes are located, and are regulated by a separate operator gene. The results of this study demonstrate that the TD and the AAS are not coordinately repressed or derepressed, indicating that, in strain 655, the TD and the AAS are regulated by separate operator genes. Therefore, if the structural genes are arranged as reported by Smith and Pattee (1967), and the TD and the AAS are located in separate operons, the remaining structural genes are either located in the same operon as the AAS, or there are at least three operons controlling the $ilv$ pathway. However, it is clear that in strain 655, as in strain K-12, multivalent repression is not solely responsible for regulating
the *ilv* pathway.

The results of this study also indicate that the inhibition of growth exerted by isoleucine or leucine does not involve the formation of false protein, or the repression or inhibition of the AAS. Leucine, by inhibiting TD activity, may interfere with isoleucine biosynthesis. This is supported by the observation that valine reversed the inhibitory effects of leucine on the growth of strain 655 and on TD activity. By analogy, isoleucine may inhibit growth by interfering with leucine biosynthesis, but no data have been obtained in this study to support this possibility. It is also possible that the inhibition of growth observed in the presence of isoleucine or leucine may involve the remaining enzymes of the *ilv* pathway, or may result from effects which are, as yet, unknown. However, further studies are necessary to determine: a) whether the remaining enzymes are affected by any or all of the three amino acids; b) the number of operons controlling the *ilv* pathway; c) the effects of KV on the growth of strain 655 and on the AAS; d) whether isoleucine affects any of the enzymes of the leucine biosynthetic pathway.
SUMMARY

The growth of strains 655, U9 and 152 of *Staphylococcus aureus* is inhibited by isoleucine and by leucine, and the inhibition is reversed by valine. Using cell-free extracts of strain 655, assays were conducted on four of the five enzymes that are involved in the biosynthesis of isoleucine and valine in an attempt to determine whether the inhibition exerted by isoleucine and leucine involves inhibition of enzyme activity or repression of enzyme formation.

The assays of reductoisomerase activity were not successful because non-specific oxidases in the cell-free extracts reacted with the cofactor in the presence of several compounds. The assays of dihydroxy acid dehydrase activity indicated that the enzyme is specific for the L-isomer of DHV and is inhibited by the D-isomer. However, because the quantity of L-DHV was very limited, only preliminary studies were possible concerning the mechanism by which the dehydrase might be regulated.

The assays of threonine deaminase (TD) activity demonstrated that the TD is stable in 1 M phosphate buffer, has a pH optimum of 8.5 and is subject to feedback inhibition by isoleucine and, to a lesser extent, by leucine. Valine, which is weakly inhibitory at relatively high concentrations, reversed the inhibition exerted by isoleucine and leucine.

Growth of the cells in the absence and in the presence of isoleucine, valine and leucine demonstrated that the TD is maximally repressed in cells grown in synthetic media containing all three amino acids, suggesting that synthesis of the TD is controlled by multivalent repression. However, the synthesis of the AAS is not significantly affected by any
one of the three amino acids, or by a combination of the three amino acids and pantothenate. This observation indicates that the AAS is not controlled by multivalent repression, and suggests that the TD and the AAS are regulated by separate operator genes.

The results of this study do not indicate the manner in which isoleucine inhibits the growth of *S. aureus*. It has been demonstrated that leucine inhibits TD activity, and this inhibition could result in deprivation of isoleucine, thereby preventing growth. While it is attractive to speculate that isoleucine may act as an analogue of leucine and inhibit an enzyme involved in leucine biosynthesis, no evidence has yet been obtained to support this concept.
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