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Nutritional studies with Acetobacter suboxydans indicated that when isoleucine, lysine, methionine, serine and arginine were singly omitted from a complete amino acid mixture, no growth was obtained. However, the organism could grow on single amino acids like glutamic acid, histidine and proline. No growth was obtained with ammonium sulfate as the sole nitrogen source. An investigation on the cause for the apparent requirement for isoleucine was made. When valine was added to a synthetic medium containing histidine, glutamic acid, proline and ammonium sulfate no growth was obtained. Valine appeared to inhibit growth, an effect which could be reversed by addition of isoleucine. Cell-free extracts of the organism synthesize both valine and isoleucine from acetolactate and acetohydroxybutyrate respectively. The amounts of the two amino acids synthesized from different intermediates were determined.

A study of the mechanism of growth inhibition due to valine was initiated. U-C^{14} threonine was incorporated into isoleucine by growing cells. The first step in the biosynthesis of isoleucine from threonine

is the deamination of the latter to α-ketobutyrate. This enzyme, threonine deaminase, was precipitated from cell-free extracts at 50 percent saturation with ammonium sulfate. Using this fraction, it was found that the activity of the deaminase was competitively inhibited by valine and isoleucine. Although isoleucine repressed the synthesis of the deaminase, valine did not. Therefore one mechanism by which valine inhibits growth of the organism is by feed-back inhibition of the threonine deaminase thereby limiting isoleucine biosynthesis. This is evident by an apparent requirement for isoleucine when valine is present in the growth medium. No inhibition of the transport of isoleucine across the cell wall was observed.

Mutants of Acetobacter suboxydans have been isolated which are prototrophic towards isoleucine and resistant to growth inhibition by valine. Some of these mutants possess a threonine deaminase which is not sensitive to valine or isoleucine.

AMINO ACID METABOLISM IN *ACETOBACTER SUBOXYDANS*

by

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INTRODUCTION

Acetobacter suboxydans was isolated by Kluyver and de Leeuw in 1924 from Dutch beer (17). A large number of substrates are incompletely metabolized by this organism, the process usually stopping after a single oxidative step (10, 15). This property of A. suboxydans has been used to produce commercially valuable products such as dihydroxyacetone, sorbose, gluconic and keto-gluconic acids.

Oxidation of glucose is of special interest, since two mechanisms are involved. Glucose is primarily catabolized to gluconic and keto-gluconic acids, however part of it is oxidized via the pentose cycle (6). Available evidence suggests that glycolysis does not play a role in the dissimilation of glucose in this organism (6). Unlike most other obligatory aerobic organisms, A. suboxydans lacks the tricarboxylic acid cycle. Several lines of evidence support this. Cells or cell-free extracts are not able to oxidize succinate, fumarate, α-keto glutarate and malate (6). The oxaloacetic acid-acetyl-coenzyme A condensing enzyme leading to the formation of citrate has not been detected in extracts (16). Radiorespirometric data also indicate the absence of an active tricarboxylic acid cycle (6).

The absence of a tricarboxylic acid cycle leads to the important question as to how the dicarboxylic amino acids, glutamic acid and aspartic acid are synthesized. Keeping this objective in mind, a survey on the nutritional requirements of this bacterium was made to

ascertain whether or not A. suboxydans could biosynthetically supply for growth all of the aspartate and glutamate families of amino acids. A study on the amino acid requirements previously had been made by Stokes and Larsen (36). Those investigators reported that valine was essential for growth. Their experiments indicated that when valine was omitted from an otherwise complete medium, no growth was observed. Our experiments failed to detect such a requirement. In order to obtain definitive evidence on this point with our organism, cell-free extracts were tested for the enzymes involved in the biosynthesis of valine. The formation of valine from acetolactate, α,β -dihydroxyisovaleric acid and α-ketoisovaleric acid has been demonstrated suggesting identity of mechanism for valine synthesis in A. suboxydans as with Torulopsis (39). Acetolactate has been shown to be a precursor of valine in E. coli (49), S. typhimurium (56) and Neurospora (57).

Although single amino acid omission from otherwise complete incubation mixtures of amino acids showed a requirement of isoleucine for growth, single amino acid growth experiments indicated that isoleucine was not essential. Growing cells could incorporate labeled threonine into isoleucine offering presumptive evidence for a mechanism of isoleucine synthesis. Examination of cell-free extracts substantiated that this amino acid could be synthesized from α-ketobutyrate and pyruvate (acetaldehyde) via α-acetohydroxybutyrate. Such a pathway has been reported for isoleucine synthesis in E. coli (20).

In order to determine whether exogenous valine would stimulate growth, experiments were conducted in a synthetic medium to which valine

had been added. Excess valine inhibited growth; growth was initiated on the addition of isoleucine. Therefore, valine appeared to inhibit synthesis of isoleucine accounting for a growth requirement of isoleucine in the incubation mixtures containing valine but not isoleucine.

Valine-isoleucine antagonism has been studied extensively in the E. coli K-12 system by Umbarger and Brown (45). The similarity between the phenomenon reported by these authors and the one presented in this thesis warranted an investigation of the regulatory activities of valine in A. suboxydans.

It is very essential for the economy of the cell to have a rigorous control of its metabolism. In biosynthetic pathways this may be accomplished via feed-back inhibition and repression (44). In feed-back inhibition the end product inhibits the activity of the first enzyme in its biosynthetic sequence. Feed-back inhibition has been described for a variety of amino acids: isoleucine (43); valine (44); histidine (25); serine (4); arginine (42); tryptophan (26); lysine and threonine (33). Enzyme repression has been defined as a relative decrease, resulting from the exposure of cells to a given substance, in the rate of synthesis of a particular apoenzyme (55). Repression of the arginine pathway has been well studied by Vogel in the E. coli system (55).

The inhibitory effect of valine in A. suboxydans can be explained as follows: Valine appears to mimick isoleucine in as much as it inhibits the activity of threonine deaminase, the first enzyme of isoleucine biosynthesis. Although isoleucine can repress threonine

deaminase, valine does not have any affect on the synthesis of this enzyme. The study reported here presents a detailed examination of valine and isoleucine biosynthesis in A. suboxydans. A mechanism of growth inhibition by valine is demonstrated.

EXPERIMENTAL

Acetobacter suboxydans ATCC 621 strain was used in all experiments. For comparative purposes, strain MB 416 used by Stokes and Larsen (36) was obtained from Merck and Company. Both organisms were maintained on yeast extract agar slants. For large scale culture the organisms were grown in 100-liter quantities at 30 for 24 hours in a pilot-plant fermenter in yeast extract medium of the following composition:

Glycerol	5%
Difco Yeast extract	0.5%
KH_2PO_4	0.25%

pH adjusted to 6.0 with 1 N NaOH

For biosynthetic experiments, cells were grown in ten liter carboys using the synthetic medium of Underkofler et al. (54) as modified by Shamberger (31) containing all designated amino acids except valine and isoleucine. After growth, cells were washed twice in phosphate buffer pH 6.0 (0.1M) and starved in buffer for 1 hour. The cells were then lyophilized and stored at 0° C.

Nutritional experiments were carried out in the following manner: cells grown in yeast extract medium were harvested in sterile centrifuge tubes. They were then washed twice after which they were resuspended in sterile isotonic saline to an optical density of 0.1 (Coleman junior spectrophotometer). This cell suspension served as an inoculum for the nutritional experiments. A double strength synthetic medium of Shamberger (31) using glycerol as carbon source was prepared. Five ml. of this medium were dispensed in 25 ml. Erlenmeyer flasks. Two mg. of

each amino acid to be tested were added and the total volume in each flask brought to ten ml. after adjusting the pH to 6.0. The flasks were sterilized and innoculated with ca. 0.05 ml. of the prepared innoculum. Culture vessels were incubated at 30° C. for different periods (as indicated) with agitation, after which growth was measured at 625 mμ in a Coleman junior spectrophotometer. Single amino acid growth experiments were also carried out in this manner.

Comparative studies on the oxidation of different substrates by strain 621 and MB 416 were carried out manometrically (53).

Radiorespirometric studies on strain MB 416 were performed by the microscale method of Wang et al. (58). Sodium hydroxide (5 N) was used as a trap for the $C^{14}O_2$ liberated. The $C^{14}O_2$ was converted to $BaC^{14}O_3$ and counted on planchets in a thin window Geiger Müller counter. Corrections due to background and self-absorption were made.

For preparation of cell-free extracts ten gms of lyophilized cells were suspended in 100 ml. phosphate buffer pH 6.0. Ten ml. of the cell suspension were then disintegrated in a sonic oscillator (Raytheon Model DF 101) for 30 minutes without interruption. Ice-cold water was circulated through the inducer apparatus to prevent temperature increases. The disintegrated cell suspension was centrifuged at 105,000 x G for 30 minutes in a Spinco preparative centrifuge. The supernatant cell-free extract was dialyzed against pH 6.0 (0,1M) phosphate buffer overnight at 4° C. with circulating buffer.

Protein determinations unless otherwise stated were by the method of Lowry et al. (23).

Activities of acetolactate and acetohydroxybutyrate reductoisomerase were determined in a Cary recording spectrophotometer by following the disappearance of the absorption band of reduced pyridine nucleotide at 340 mμ. Acetoin determinations were carried out by the method of Westerfeld (59).

Using cell-free extracts of A. suboxydans, α-ketoisovaleric acid was isolated from acetolactate in the following manner:

One ml. of cell-free extract (34 mg. protein) was incubated with 1 mmole of acetolactate, 10 μmoles each of NADPH/1, GSH and ATP, 1 μmole of CoASH and 1 mmole phosphate buffer pH 7.6 (0.1M). The system was incubated for eight hours. α-keto isovaleric acid was isolated by the method of Friedemann and Haugen (9) as a 2,4 dinitrophenylhydrazone.

The amounts of valine formed from different intermediates were determined by circular paper chromatography using the method of Giri (11) and a solvent system of butanol:acetic acid:water (40:10:50). After developing the paper, it was sprayed with 2 percent ninhydrin in 95 percent acetone. The paper was again dried and heated for fifteen minutes at 65° C. Streaks due to valine were excised and the color eluted in five ml. of 50 percent (v/v) n-propanol in water. The

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/1 The following abbreviations have been used in this thesis:
NADPH--reduced nicotinamide adenine dinucleotide phosphate
GSH--glutathione
ATP--adenosine triphosphate
CoASH--coenzyme A
NADH--reduced nicotinamide adenine dinucleotide
TPP--thiamine pyrophosphate
AMP--adenosine monophosphate
NAD--nicotinamide adenine dinucleotide
NADP--nicotinamide adenine dinucleotide phosphate

tubes were allowed to set for twenty minutes and the colored solution in each tube clarified by centrifugation. The clear colored solution was then read at 570 mμ in a Coleman junior spectrophotometer.

Valine standards were spotted along with the experimental samples to check the accuracy of the method and to aid in the identification of valine.

Incorporation of labeled threonine into isoleucine in growing cells was determined as follows: five μc of U-C^{14} threonine were added to a synthetic growth medium containing glutamate, histidine and proline each at a concentration of two mg. The total volume was ten ml. The system was then inoculated with A. suboxydans and incubated for 72 hours at 30° C. as shake cultures. The cells were harvested and hydrolyzed with 6N HCl for eight hours at 121° C. in a sealed tube. The hydrolyzate was neutralized with sodium hydroxide and spotted on Whatman #1 paper. After development of the paper in butanol:water:ethanol system (20:4.5:1) it was scanned for radioactivity using a Vanguard model 880 strip counter.

The amount of isoleucine synthesized from different intermediates was determined microbiologically using S. faecalis R 8043 by the method of Stokes et al. (35).

α-acetohydroxybutyrate was isolated as a phenylosazone from α-ketobutyrate and pyruvate (source of acetaldehyde) in the following manner: 1×10^{-3} M. each of pyruvate and α-ketobutyrate were incubated with two ml. cell-free extract (32 mg. protein), 10 μmoles each of ATP and GSH, 1 μmole CoASH, 100 μmoles Mg^{++}, 10 ml. phosphate buffer

pH 6.0 (0.1 M.). The acetohydroxybutyrate was isolated as a phenylosazone by the method of Juni (14) and Leavitt and Umbarger (20).

For the valine-isoleucine antagonism studies two and one half ml. of synthetic medium was dispensed in rimless test tubes, and two mg. each of histidine glutamate, proline, and five mg. ammonium sulfate were added. Different concentrations of valine and/or isoleucine were added. Total volume was then made to five ml. After sterilization, inoculum prepared in a manner identical to that for nutritional studies was then added. After incubation at 30° C. growth was measured at 625 mμ in a junior Coleman spectrophotometer.

Since cell-free extracts contained an active α-ketobutyrate oxidase, estimation of threonine deaminase by the method of Friedemann and Haugen (9) was not practicable. Activity of threonine deaminase was estimated by two independent methods. One was dependent on the estimation of free ammonia liberated in the reaction by the method of Lang (19) using Nessler's reagent.

In the other method, the activity of the deaminase was coupled with that of α-ketobutyrate oxidase, and the $C^{14}O_2$ liberated from U-C^{14} threonine was absorbed in one ml. of sodium hydroxide (5N). One half ml. of the sodium hydroxide solution was then pipetted into scintillation vials and counted using the solvent system of Reed (29). The vials were counted in a Packard scintillation counter. Efficiency of counting was about 41 percent.

Threonine deaminase could be separated from α-ketobutyrate oxidase by ammonium sulfate fractionation of the cell-free extract. At 50

percent saturation of crude cell-free extract, threonine deaminase was quantitatively precipitated and could be removed by centrifugation at 105,000 x G for 30 minutes. α-ketobutyrate oxidase remained in solution until 100 percent saturation with ammonium sulfate.

The effects of valine, leucine and isoleucine on the activity of threonine deaminase was measured by adding the respective amino acids to the incubation mixture. Effect of isoleucine on the synthesis of threonine deaminase was determined by growing the cells in a synthetic medium containing all amino acids except valine. Since valine inhibited growth of the organism, the effect of valine on the synthesis of threonine deaminase was measured indirectly. Cells were grown in the presence of isoleucine but no valine. Part of the medium was harvested and the threonine deaminase activity in cell-free extract of these cells determined. The remainder of the medium was used as an inoculum for 3 different flasks. Flask 2 contained valine synthetic medium; flask 3 contained synthetic medium without valine or isoleucine; flask 4 contained isoleucine synthetic medium. These flasks were incubated for 48 hours and the cells from each flask were harvested and the activity of threonine deaminase determined.

Conversely, cells were grown in the absence of isoleucine and valine. After measuring threonine deaminase activity in these cells, the bulk of the medium was innoculated into 3 separate flasks. Flask 6 contained valine synthetic medium; flask 7 contained isoleucine synthetic medium; flask 8 contained synthetic medium without valine or isoleucine. After 48 hours cells were harvested and the activity of

threonine deaminase determined. As a control for each system, alcohol dehydrogenase activity was measured using NAD^+ as a cofactor.

Mutants resistant to growth inhibition by valine and prototrophic toward isoleucine were isolated in the following manner: Wild-type cells were exposed to ultraviolet light from a distance of 7½ inches for 45 seconds. These were then inoculated into yeast extract-glycerol medium and incubated in the dark at 30° C. for 72 hours with agitation. This step helped to build up the mutant population. Cells were harvested under aseptic conditions and washed with sterile 0.9 percent NaCl and inoculated into a synthetic medium containing all amino acids except isoleucine. After 48 hours incubation, they were plated on agar plates containing synthetic medium plus valine but no isoleucine; colonies which grew were resistant to growth inhibition by exogenous valine. Fifteen of these colonies were purified and were grown in one liter quantities. The cells were harvested and threonine deaminase sensitivity towards valine was tested in each of the fifteen mutant cultures. Mutant colonies containing threonine deaminase which did not exhibit sensitivity toward valine were grown on a large scale (Mutants 1, 4, and 15) for enzymic analyses.

Experiments on transport of valine and isoleucine across the cell membrane were carried out in the following manner: two and one half mg. (dry weight) of cells were washed with phosphate buffer (pH 6, 0.1M). After several washings the cells were suspended in two ml. of this buffer. Fifty mg. glucose were added and the volume brought to 4.9 ml. with buffer. The flasks were shaken at 30° for 15 minutes.

Uniformly labeled isoleucine of known specific activity was then added. The total volume in each flask was 5 ml.

At definite time intervals one ml. aliquots from each flask were rapidly filtered through a Millipore filter of 0.65μ pore size. The cells were washed twice on the filter with cold phosphate buffer and the dried filter placed in scintillation vials and counted as previously described (29).

The effect of valine on the rate of transport of isoleucine was measured by adding equimolar concentrations of cold valine to the incubation mixture.

RESULTS AND DISCUSSION

Nutritional Studies

Omissions of isoleucine, serine, arginine, methionine and lysine singly from a mixture of 20 amino acids greatly reduced cell yield (Table I). This is in contrast with the results of Stokes and Larsen (36) who found that when single eliminations were made from a mixture of 20 amino acids, only valine appeared to be essential, although the removal of isoleucine, alanine or histidine reduced the growth rate.

In the present study, growth could also be permitted by any one of a few amino acids when used singly. Glutamic acid, histidine, proline, or glutamine along with ammonium sulfate to supplement the nitrogen source could permit growth of this organism: α-keto glutarate along with ammonium sulfate could also permit growth (Table II). This is in contrast to the previous experiment in which single amino acids had been omitted from a complete mixture: isoleucine, serine, arginine, methionine and lysine all were needed. However, since the organism could grow on single amino acids, the essential nature of the above five amino acids is doubtful. An important feature of the single amino acid growth experiments is the relationship of all to glutamic acid. Proline-glutamic acid relationship has been shown by many workers (34, 51) as has the conversion of histidine to glutamic acid (40, 41). The biosynthesis of glutamic acid from oxaloacetate and glyoxalate *via* oxalomalate from citramalate has been shown to be operative in cell-free extracts of *A. suboxydans* (30).

A comparative study on strain 621 and strain MB 416 was made. The oxygen uptake using different substrates was measured using these two strains. The results are shown in Table III. The important difference between the two strains lies in the capacity of MB 416 to oxidize sorbitol and glucose to a greater extent. On the other hand strain 621 can oxidize glycerol probably a step further than MB 416. Both strains are gram negative, cannot grow on nutrient agar and are morphologically similar.

A time-course study on the oxidation of differently labeled glucose by strain 416 was made. The radiorespirometric pattern is shown in Figure 1. Comparing the yield of $C^{14}O_2$ from glucose-1 and glucose-6-C^{14}, it is quite likely that MB 416 strain oxidizes glucose via pentose cycle. However, the operation of an Entner-Doudoroff mechanism cannot be ruled out using these data. On the other hand, strain 621 oxidizes glucose exclusively via pentose cycle (6). These experiments suggest that the strain used by Stokes and Larsen (36) is different from the strain used in the present study.

Biosynthesis of Valine and Isoleucine

Since valine and isoleucine appeared to be dispensable, it was of interest to study their biosynthesis. Cell-free extracts converted acetolactate, α,β-dihydroxyisovaleric acid and α-ketoisovaleric acid into valine (Table IV).

Since the early step in utilization of acetolactate for valine biosynthesis is its reduction and isomerization to α,β-dihydroxyisovaleric

acid, the activity of acetolactate reductoisomerase was measured using NADPH as a cofactor (Figure 2). NADPH could not be replaced by NADH. The optimum activity was observed at pH 7.6 (Figure 3). The K_m of the enzyme, determined by the method of Lineweaver and Burk (22), at pH 7.6 and at constant NADPH concentration was 7.14×10^{-2} M. (Figure 4). The rate of NADPH oxidation using acetolactate as substrate was linear with respect to acetolactate concentration (Figure 5).

The specific activity of acetolactate reductoisomerase is shown in Table V. The same table also shows the effect of valine on the specific activity of acetolactate reductoisomerase. From this table it is apparent that the activity of this enzyme is not inhibited by valine.

Acetolactate can be decarboxylated to acetoin in the presence of TPP and cell-free extracts of A. suboxydans. The latter can be reduced by NADH or NADPH to 2,3-butanediol. Contamination of acetolactate samples with traces of acetoin would thus affect acetolactate reductoisomerase measurements. It was essential therefore to test acetolactate samples with NADH to make sure that acetolactate was free of any acetoin. This was routinely performed on all reductoisomerase determinations.

Metabolism of Acetoin

Two separate pathways exist for acetoin formation in A. suboxydans: it can be formed (a) by decarboxylation of acetolactate, formed from pyruvate and acetaldehyde, using TPP as a cofactor, or (b) by condensation

of two moles of acetaldehyde derived from pyruvate. Table VI shows the amount of acetoin formed from pyruvate. The determination of acetolactate involves its conversion by added H_2SO_4 to acetoin and colorimetric estimation of the latter (59). Great difficulty was observed in measuring the formation of acetolactate from pyruvate and acetaldehyde by a ketol-type condensation because of the presence of the second pathway.

Difficulties involved in the measurement of acetolactate prevented determining the effect of valine on its formation. Acetolactate forming enzyme is sensitive to feed-back inhibition by valine in E. coli K-12 (44). Although the biosynthesis of valine in A. suboxydans is similar to that in the enteric bacteria (49), it was not possible to determine whether the formation of acetolactate in the A. suboxydans system was also sensitive to valine.

The metabolic fate of acetoin has been investigated. It has only a transient existence, due to the presence of a very active acetoin reductase, which reduces it in the presence of NADH to 2,3-butanediol. At 25-50 percent ammonium sulfate saturation, acetoin reductase is precipitated from cell-free extracts. The pH optimum for the reductase extends from 4.2 to 7.5. The K_m of the reductase was 3.85×10^{-3} M. The enzyme is inhibited by pCMB. Acetoin could not protect the enzyme from inhibition by pCMB.

The conversion of acetolactate to α,β -dihydroxyisovaleric acid occurs through a pinacol rearrangement followed by reduction. If a pinacol rearrangement took place first, then the product of such a

reaction would be an α-keto β-hydroxyisovaleric acid. If the reduction of acetolactate were to take place first, the product would then be α, β, dihydroxy- β-methyl butyric acid. Stereoisomers of the latter compound were tested by Strassman et al. (37), and they found in yeast that these compounds could not be converted to α-ketoisovaleric acid.

Wagner, Radhakrishnan and Snell (57) have reported that synthetic α-keto- β-hydroxyisovaleric acid can be reduced by a reductase present in cell-free preparations of N. crassa mutants. α, β-dihydroxyisovaleric acid was the product of this reaction. Free α-keto-β-hydroxyisovaleric acid has not been isolated from cultures of E. coli or N. crassa.

Umbarger et al. (50) have proposed that a single enzyme is responsible for the rearrangement and reduction of acetolactate to α, β-dihydroxyisovaleric acid. Such an enzyme, reductoisomerase (isomeroreductase) has been isolated. The reductoisomerase converts acetolactate to α, β-dihydroxyisovaleric acid without formation of an α-keto- β-hydroxyisovaleric acid. The reductoisomerase is not able to reduce α-keto- β-hydroxyisovaleric acid. Radhakrishnan, Wagner and Snell have proposed that this ketohydroxy acid may not be an intermediate. If it is one, it may be enzyme bound (28).

Armstrong and Wagner (3) have isolated the reductoisomerase free from reductase from E. coli and S. typhimurium. Loss of reductoisomerase activity resulted in concurrent appearance of reductase activity. These authors propose that the reductase may be an altered form of the reductoisomerase. The reduction of α-keto-β-hydroxyisovaleric acid

was followed in the A. suboxydans system using NADPH as cofactor. In all experiments, there was a lag period of about 100 seconds during which time no oxidation of the NADPH took place. After 100 seconds, the reduced pyridine nucleotide appeared to be oxidized.

If the α-keto-β-hydroxyisovaleric acid were incubated with cell-free extract in the absence of NADPH for 100 seconds and at the end of this period, NADPH was added, oxidation of the reduced pyridine nucleotide took place without lag (Figure 6). In both instances, α-ketoisovaleric acid could be detected by paper chromatography of the phenylhydrazone using the method of Strassman et al. (37). The presence of a lag period prior to oxidation of NADPH suggests that the α-keto-β-hydroxyisovaleric acid had to be activated to an unknown compound before reduction was accomplished. Attempts to identify this compound were not successful.

The α-ketoisovaleric acid 2,4-dinitro phenylhydrazone derivative isolated from acetolactate, as explained in the experimental section, melted at 196°-197° C. Authentic derivative melts at 197° C. The observed mixed melting point was 195°-197° C. The isolated phenylhydrazone was then reduced with H_2 and palladized asbestos in a Warburg vessel. The reduced derivative was identified as valine by paper chromatography using butanol:acetic acid:water (40:10:50) system.

Figure 7 summarizes the biosynthesis of valine in A. suboxydans. The pathway presented is identical to the one reported by Strassman et al. (39) in yeast; Umbarger et al. in E. coli (49); Wagner et al. in Neurospora (57).

Since U-C^{14} threonine could be incorporated into isoleucine (see Experimental Section), it was necessary to determine whether the pathway for isoleucine biosynthesis in A. suboxydans was the same as that in E. coli (20), yeast (38), Neurospora (57). Cell-free extracts were incubated with α-ketobutyrate and pyruvate (as source of acetaldehyde). In a separate experiment, α-acetohydroxybutyrate was incubated with cell-free extract along with cofactors listed in Table VII. The amount of isoleucine synthesized from these two substrates is also shown in Table VII.

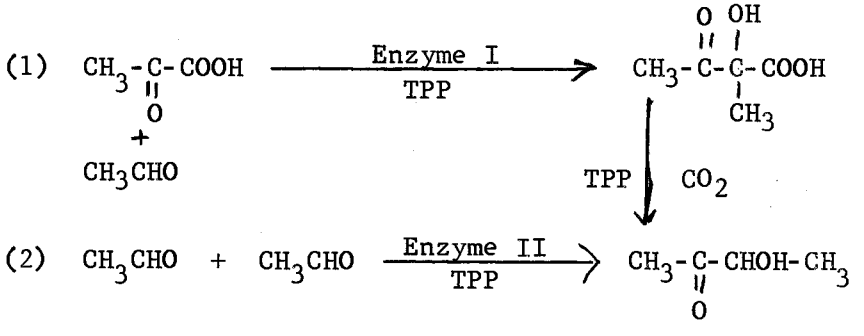
In order to prove that α-acetohydroxybutyrate is an intermediate in isoleucine biosynthesis in this organism, acetohydroxybutyrate was isolated from α-ketobutyrate and pyruvate (as source of acetaldehyde) using cell-free extracts of A. suboxydans as shown in the experimental section. The melting point of the derivative was 168° C. Authentic α-acetohydroxybutyrate phenylosazone melted at 167° C. Activity of acetohydroxybutyrate reductoisomerase in cell-free extract was also measured. The specific activity of the enzyme is shown in Table VIII.

Though other proposed intermediates of isoleucine biosynthesis have not been tested, it appears that the biosynthetic pathway of this amino acid is identical to that proposed in E. coli K-12 and N. crassa. This pathway is represented in Figure 8.

Valine-Isoleucine Antagonism

Since the experiments of Stokes and Larsen (36) had indicated that valine was essential, different concentrations of valine were added to

the synthetic medium to determine its effect on growth. Results of this experiment are shown in Table IX. From this table it is apparent that concentrations of valine higher than 1.3×10^{-3} M. appear to inhibit growth. Growth inhibition due to valine is completely reversed by the addition of isoleucine. Isoleucine alone did not stimulate growth. Since excess valine is growth inhibitory, it is essential that the cell control synthesis of this amino acid. Umbarger and Brown (48) have proposed that valine controls its synthesis in the enteric bacteria by competitively inhibiting the formation of acetolactate. Studies of Radhakrishnan *et al.* (27) show that *E. coli* possesses two acetolactate forming enzymes with different pH optima. The enzyme with pH optimum of 8.0 appears to be the one that provides acetolactate for valine biosynthesis; this enzyme is sensitive to inhibition by valine (48). In *A. suboxydans* two enzymes are present for the synthesis of acetoin (5).



Enzyme I provides the acetolactate necessary for valine biosynthesis. Enzyme II condenses acetaldehyde formed from pyruvate, in the presence of TPP, to form acetoin, a neutral product. Acetoin does not appear to participate in any biosynthetic reaction. Enzyme I may therefore be classified as a biosynthetic enzyme while Enzyme II as a catabolic enzyme.

Valine-isoleucine and leucine antagonism had been observed by Gladstone in anthrax bacillus (12). Umbarger and Mueller (52) observed antagonism between valine and isoleucine in E. coli K-12. Umbarger and Brown (45) suggested that in strain K-12 valine inhibits the formation of isoleucine. Refining this work, Leavitt and Umbarger (21) have found that valine inhibits formation of acetohydroxybutyrate, an intermediate in isoleucine biosynthesis.

Valine-isoleucine antagonism studies in the A. suboxydans system suggested two possibilities:

(1) Valine inhibits synthesis of isoleucine.

(2) Presence of valine may inhibit the incorporation of isoleucine into cellular protein.

The latter appears to be unlikely since it has been shown that the amino acid activating enzymes and the transfer enzymes are quite specific in other bacteria (32). Since the second possibility seems less likely, a detailed study on the effect of valine on isoleucine biosynthesis was undertaken.

Threonine has been proposed to be a precursor of isoleucine (1). The effect of valine on threonine deaminase, an enzyme obligatory for conversion of threonine to α-ketobutyrate, was studied, since this was the most logical enzyme to test first because inhibition of several of the other enzymes would prevent formation of leucine or threonine.

The formation of α-ketobutyrate from threonine could not be measured directly because of the presence of an α-ketobutyrate oxidase in cell-free extracts, two separate assay systems were devised. Agreement between the two assay methods was observed (Table X).

Table XI shows the effect of valine, isoleucine and leucine on the activity of α-ketobutyrate oxidase. Because these three amino acids did not affect α-ketobutyrate oxidase activity, it was possible to study the effect of these three amino acids on the activity of threonine deaminase by coupling it with α-ketobutyrate oxidase and measuring the $C^{14}O_2$ evolved using U-C^{14} threonine. Since threonine deaminase could be precipitated at 0-50 percent saturation of cell-free extracts with ammonium sulfate, almost all studies were carried out with this fraction.

The cofactor requirements of threonine deaminase was determined as shown in Table XII; Mg^{++}, pyridoxal phosphate, glutathione, and AMP are all essential for maximum activity. In crude cell-free extracts, AMP could be replaced by ATP. Cofactor requirements of threonine deaminase isolated from sheep liver have been studied by Greenberg et al. (13).

The effect of threonine on the activity of threonine deaminase in the 0-50 percent ammonium sulfate precipitated fraction was measured. Excess threonine appeared to inhibit the activity of the enzyme. Using a double reciprocal plot, the K_m of the deaminase was found to be 3.12×10^{-4} M. (Figure 9).

A study was made on the effect of valine, isoleucine and leucine on threonine deaminase activity. From the values reported in Table XIII, it is evident that valine and isoleucine inhibit threonine deaminase, whereas leucine has no effect.

A similar study on the effect of valine, isoleucine and leucine was made by Umbarger and Brown (47) on threonine deaminase in E. coli K-12 system. They found that isoleucine and to some extent, leucine inhibited threonine deaminase activity. Valine had no effect. In the present study with A. suboxydans valine and isoleucine inhibit the activity of threonine deaminase, but leucine has no effect.

A study of the type of inhibition due to valine and isoleucine on threonine deaminase was initiated. Figure 10 shows a double reciprocal plot of the deaminase in the presence of valine and isoleucine. The K_i values for valine and isoleucine as determined by the graphical method of Dixon (7) are as follows: K_i valine = 2.4×10^{-4} M., K_i isoleucine = 1.04×10^{-4} M. From Figure 10 it is clear that valine and isoleucine competitively inhibit the activity of threonine deaminase. Isoleucine appears to be a stronger competitive inhibitor than valine.

Since both valine and isoleucine appear to inhibit the activity of threonine deaminase, it was of interest to determine whether there might be two threonine deaminases, one sensitive to valine and the other sensitive to isoleucine. If this were true, the total inhibition due to valine and isoleucine would be the sum of that observed for each independently. The combined effects of valine and isoleucine on threonine deaminase were compared with individual effects of these two amino acids on the enzyme. As shown in Table XIV, the total inhibition is not additive, and it is postulated that only a single threonine deaminase, sensitive to both valine and isoleucine, exists

in A. suboxydans. Stadtman et al. (33) have shown in E. coli K-12, two separate aspartokinases, one sensitive to lysine and the other sensitive to threonine. When threonine and lysine were added simultaneously, the total inhibition was approximately the sum of that observed for each independently. Umbarger and Brown (46) have shown that in E. coli K-12, two threonine deaminases are synthesized by the organism. One of them, the biosynthetic deaminase, requires pyridoxal phosphate as a cofactor and is sensitive to feed-back inhibition by isoleucine. The other threonine deaminase has a catabolic function, and is an adaptive enzyme. The catabolic threonine deaminase is formed only under anaerobic conditions in the absence of a fermentable energy source. It is sensitive to repression by carbohydrate and by aeration. Pyridoxal phosphate, glutathione, and adenylic acid are cofactors (44).

Changeux (5) has shown that the biosynthetic threonine deaminase in E. coli K-12 can be desensitized towards isoleucine by heat treatment or treatment with pCMB. Either isoleucine or threonine appear to protect the enzyme from desensitization, if the enzyme is heated in their presence. When the 0-50 percent ammonium sulfate fraction was heated to 60° C., the activity of the deaminase was reduced by about 50 percent. The deaminase was completely inactivated at 80° C. Though the untreated 0-50 percent ammonium sulfate fraction was sensitive to valine and isoleucine, the 60° C. treated fraction was insensitive to isoleucine but had retained its sensitivity towards valine as shown in Table XV.

The observation that heat treated threonine deaminase of A. suboxydans is sensitive to inhibition by valine but insensitive to isoleucine suggests that there might be three separate sites independent of each other, for isoleucine, valine and threonine. Such an interpretation is similar to the one arrived at by Monod, Jacob and Changeux (24).

The effect of valine on the synthesis of threonine deaminase was measured indirectly (see Experimental Section). The results of this experiment are shown in Table XVI. It is clear from this table that valine does not repress the synthesis of threonine deaminase.

The observation that isoleucine alone can repress synthesis of threonine deaminase in A. suboxydans is in contrast to the observation of Freundlich et al. (8) who have shown that in mutants of Salmonella typhimurium valine, isoleucine and leucine are all essential to repress synthesis of threonine deaminase. Such a phenomenon has been called multivalent repression. Using wild type Salmonella typhimurium, Armstrong et al. (2) have observed no multivalent repression, however. Leavitt and Umbarger (21) have shown that each of the last four steps in valine and isoleucine synthesis is catalyzed by the same enzymes in E. coli and S. typhimurium. Excess of either valine or isoleucine will not affect synthesis of the enzymes of the other amino acids due to multivalent repression. Since enzymes of neither valine nor isoleucine pathway in A. suboxydans have been purified and studied, no explanation can as yet be offered as to why multivalent repression is not observed.

The inhibitory effect of valine can now be explained. Valine inhibits the activity of threonine deaminase, thereby affecting the biosynthesis of isoleucine. By limiting the synthesis of isoleucine, valine causes an apparent requirement for isoleucine. Addition of isoleucine to the synthetic medium containing excess valine, elevates the internal concentration of isoleucine, thereby reversing valine-inhibition.

No competition between valine and isoleucine appears to exist at the permease level (Figure 11), therefore, the transport of isoleucine is not inhibited by valine.

Genetic Experiment

Threonine deaminase of mutants of A. suboxydans which could grow in the presence of valine were tested for feed-back sensitivity toward valine and isoleucine. The result of such an experiment is shown in Table XVII. These mutants appear to produce an altered form of threonine deaminase, which was activated by both isoleucine and valine. Activation of the deaminase was greater by valine than by isoleucine.

Monod, Changeux and Jacob (24) have proposed that the binding of the feed-back inhibitor (allosteric effector) produces a reversible conformational alteration of the enzyme (allosteric transition). Such an alteration results in inhibition of enzymic activity. Such a mechanism is comparable to the "induced fit" theory of Koshland (18).

The results of threonine deaminase activation by valine and isoleucine obtained from mutants of A. suboxydans can be interpreted as follows:

1. The altered form of the deaminase has either lost the valine and isoleucine sensitive sites or the structure of the enzyme is altered in a manner so that valine and isoleucine no longer bind with the enzyme. If this is true, it is difficult to explain the stimulatory effect of the enzyme in the presence of the two amino acids.

2. Valine and isoleucine bind with the altered enzyme, and in doing so alter the conformational structure of the enzyme so that the affinity of the enzyme for the substrate is increased. This is in contrast to the explanation of Monod, Changeux and Jacob (24). The latter explanation appears to be more likely.

SUMMARY

Nutritional studies with Acetobacter suboxydans ATCC 621 indicated that valine was not essential for growth. This was in contrast to an identical study made by Stokes and Larsen (36). These authors had found that valine was essential for growth. Subsequent comparative study made on the two strains indicated that they may not be identical.

To substantiate the observation that valine was not essential cell-free extracts of the bacterium was examined for the enzymes leading to valine synthesis from 2 and 3 carbon precursors. Valine appears to be synthesized from pyruvate and acetaldehyde via acetolactate. The amounts of valine synthesized from different intermediates was also determined.

Isoleucine is also synthesized by the organism from threonine via acetohydroxybutyrate. The latter has been isolated from α-ketobutyrate and pyruvate using cell-free extracts. The amounts of isoleucine synthesized from α-ketobutyrate and pyruvate as also from acetohydroxybutyrate has been determined.

In order to determine whether valine would stimulate growth, different concentrations of valine were added to a synthetic medium containing histidine, glutamic acid, proline and ammonium sulfate. Valine appeared to inhibit growth; isoleucine when added, could reverse valine inhibition.

Valine and isoleucine competitively inhibit the activity of threonine deaminase, an enzyme obligatory for isoleucine synthesis

from threonine. Valine does not repress the synthesis of this enzyme. Heat treatment of threonine deaminase desensitizes the enzyme towards isoleucine but not valine. Only a single threonine deaminase appears to be present in the cell.

Mutants of A. suboxydans have been isolated; some of these possess a threonine deaminase which is no longer sensitive to valine or isoleucine inhibition.

Thus one mechanism by which valine inhibits growth is by inhibiting the activity of threonine deaminase thereby limiting the amount of isoleucine synthesis from its precursor. This is further substantiated by the observation that valine inhibition of growth can be reversed by isoleucine.

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APPENDIX

TABLE I

Effects of omission of single amino acids from a mixture of twenty amino acids on the growth of Acetobacter suboxydans

Omissions	Optical Density
None	0.52
Valine	0.22
Isoleucine	0.02
Histidine	0.29
Glutamic acid	0.19
Aspartic acid	0.19
Lysine	0.06
Proline	0.30
Methionine	0.06
Arginine	0.05
Leucine	0.15
Alanine	0.35
Glycine	0.51
Tyrosine	0.34
Threonine	0.10
Serine	0.09
Cysteine	0.21
Phenylalanine	0.19
Tryptophan	0.39
Hydroxyproline	0.35
Cystine	0.18

Cultures incubated at 30° for 72 hours with agitation.

TABLE II

Growth of Acetobacter suboxydans on single amino acids

	Optical density at 625 mμ
All twenty amino acids	0.22
Proline	0.10
Histidine	0.11
Glutamic acid	0.14
Valine	0.0
Isoleucine	0.0
α-ketoglutaric acid	0.11
Glutamine	0.12
Aspartic acid	0.02
Methionine	0.01
Ammonium sulfate	0.0

Incubated for 144 hours at 30° C with agitation. Optical density measured in a Colemen Junior spectrophotometer.

TABLE III

Comparative study on oxidation of different substrates by Acetobacter suboxydans (ATCC 621) and (MB-416)

Substrate	Amount µmoles	Oxygen uptake in ATCC 621 µliters	ATCC 621 µatoms	MB-416 µliters	MB-416 µatoms
Ethyl alcohol	10.6	205.92	18.38	205.67	18.38
Erythritol	10.0	106.50	9.50	118.15	10.50
Sorbitol	10.0	70.40	6.30	144.45	10.20
Citrate	10.0	0	0	0	0
Glucose	10.0	367.50	32.80	624.8	55.70
α-ketoglutarate	10.0	0	0	0	0
Glycerol	10.7	332.0	29.60	231.0	20.60

Incubation system: 10 mgs. lyophilized cells; 150 µmoles phosphate buffer pH 6.0; 10 µmoles Mg^{++}; 10% KOH (in center well) 0.1 ml.; substrate concentration as indicated, water to make 3 ml.

TABLE IV

Amount of valine synthesized from different intermediates by cell-free extract of *acetobacter suboxydans*

Substrates	Valine synthesized μmoles/mg. protein
Acetolactate	0.29
α,β-dihydroxyisovaleric acid	0.50
α-ketoisovaleric acid	0.68

0.5 ml. cell-free extract (protein 2.4 mgs.), 2 μmoles of substrate as indicated, pyridoxal phosphate 10 μgm., 10 μmoles glutamic acid, 10 μmoles Mg^{++}, 20 μmoles phosphate buffer pH 7.6, (acetolactate flask contained in addition to above, 0.2 μmoles NADPH). Incubated at 30° for 8 hours.

TABLE V

Specific activity of acetolactate reductoisomerase

Experimental	Specific activity expressed as △OD/mg. protein/minute
Complete system*	0.014
Complete system plus 10 μmoles valine	0.013

* Complete system: 100 μmoles phosphate buffer pH 7.6, 10 μmoles Mg^{++}, 1 μmole acetolactate, 0.1 μmole NADPH, 0.1 ml. cell-free extract (0.4 mgs. protein), water added to make total volume - 3 ml.

TABLE VI

Formation of acetoin in cell-free extracts of Acetobacter suboxydans

Experimental	µg. Acetoin formed
Complete system*	14.03
Complete system plus 10 µmoles valine	13.49

* Complete system: 0.1 ml. cell-free extract (1.4 mg. protein), 0.1 ml. water, 10 µmoles pyruvate, 0.5 ml. phosphate buffer, 30 µgms. TPP, 10 µmoles Mg^{++}. Incubate for 20 minutes at 37° C.

TABLE VII

Isoleucine synthesized from α-ketobutyrate and pyruvate and acetohydroxybutyrate in cell-free extracts of Acetobacter suboxydans

Experimental	μmoles isoleucine synthesized/ mg. protein
α-ketobutyrate and pyruvate	0.16
Acetohydroxybutyrate	0.15

System: Dialyzed cell-free extract 1 ml. (3.64 mg. protein), NADH 2 μmoles, CoASH and ATP - 1 μmole each, phosphate buffer pH 6.0, 10 μmoles Mg^{++}, 10 μmoles, alanine 10 μmoles, pyridoxal phosphate 10 μg. Substrate concentration 2 μmoles each. Incubated at 30° C. for 8 hours.

TABLE VIII

Activity of α-acetohydroxybutyrate reductoisomerase in cell-free extracts

Experimental	Specific activity △ O.D./mg. protein/minute
Complete system*	0.38
Complete system plus 10 μmoles valine	0.32

* Complete system: 2 μmoles α-acetohydroxybutyrate, 0.1 dialyzed cell free extract (0.48 mg. protein), 0.1 μmole NADH, 10 μmoles Mg^{++}, 40 μmoles phosphate buffer pH 6.0. Total volume 3 ml.

TABLE IX

Effects of valine and isoleucine
on growth of Acetobacter suboxydans

mgs. valine	mgs. isoleucine	O.D.
0.0	0.0	0.1
0.3	0.0	0.1
0.7	0.0	0.1
0.8	0.0	0.02
1.0	0.0	0.01
1.4	0.0	0.01
1.5	0.0	0.0
2.0	0.0	0.0
0.8	0.2	0.1
1.0	0.2	0.08
1.0	0.6	0.1
1.5	0.2	0.1
2.0	0.2	0.02
2.0	0.4	0.09
2.0	0.6	0.1

Composition of growth medium: Shamberger basal medium containing 2 mgs. each of histidine, proline and glutamic acid and 5 mgs. of $(NH_4)_2SO_4$. Total volume 5 ml. 84 hours shake culture incubated at 30° C.

TABLE X

Comparison of threonine deaminase estimations
by the two methods

Ammonia method	
	μmoles NH_3 liberated
Blank	0.93
Experimental I	3.43
Experimental I (duplicate)	3.12
Average: (Experimental minus blank)	
2.35 μmoles NH_3 liberated	
CO_2 Method	
	μmoles CO_2 liberated
Blank	1.80
Experimental I	4.70
Experimental I (duplicate)	4.5
Average: (Experimental minus blank)	
2.6 μmoles CO_2 liberated	

Experimental system: 10 μmoles threonine, 10 μmoles Mg^{++}, 10 μgm. pyridoxal phosphate, 1 μmole each of GSH and AMP, 1 ml. crude cell-free extract, 500 μmoles phosphate buffer pH 6.0. Blank did not contain any threonine. Incubation at 30° for 20 hours in the presence of 100 μg. Chloramphenicol.

TABLE XI

Oxidation of α-ketobutyrate by crude cell-free extracts of Acetobacter suboxydans

	μliters O_2 consumption	μliters CO_2 production
Complete system* (A)	101	165
(A) plus 10 μmoles valine	104	171
(A) plus 10 μmoles isoleucine	98	159
(A) plus 10 μmoles leucine	106	170

* Complete system: 10 μmoles α-ketobutyrate, 1 ml. cell-free extract (protein 2.8 mg ./ml.), phosphate buffer pH 6.0 (0.1M) 100 μmoles, Mg^{++} 10 μmoles, total volume 3 ml. Time of incubation 20 minutes at 30° C.

TABLE XII

Cofactor requirements of threonine deaminase

	Percent threonine deaminase activity
Complete system* (A)	100
minus Mg	44.5
minus AMP	1.2
minus GSH	16.0
minus pyridoxal phosphate	65.6
minus enzyme	0

* Complete system: 5 μmoles L-threonine, 10 μg. pyridoxal phosphate, 10 μmoles Mg^{++}, 1 μmole each of GSH and AMP, 100 μmoles phosphate buffer pH 6.0, 0.2 ml. 0-50 percent dialyzed $(NH_4)_2SO_4$ fraction. (Protein 2.8 mg./ml.).

TABLE XIII

Effect of valine, isoleucine and leucine on the activity of threonine deaminase in Acetobacter suboxydans

	Percent threonine deaminase activity
Complete system* (A)	100.0
(A) plus 10 μmoles L-valine	19.6
(A) plus 10 μmoles L-isoleucine	8.3
(A) plus 10 μmoles L-leucine	109.0

* Complete system: 1 μmole U-C^{14} threonine containing 1 μcurie, 100 μmoles phosphate buffer pH 6.0, 10 μmoles Mg^{++}, 1 μmole each of ATP and GSH, 10 μg. pyridoxal phosphate, 1 ml. cell-free extract (protein 2.8 mg./ml.). Final volume 3.0 ml.

TABLE XIV

Threonine deaminase activity
of Acetobacter suboxydans extracts

	Specific activity cpm/mg. protein/hour
Complete system* (A)	146.0
(A) plus 10 μmoles valine	71.4
(A) plus 10 μmoles isoleucine	68.4
(A) plus 10 μmoles valine and 10 μmoles isoleucine	62.8

* Complete system: 5 μmoles of 0-5 μc U-C^{14} threonine, 100 μmoles phosphate buffer pH 6.0, 10 μmoles Mg^{++}, 1 μmole each of AMP and GSH, 10 μg. pyridoxal phosphate, 0.5 ml. 0-50 percent $(NH_4)_2SO_4$ extract (2.6 mgs. protein).

TABLE XV

Effect of heat treatment on the sensitivity of threonine deaminase towards valine and isoleucine

	Specific activity cpm/mg./hr.
(A) Untreated extract	146.0
(B) treated 60° at 15 minutes	63.7
(B) plus 10 μmoles valine	25.5
(B) plus 10 μmoles isoleucine	69.5
(C) treated at 70° at 15 minutes	57.9
(C) plus 10 μmoles valine	30.7
(C) plus 10 μmoles isoleucine	41.7
(D) treated 80° C. at 15 minutes	0

System: 5 μmoles of 0.5 μc U-C^{14} threonine, 100 μmoles phosphate buffer pH 6.0, 10 μmoles Mg^{++}, 1 μmole each of AMP and GSH, 10 μg pyridoxal phosphate, 0.5 ml. 0-50 percent $(NH_4)_2SO_4$ extract (2.6 mgs. protein).

TABLE XVI

Repression of threonine deaminase by isoleucine in Acetobacter suboxydans

	Specific activity	
	threonine deaminase*	alcohol dehydrogenase**
1. Cells grown in the presence of isoleucine.	16.8	0.21
2. Cells grown in the presence of isoleucine as in (1) transferred into valine synthetic medium and aerated for 48 hours.	104.9	0.24
3. Cells grown in the presence of isoleucine as in (1) transferred into a synthetic medium without isoleucine or valine and aerated for 48 hours.	612.4	0.18
4. Cells grown in the presence of isoleucine as in (1) transferred to a synthetic medium containing isoleucine and aerated for 48 hours.	18.5	0.21
5. Cells grown in the absence of isoleucine as in (1) transferred	986.4	0.18
6. Cells grown in the absence of isoleucine as in (5) transferred to a valine synthetic medium and aerated for 48 hours.	1061.2	0.25
7. Cells grown in the absence of isoleucine as in (5) transferred to a synthetic medium containing isoleucine and aerated for 48 hours.	36.9	0.22
8. Cells grown in the absence of isoleucine as in (5) transferred to a synthetic medium containing no isoleucine and aerated for 48 hours.	1004.1	0.20

* cpm/mg. protein/hour of $C^{14}O_2$ recovered
** OD/mg. protein/min. measured at 340 mμ

Assay system for alcohol dehydrogenase: 5 μmoles alcohol, 100 μmoles phosphate buffer pH 7.0, crude cell-free extract 0.5 ml., 0.5 μmoles NAD^+, 10 μmoles Mg^{++}, total volume made up to 3 ml. with water.

TABLE XVII

Effect of valine and isoleucine
on threonine deaminase of mutants of Acetobacter suboxydans

Mutant Number	Specific activity of threonine deaminase*	Stimulation
I	1.37	x
I plus 10 μmoles valine	44.09	32.19x
I plus 10 μmoles isoleucine	30.96	22.6x
IV	74.5	y
IV plus 10 μmoles valine	445.0	5.97y
IV plus 10 μmoles isoleucine	271.0	3.64y
XV	1.89	z
XV plus 10 μmoles valine	57.20	30.26z
XV plus 10 μmoles isoleucine	37.21	19.6z

* Incubation system: 0.5 ml. of cell-free extract of mutant; 5 μmoles containing 0.5 μc U-C^{14} threonine, 1 μmole each of AMP and GSH, 10 μg. pyridoxal phosphate, 100 μmoles phosphate buffer pH 6.0, 10 μmoles Mg^{++}. Incubate at 30° C. for 8 hours. Specific activity expressed as cpm/mg. protein/hr.

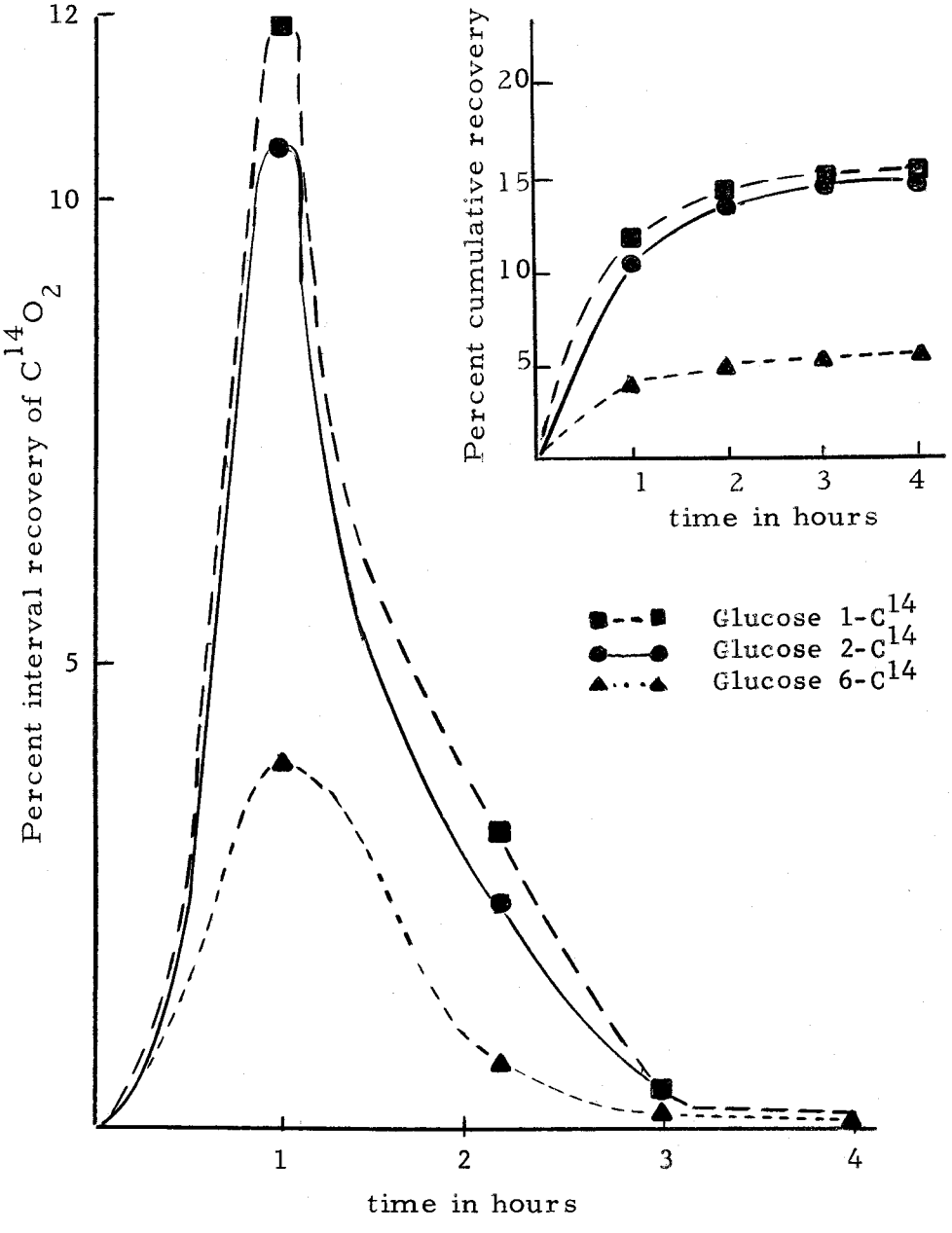

Figure 1. Time course study on the recovery of $C^{14}O_2$ produced from differently labelled glucose by Acetobacter suboxydans MB-416. Each vessel contained 0.1 μcurie specifically labelled glucose, 10 mg . carrier glucose, 10 μmoles Mg^{++} and 10 mg . lyophilized cells.

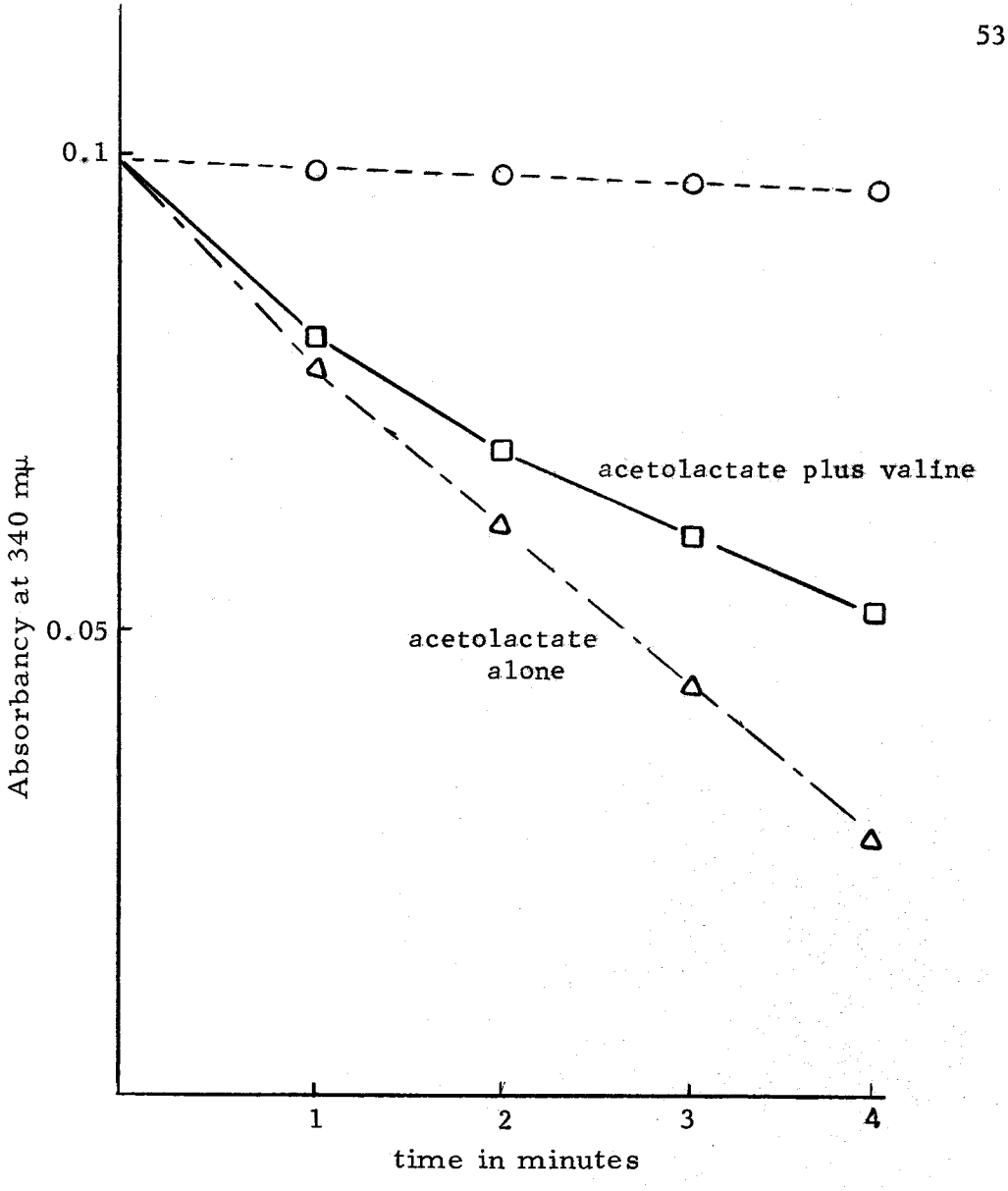

Figure 2. Oxidation of NADPH by cell-free extracts in the presence of acetolactate. Each system contained 100 μmoles phosphate buffer pH 7.6, 10 μmoles Mg^{++}, 0.1 μmole NADPH, 0.1 ml. cell-free extract (0.4 mg. protein) and water to 3 ml., plus where indicated 1 μmole acetolactate and 10 μmoles valine.

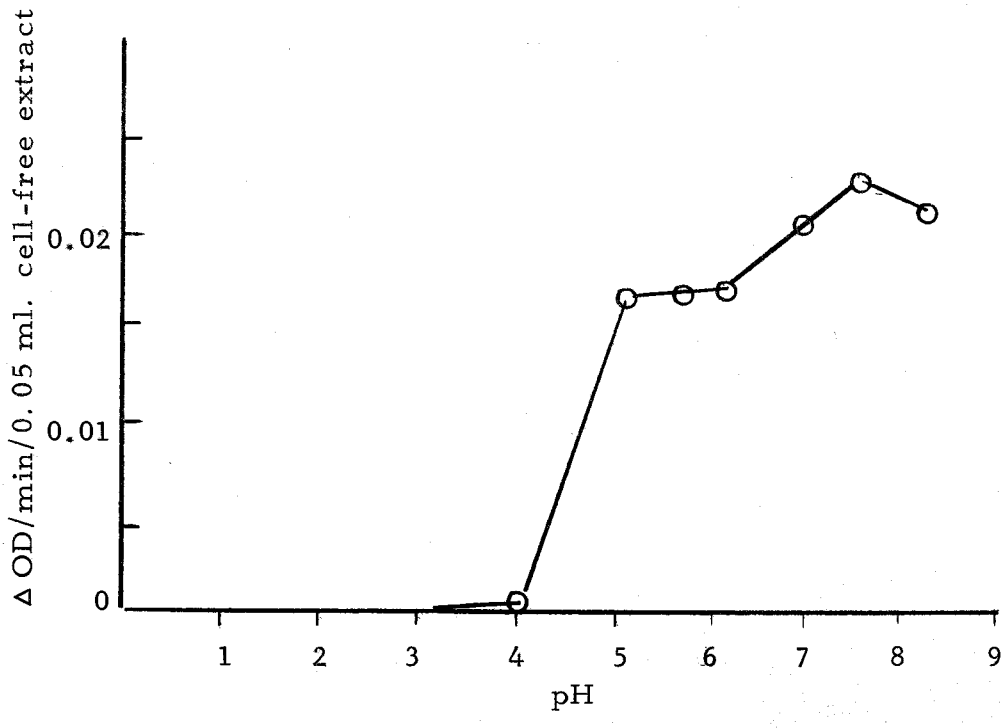

Figure 3. Effect of pH on the activity of acetolactate reductoisomerase. Each system contained 250 μmoles of phosphate buffer (pH as indicated), 0.05 ml. cell-free extract, 10 μmoles Mg^{++}, 0.1 μmole NADPH, 0.2 μmole acetolactate plus water to 3 ml.

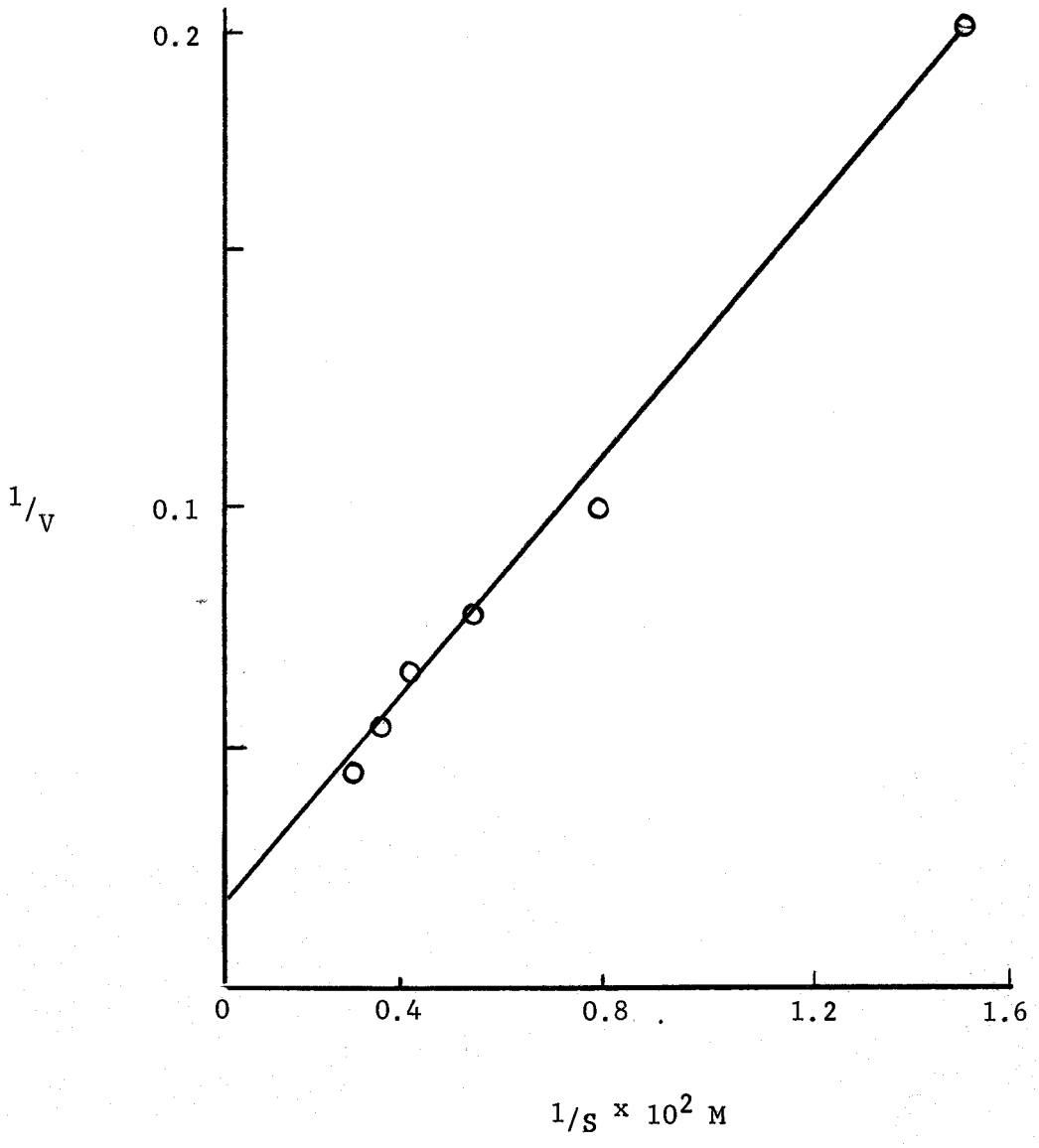

Figure 4. Lineweaver-Burk plot of acetolactate reductoisomerase.

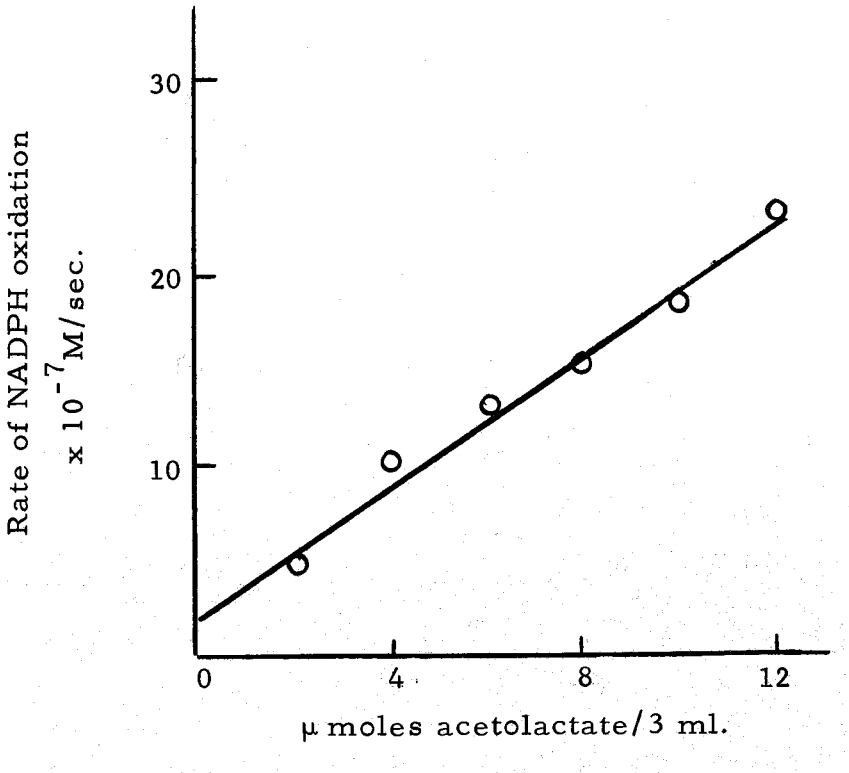

Figure 5. Rate of oxidation of NADPH at different concentrations of acetolactate using cell-free extract of Acetobacter suboxydans. Each system contained 250 μmoles of phosphate buffer pH 7.6, 10 μmoles Mg^{++}, 0.05 ml. cell-free extract (0.42 mg. protein), 0.1 μmole NADPH plus acetolactate as indicated. Total volume = 3 ml.

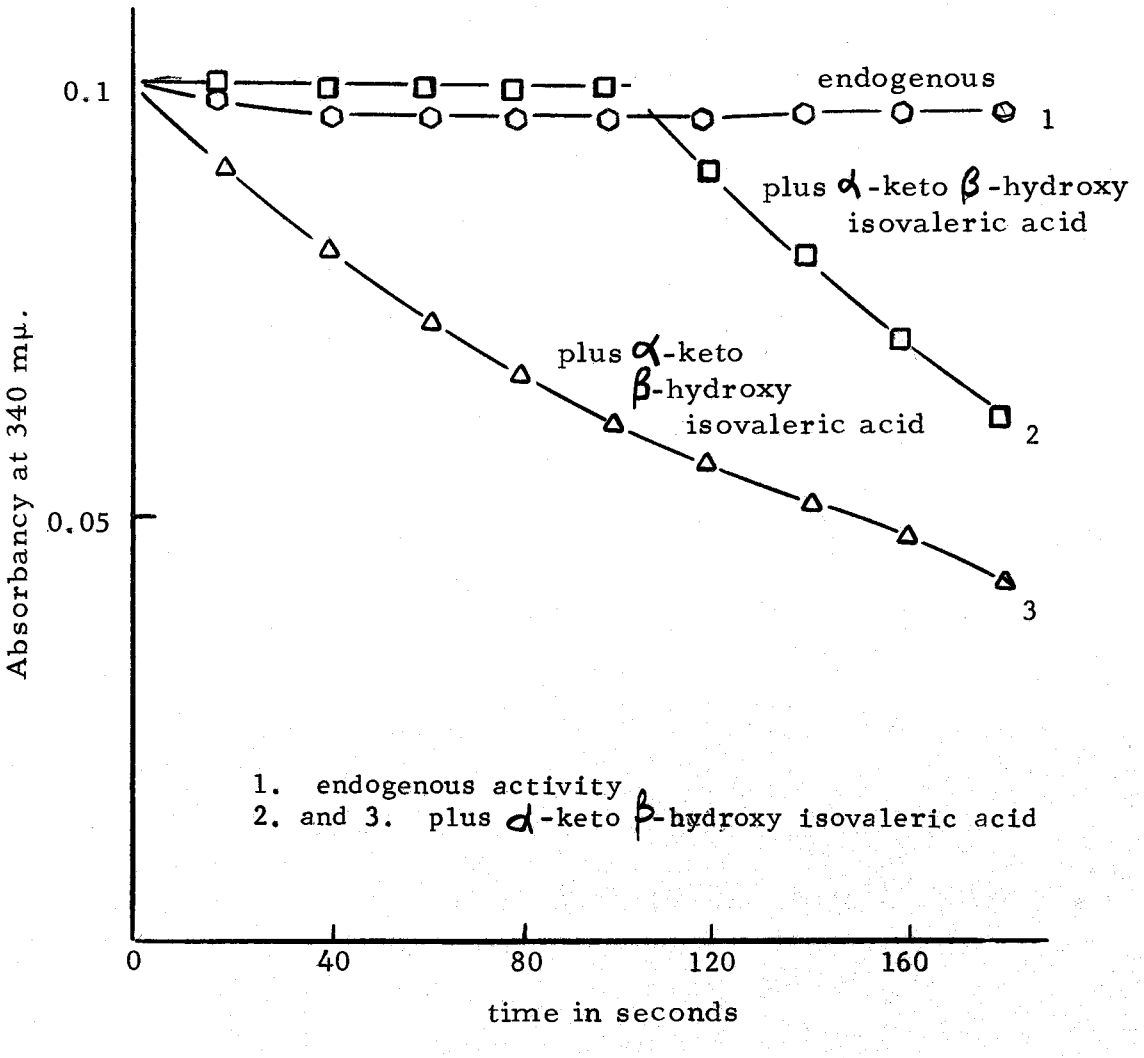

Figure 6. Oxidation of NADPH by cell-free extracts in the presence of α-keto β-hydroxy isovaleric acid. Each system contained 250 μmoles phosphate buffer pH 7.6, 0.1 ml. cell-free extract (0.64 mg. protein), 10 μmoles Mg^{++}, 0.2 μmole NADPH, plus where indicated 2 μmoles α-keto β-hydroxy isovaleric acid. System 3: cell-free extract preincubated with α-keto β-hydroxy isovaleric acid and other cofactors before addition of NADPH. System 2: No preincubation. System 1: endogenous NADPH oxidation.

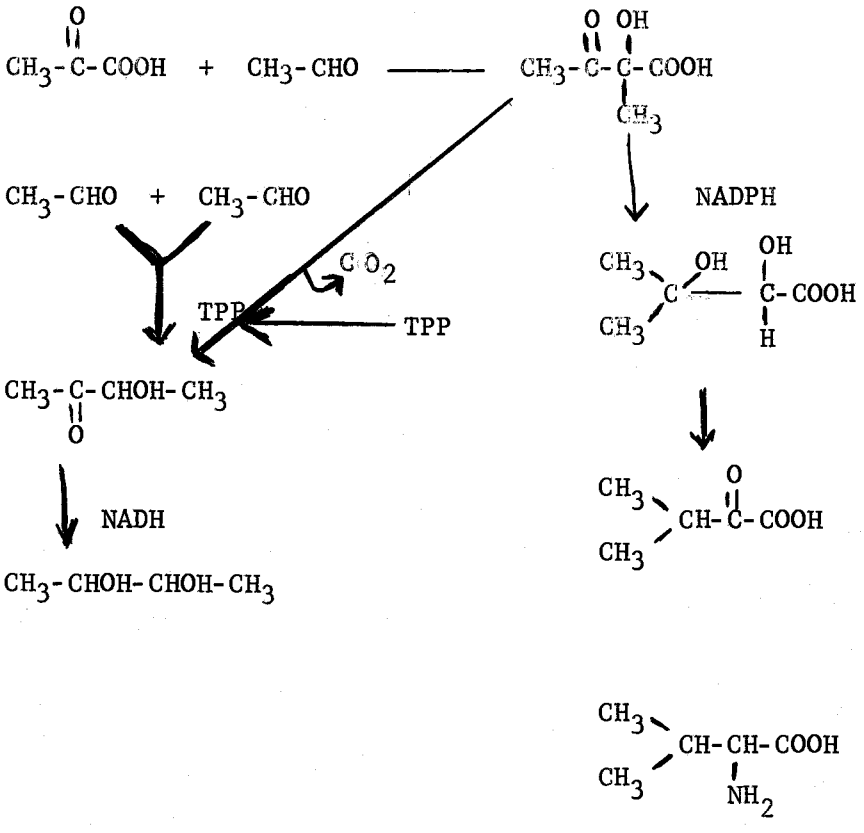

Figure 7. Suggested biosynthesis of valine in Acetobacter suboxydans.

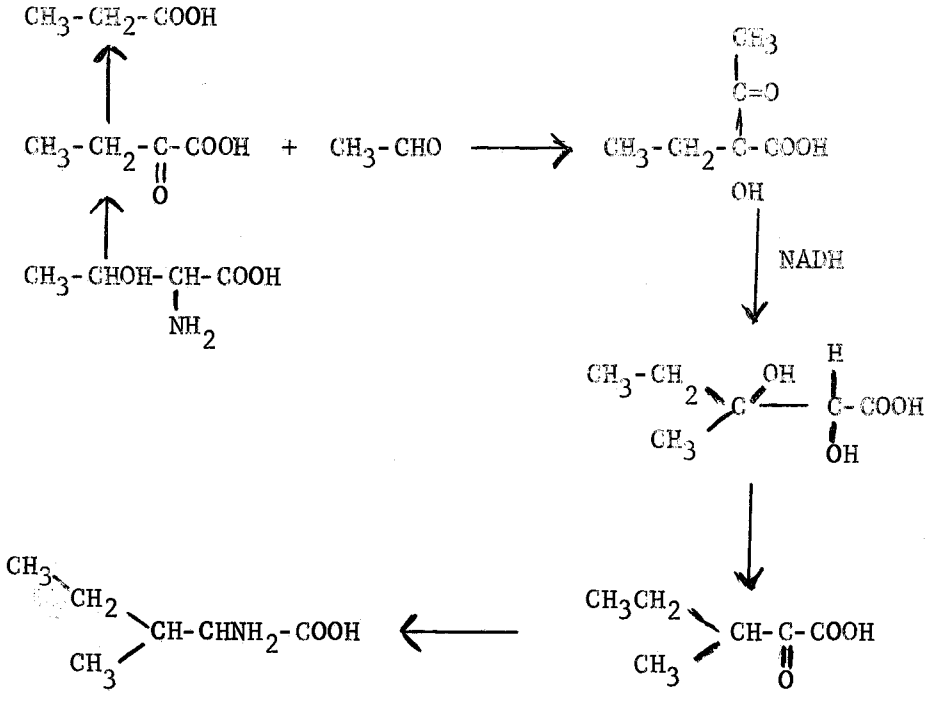

Figure 8. Suggested biosynthesis of isoleucine in Acetobacter suboxydans.

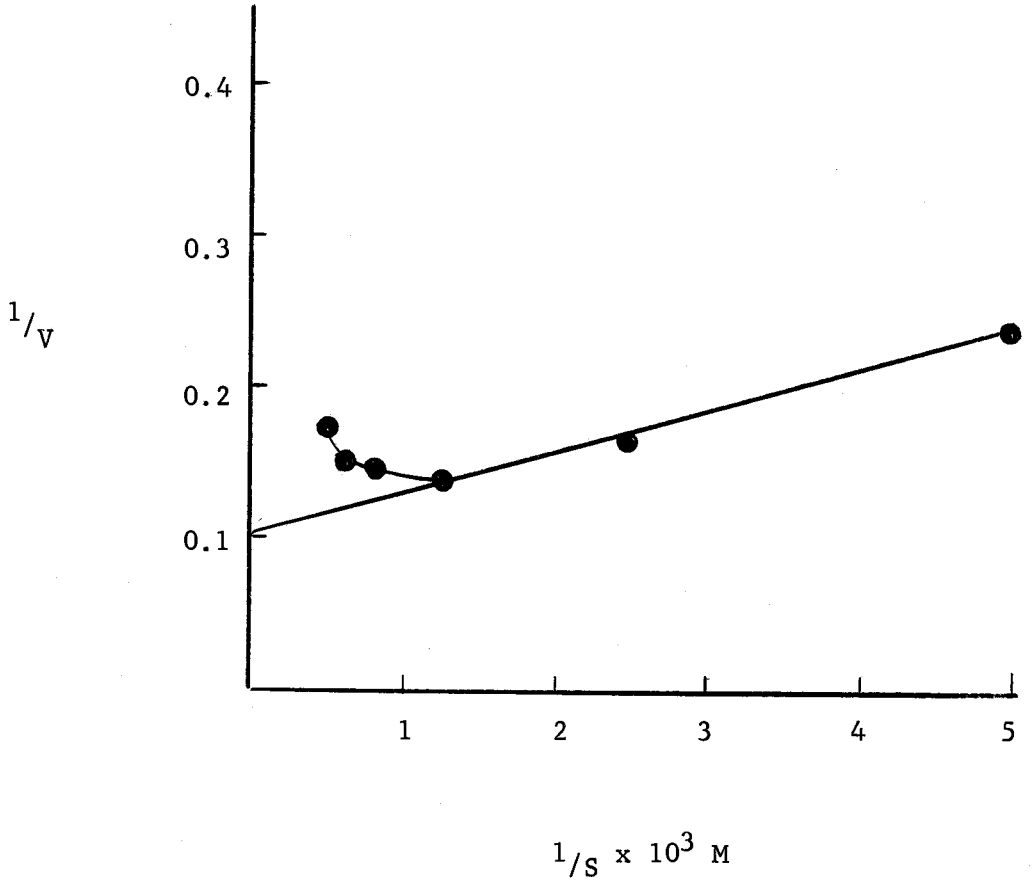

Figure 9. Lineweaver-Burk plot of threonine deaminase.

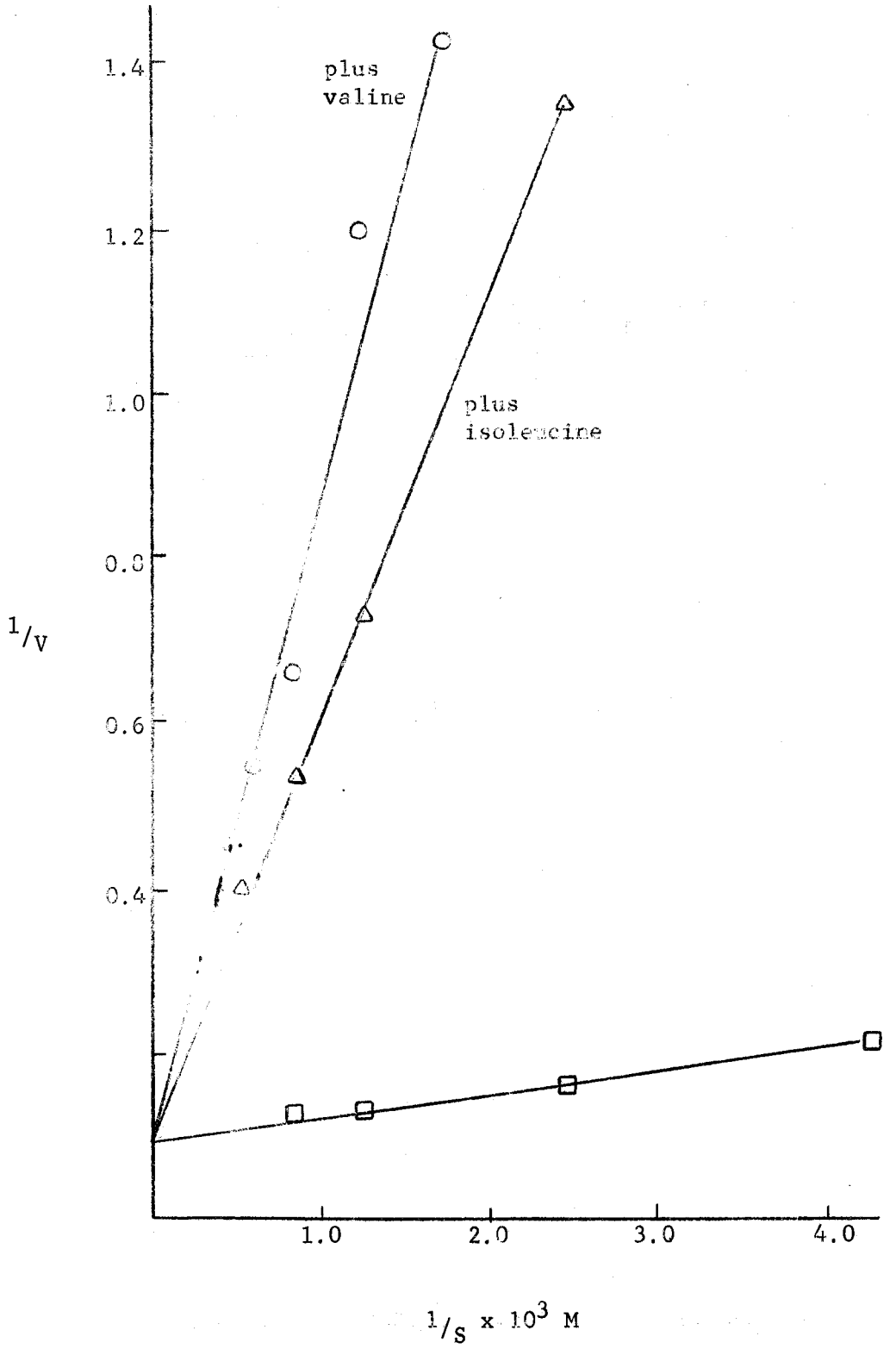

Figure 10. Lineweaver-Burk plot of threonine deaminase showing the nature of inhibition of the enzyme activity by valine and isoleucine.

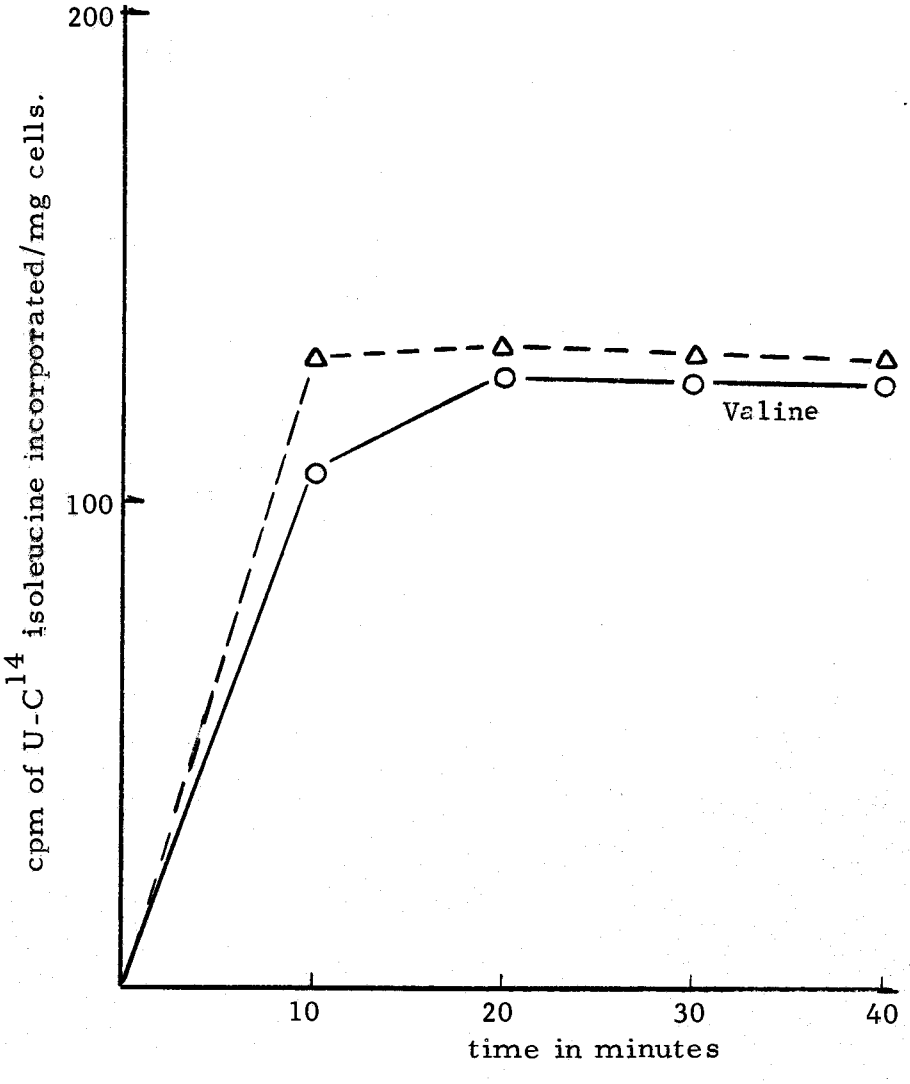

Figure 11. Effect of valine on the transport of isoleucine in cells of Acetobacter suboxydans. Each flask contained 2.5 mg. lyophilized cells, 50 mgs. glucose, 0.3 µmole isoleucine U-C^{14} (0.1 µc), 400 µmoles phosphate buffer pH 6.0, water to 5 ml., plus where indicated 0.3 µmole valine.