Studies in Valine Biosynthesis
VIII. Dihydroxyacid Dehydratase Activity in Microorganisms with Diverse Fermentation Patterns[1] (35563)

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The elucidation of the microbial pathways for amino acid biosynthesis in *Escherichia coli* and *Neurospora crassa* developed from the analysis of mutants, radioisotope studies, and then extensive enzymatic investigations (1). However, the intermediate biosynthetic steps in *E. coli* are now known to differ from the intermediates for the biogenesis of: serine in various biological systems (1); for lysine in higher fungi (2); for glutamate in *Acetobacter suboxydans* (3) and *Clostridium kluyveri* (4, 5); for alanine in *C. kluyveri* (6) and *C. pasteurianum* (7); and for cysteine in *Aspergillus nidulans* [cf. (8)]. With respect to valine, isoleucine, and leucine formation, *Ruminococcus flavefaciens* and several other rumen anaerobes are different from *E. coli* in their reliance on a reductive carboxylation of a branched chain fatty acid and amination to produce the corresponding amino acid [(9, 10) and earlier references cited therein]. Thus the *a priori* transfer of a biosynthetic pathway from one organism to another must be replaced by direct experimental inquiry to determine whether biological unity or diversity prevails.

To screen various biological systems for a specified pathway, investigators have used the incorporation of radioactive aspartate into lysine (2) as a marker of the routes of lysine biosynthesis. Similarly the demonstration of 5-dehydroquinase (11) and shikimic acid kinase (12) were initial clues of the path of biosynthesis of aromatic amino acids in a variety of microorganisms or plants. The presence of dihydroxyacid dehydratase (2, 3-dihydroxyacid hydrolyase, E.C.4.2.1.9.) was employed as one of several indicators of a specific sequence in valine–isoleucine biosynthesis with glucose-grown bacteria (13, 14), higher fungi (15), and higher plants (16, 17). The selection of the dehydratase was based on its unique substrates (13, 17) and its catalysis of the conversion of α,β-dihydroxyisovalerate to α-ketoisovalerate, and α,β-dihydroxy-β-methyl-*n*-valerate to α-keto-β-methyl-*n*-valerate, which are intermediates in valine and isoleucine formation. These overall pathways utilize pyruvate or α-ketobutyrate in a parallel series of steps mediated by four common enzymes (*i.e.*, acetohydroxy acid synthetase, isomeroreductase, dihydroxyacid dehydratase and aminotransferase) (1). The focus of this report is the identification of the dehydratase in microorganisms with a wide variety of fermentation patterns (18).

Materials and Methods. Source, growth, and rupture of microorganisms. Stock cultures of *Clostridium acetobutylicum*, ATCC 824, and *Clostridium butyricum*, ATCC 6015, were carried on fluid thioglycolate medium (Difco), and grown at 37° on defined media (19, 20) as modified later. The cells of *C. kluyveri*, ATCC 8527, were grown at 37° on the usual ethanol–acetate

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TABLE I. Dehydratase Activity of Various Heterotrophic Microorganisms.

Main type of fermentation	Specific organism	N source for growth (other than vitamins)	Dehydratase specific activity (units/mg of protein)
Hexose diphosphate pathways (Embden-Meyerhof glycolytic system)			
Ethanolic fermentation	*Saccharomyces cerevisiae*	$(NH_4)_2SO_4$	0.36[a]
	Brewer's yeast	$(NH_4)_2SO_4$	0.16[a]
	Torulopsis utilis	$(NH_4)_2SO_4$	0.47[a]
Butyric acid and solvent-producing fermentations	*Clostridium butyricum*	L-Glu	1.51
	Clostridium acetobutylicum	L-Glu + L-Asn	1.68
Mixed acid fermentation	*Escherichia coli*	$(NH_4)_2SO_4$	0.68[a]
	Proteus morganii	$(NH_4)_2SO_4$ + Met + Cys	5.64[a]
	Arthrobacter globiformis	NH_4Cl	1.86
	Arthrobacter JG-9	NH_4Cl	1.26
	Penicillium chrysogenum	NH_4 acetate	0.19[a]
Butylene glycol fermentation	*Aerobacter aerogenes*	$(NH_4)_2SO_4$	1.83[a]
	Serratia marcescens	$(NH_4)_2SO_4$	0.17[a]
	Bacillus subtilis	$(NH_4)_2SO_4$	0.43[a]
	Staphylococcus aureus	17 amino acids	2.60[a]
Hexose monophosphate pathway (Warburg-Dickens-Horecker pathway)			
Via pentose cycle	*Acetobacter suboxydans*	L-Glu	0.14[a]
	Acetobacter melanogenum	$(NH_4)_2SO_4$	0.21[a]
Via 3–2 cleavage of pentose—P	(heterolactic fermentation)		
	Streptococcus bovis	$(NH_4)_2SO_4$	0.64
Via 2-keto-3-deoxy-6-P-gluconate	(Entner-Doudoroff pathway)		
	Pseudomonas fluorescens	NH_4NO_3	0.85
	Pseudomonas aeruginosa	$(NH_4)_2SO_4$	0.78[a]
Multiple pathways of hexose utilization			
Propionate fermentation	*Propionibacterium shermanii*	L-Glu	1.72
	Propionibacterium arabinosum	L-Glu	0.54
	Propionibacterium pentosaceum	$(NH_4)_2SO_4$	0.37
Organic acid fermentation			
Ethanol–acetate fermentation	*Clostridium kluyveri*	$(NH_4)_2SO_4$	0.57
Formate assimilation	*Pseudomonas* AM 1	$(NH_4)_2SO_4$	0.26
Oxalate assimilation	*Pseudomonas oxalaticus*	$(NH_4)_2SO_4$	0.58

[a] Earlier reported detection of the dehydratase in cell-free extracts (13, 14).

medium (21). To obtain sufficient cells for enzyme assays, these microorganisms were grown in 1-liter Erlenmeyer flasks, or filled, glass-stoppered bottles, harvested by centrifugation at a turbidity reading that was earlier shown to be in the exponential phase, and then ruptured. Stock cultures of *Propionibacterium shermanii*, ATCC 9614, *Propionibacterium arabinosum*, ATCC 4965, and *Propionibacterium pentosaceum,* ATCC 4875, were maintained on tomato juice agar and grown at 30° under stationary, semi-anaerobic conditions on a defined glucose–salts–vitamin medium (22) with the main nitrogen source indicated in Table I and harvested in the exponential phase. The sonication time (Branson Sonifier, model S-125, Branson Instruments, Inc., Danbury, Conn.) for optimal release of the dehydratase was shown to be 10 min for each of the three *Clostridia* and *P. pentosaceum* and 5 min for the *P. shermanii* and *P. arabinosum.*

The culture of *Streptococcus bovis*, ATCC 9809, was maintained and grown semianaerobically on media with $(NH_4)_2SO_4$ as the nitrogen source (23). Since most lactic acid bacteria have amino acid requirements, *S. bovis* is unique in its ability to grow on ammonium salts. Duplicate or more batches of cells for each of the above seven organisms were harvested, ruptured, and assayed to give the averages reported in Table I.

The cell-free extract of *Pseudomonas AM 1*, grown on methanol as the sole carbon source, was a gift by Drs. J. R. Quayle and Patricia A. Johnson, Dept. of Biochemistry, University of Oxford, Oxford, England (24). The strain of *Pseudomonas oxalaticus* (OX 1), also a gift from Dr. Quayle, was grown on their earlier described medium (25). Extracts of *Arthrobacter globiforms* and *Arthrobacter JG-9* were a gift of Dr. Bruce F. Burnham, Dept. of Biochemistry, University of Oxford, and were grown as earlier described by Morris (26).

Enzyme assays. The assay system for dihydroxyacid dehydratase contained: 0.10 *M* Tris–HCl buffer, pH 8.0, 0.02 *M* $MgCl_2$, 0.02 *M* DL-α,β-dihydroxyisovalerate and 0.05–0.20 ml of a cell-free extract, added last, in a final volume of 1.0 ml. After incubation for 30 min in a 37° water bath, the reaction was terminated with trichloroacetic acid and analyzed for keto acid by the dinitrophenylhydrazone color reaction. The details of synthesis of substrate (13), keto acid measurement (14), and determination of protein concentration (13) are described in earlier reports. The specific activity of the dihydroxyacid dehydratase are reported, as before, in micromoles of keto acid enzymatically formed per 30 min per mg of protein.

The enzyme, isomeroreductase (also known as reductoisomerase), catalyzes the conversion of α-acetolactate to α,β-dihydroxyisovalerate (27, 28). The simultaneous oxidation of NADPH (Sigma Chemical Co., St. Louis, Mo.) was followed in a Beckman DU-2 spectrophotometer at 25°. The components of the assay followed that described earlier (27). DL-α-Acetolactate was obtained by the alkaline hydrolysis of methylacetoxyetylacetolactate (a gift from Dr. Elliot Juni, Emory University, Altanta, Ga.) at room temperature for 40 min with two equivalents of NaOH.

Thin-layer chromatography. The keto acid unknowns and standards were treated with 2,4-dinitrophenylhydrazine (29) in the dark for 1 to 3 hr until the hydrazone precipitated. The oxaloacetate derivative took several days to sediment. The hydrazones were centrifuged, washed with cold water, recentrifuged, dissolved in 2.0 ml of 95% ethanol, and spotted on 8 × 8-in. glass plates coated with silica gel G (Research Specialties Co., Richmond, Calif.). The TLC plates were prepared by spreading a smooth 30% (w/v) aqueous slurry of silica gel G with a glass rod to a standard thickness (about 0.25 mm, controlled by a layer of masking tape at the edges of the plate), dried in air, activated for 1 hr at 100° and stored in a desiccator until used. The solvent systems used for development are indicated in Table II (29). The keto acid standards were purchased from California Corp. for Biochemical Research, Los Angeles, Calif. Glyoxylic acid was synthesized by the periodate oxidation of tartaric acid (30).

Results. Elucidation of the various classical patterns of glucose utilization through the hexose diphosphate path, the hexose monophosphate steps or both has developed from the experiments of many investigators (*cf.* review, 18). Rearrangement of our previous dehydratase data from a morphological classification to one based on fermentation pathways (18) indicated some gaps and led to the search in the new groups in Table I. Dehydratase activity was found in members of the *Clostridia*, *Arthrobacteria*, a *Streptococcus*, *Propionibacteria*, and *Pseudomonads* and their respective types of fermention. With the known differences in media composition, it is not possible to decipher whether the range of values in Table I was due to nutritional, genetic, or regulatory factors *Pseudomonas fluorescens*, ATCC 11250, was grown on an inorganic salts medium, harvested in the log phase and assayed; the resulting specific activity in Table I is 56-fold higher than that earlier reported with a commercial source of cells

TABLE II. Identification of Product of Dehydratase Reaction by Thin-Layer Chromatography.

	Solvent systems for development		
Solvent system	Isoamyl alcohol–0.25 N NH_4OH (20:1)	n-Butanol–ethanol–0.5 N IH_4OH (7:1:2)	Ethyl acetate–benzene–ethanol–acetic acid (12:8:3:1)
Time for development (hr):	5.0	3.0	0.75
	R_s values with α-ketoisovalerate at 1.00		
Source of enzyme for formation of unknown keto acid(s)			
C. acetobutylicum extract grown on glutamate	0.96	1.00	0.98
C. butyricum extract grown on glutamate	0.96	0.98	0.99
C. kluyveri extract grown on $(NH_4)_2SO_4$	1.00	0.98	1.01
P. shermanii extract grown on glutamate	0.98	1.03	1.01
P. arabinosum extract grown on glutamate	1.01	1.05	1.01
Standards			
α-Ketoisovaleric acid (R_s)	1.00	1.00	1.00
α-Ketoisovaleric acid (R_f)	0.46	0.78	0.89
α-Ketoisocaproic acid	0.47, 1.05	0.85, 1.04	1.01, 1.08
Glyoxylic acid	0.34, 0.59	0.70, 0.89	0.82, 0.92
Pyruvic acid	0.23, 0.62	0.68, 0.88	0.90, 1.01
α-Ketobutyric acid	0.28, 0.66	0.73, 0.91	0.96, 1.06
α-Ketoglutaric acid	0.08	0.28	0.66
Oxaloacetic acid	0.00	0.26	0.34
Dinitrophenylhydrazine•HCl	2.04	1.12	1.12
Dinitrophenylhydrazine as free base in ethanol solution	1.85	1.09	1.12

(13). *Pseudomonas AM 1* was grown on methanol, which is fixed into cell substance by the route of formaldehyde, formate, and serine (24). In *Pseudomonas oxalaticus*, oxalate is coupled with coenzyme A and then reduced to glyoxylate (25) which, in turn, leads to the formation of tartronic semialdehyde and glycerate. Extracts from each of these new *Pseudomonad* cultures possessed dehydratase activity (Table I). Within certain limitations to be stated in the Discussion section, these enzymatic clues are consistent with the presence of the *E. coli* pathway of valine biosynthesis in the above taxonomic groups, but also for microorganisms with the newly studied fermentations in Table I.

Some related nutritional observations should be briefly mentioned. *C. acetobutylicum* has been earlier grown on an inorganic salts medium with glucose, $(NH_4)_2SO_4$, asparagine, biotin, and *p*-aminobenzoic acid (19). Since this medium did not support growth in our laboratory, the addition of glutamate (1.12 g of amino N/liter) was found to give consistent growth and then confirmation of the vitamin requirements. Similarly, *C. butyricum*, which has been grown on an $(NH_4)_2SO_4$ plus biotin medium (20), grew far better upon supplementation with glutamate. Extracts of *C. kluyveri* grown on ethanol–acetate (21) had a 95-fold higher specific activity than the earlier assay (13) with an extract from commercial lyophilized cells. *S. bovis* is the only reported number of this species which

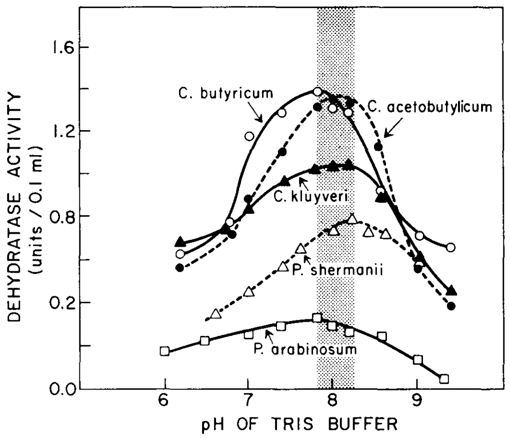

FIG. 1. Optimal pH of the dihydroxyacid dehydratase from some heterotrophic bacteria.

can use $(NH_4)_2SO_4$ as the sole nitrogen source for growth [(23) and earlier cited references]. The vitamin needs of the *Propionibacteria* were earlier demonstrated to be biotin and pantothenic acid [(22) and their earlier citations]. After confirming these vitamin requirements, the substitution of glutamate (0.70 g of amino N/liter) for the casein acid hydrolyzate in this basal medium was found to support growth. *P. pentosaceum* differed from the other two in its ability to grow on $(NH_4)_2SO_4$ as the major nitrogen source.

Several additional observations were made with these extracts. The optimal pH for the dehydratase activity in *C. butyricum, C. acetobutylicum,* and *C. kluyveri* were 7.8, 8.0, and 8.0, respectively (Fig. 1). The dehydratase of *P. shermanii* and *P. arabinosum* had an optimal pH of 7.8–8.2 (Fig. 1). This peak is similar to that previously observed for *E. coli, N. crassa, S. cerevisiae, Phaseolus radiatus,* and *Spinacea oleracea* (1, 17).

Thin-layer chromatography was used to identify the nature of the keto acid product for several of the above extracts (Table II). Five times the usual volume for each component in the dehydratase assay were incubated for 1 hr at 37°. After terminating the reaction with 2 ml of 10% trichloroacetic acid, the protein precipitate was removed by centrifugation. The yellow dinitrophenylhydrazone derivatives were prepared (29), dissolved in 95% ethanol, spotted on silica gel G thin-layer chromatography plates, and developed in the stated solvent systems (Table II). Two spots appeared for α-ketoisocaproate, α-ketobutyrate, pyruvate, and glyoxylate in all three solvent systems. These spots are probably the *syn* and *anti* forms of the dinitrophenylhydrazones, the *syn* forms being predominant (31, 32). For the α-ketoisovalerate standard and each of the five enzymatically produced keto acids, only one spot was found. Since the R_f (or R_s) values are the same in three solvent systems, the unknown keto acid was identified as α-ketoisovalerate.

Another enzyme in the valine–isoleucine biosynthetic pathway, α-acetohydroxyacid isomeroreductase (also called reductoisomerase), catalyzes an alkyl migration in and a NADPH-dependent reduction of α-acetolactate to α,β-dihydroxyisovalerate (28). This enzyme was demonstrated in *P. shermanii* and *P. arabinosum* (using the same extracts as in Table I) and had an average sp act of 0.67 and 0.46 μmoles of NADPH oxidized/min/mg of protein, respectively.

Discussion. As mentioned in the Introduction, two microbial pathways for the biosynthesis of serine, lysine, alanine, cysteine, valine, isoleucine, and leucine, and three for glutamate are presently known. Table I shows that many of the microorganisms with these reported differences in other pathways have the dihydroxyacid dehydratase, an indicator of valine–isoleucine biosynthesis. The data in Table I extends the taxonomic distribution of the dehydratase beyond that reported previously (13–15), but more importantly, demonstrates the dehydratase in microorganisms with many types of fermentation (cf. 18), which have not been hitherto studied for valine–isoleucine biosynthesis. The implication of these results is that each of the microbes has the *E. coli–N. crassa* pathway of valine–isoleucine biosynthesis. The limitations, supporting evidence, and the significance of these observations are briefly presented below.

The initial experimental evidence, whether from mutant, radioisotope, or enzyme investigations, does not necessarily establish a specific pathway in a microorganism. When deductions from several areas of evidence coincide, the degree of certainty for a specific route is greatly increased. Therefore, the presence of the dehydratase is necessary, but not sufficient, evidence to establish the *E. coli–N. crassa* pathway of valine biosynthesis in the named microorganisms. Without other evidence, these findings do not identify the nature of a possible alternative pathway, nor exclude the possibility of the simultaneous presence of two or more pathways. These restrictions do not apply for those organisms with multiple lines of evidence, namely *E. coli, N. crassa, Aerobacter aerogenes, Salmonella typhimurium, Saccharomyces cerevisiae,* and *Torulopsis utilis*, which will not be discussed further here [cf. (1)]. With regard to the less extensively studied organisms, our earlier dehydratase results (Table I) are now supplemented by subsequent enzymatic and/or mutant evidence for *Penicillium chrysogenum* (33), *Serratia marcescens* (34), *Bacillus subtilis* (35), *Staphylococcus aureus* (36), *Acetobacter suboxydans* (37), and *Pseudomonas aeruginosa* (38, 39). Thus, the combination of the present and the cited evidence eliminates the possibility of alternative pathways in these more fully studied organisms.

Where the present enzymatic data was the sole evidence of a pathway for many microorganisms (Table I), the possibility of other biosynthetic pathways within the same organism cannot be rigorously excluded. Such a possibility is probably remote, since, with the exception of serine (1) and glutamate (3), each organism has usually only one pathway for an amino acid (1, 5–9). For instance, the diaminopimelic acid pathway for lysine formation in bacteria is not found in molds, which have the aminoadipic acid pathway, and vice versa (2).

The biosynthesis of amino acids in strict anaerobes requires special comment. Earlier experiments on ^{14}C-acetate incorporation into the six amino acids in *C. kluyveri* gave a labeling pattern other than that anticipated from the tricarboxylic acid cycle (4), and suggested a condensation of acetate with CO_2. Such a CO_2 fixation reaction has been substantiated with knowledge for the ferredoxin-mediated reductive synthesis of pyruvate from CO_2 and acetyl phosphate with extracts of *Clostridium pasteurianum* (7), or from CO_2 and acetyl CoA by *C. kluyveri* (6). This pyruvate is apparently utilized for valine biosynthesis, since the dehydratase was demonstrated in three *Clostridia* (Table I). The dehydratase has also been found in photoorganotrophs (40), which are similar to *C. kluyveri* in having a C_1 plus C_2 condensation. With respect to another strict anaerobe, *Ruminococcus flavefaciens,* extracts which were prepared by Dr. Milton J. Allison, National Animal Disease Laboratory, Ames, Iowa, and mailed to us in the frozen state, were devoid of dehydratase activity. Reports from this group (9, 10) indicate a biosynthesis by a reductive carboxylation of isobutyrate and 2-methylbutyrate and then amination to yield valine and isoleucine, respectively. Thus, this and several other rumen bacteria are the sole known exceptions for an otherwise widespread taxonomic and biochemical distribution of the *E. coli* pathway of valine–isoleucine biosynthesis.

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