Acetolactate Metabolism and the Presence of a Dihydroxy Acid Dehydratase in Micro-organisms

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1. The growth characteristics of nine micro-organisms on complex broth and defined media, usually with a single nitrogen source (other than vitamins), were examined as a necessary step before growth of cells for enzyme assays. Six of these bacteria gave a positive colour test with a creatine-potassium hydroxide reagent, indicating the presence of acetoin, which other investigators have shown is formed via the intermediate, α -acetolactate. 2. Cell-free extracts of exponential-phase cells of *Bacillus subtilis*, *Staphylococcus aureus*, *Proteus morganii*, *Acetobacter rancens* (two strains), *A. kuetszingianus*, *A. acetosus*, *Acetomonas* (*Acetobacter*) *melanogenus* and *Acetomonas* (*Acetobacter*) *suboxydans* (A.T.C.C. no. 621) were found to contain the enzyme, dihydroxy acid dehydratase (2,3-dihydroxy acid hydro-lyase). 3. The specific activity of the dehydratase from organisms grown on valine- and isoleucine-deficient media was greater than those grown on a complex broth or media containing complete amino acid mixtures. The omission of valine plus isoleucine from a medium containing 19 amino acids caused an increase in the dehydratase specific activity of *Staphylococcus aureus* and *Proteus morganii*. 4. The rate of keto acid formation from $\alpha\beta$ -dihydroxyisovalerate by extracts of six of the above-named organisms was faster than, but somewhat proportional to, the similar rate from $\alpha\beta$ -dihydroxy- β -methyl-*n*-valerate as substrate. 5. These findings may be related to acetolactate synthesis, acetoin formation and valine-isoleucine biosynthesis in the above-mentioned micro-organisms.

Acetoin (acetylmethylcarbinol) is recognized as the microbial end-product responsible for a positive Voges-Proskauer test (see review by Wood, 1961). Although there are several mechanisms of acetoin formation, the formation and subsequent decarboxylation of α -acetolactate is the pathway in *Aerobacter aerogenes* and other bacteria with a butanediol type fermentation (Juni, 1952*a,b*). Radioisotope-distribution patterns in *Torulopsis utilis* led to the suggestion that α -acetolactate also was an intermediate in valine biosynthesis (Strassman, Thomas & Weinhouse, 1955). Confirmation of this function was provided by direct study of the acetolactate-forming enzyme in *Escherichia coli* (Umberger & Brown, 1958; Radhakrishnan & Snell, 1960), *Neurospora crassa* (Radhakrishnan & Snell, 1960) and *Saccharomyces cerevisiae* (Lewis & Weinhouse, 1958) and by other evidence (see review by Umberger & Davis, 1962).

After the initial formation of acetolactate, the other steps in valine biosynthesis are catalysed by

a α -hydroxy- β -oxo acid reductoisomerase (see Kanamori & Wixom, 1963, for recent references), a dihydroxy acid dehydratase (EC 4.2.1.9; 2,3-dihydroxy acid hydro-lyase; formerly called dihydroxy acid dehydrase) and an aminotransferase. The dehydratase catalyses the dehydration of $\alpha\beta$ -dihydroxyisovaleric acid to α -oxoisovaleric acid, and $\alpha\beta$ -dihydroxy- β -methyl-*n*-valeric acid to its keto analogue. This enzyme has been demonstrated in extracts of *E. coli* and *N. crassa* (Myers & Adelberg, 1954), *S. cerevisiae* (Wixom, Shatton & Strassman, 1960), other micro-organisms (Wixom, Wikman & Howell, 1961) and higher plants (Wixom & Hudson, 1961; Kanamori & Wixom, 1963). To provide information on the pathway of valine biosynthesis in as yet untested micro-organisms, the presence of the dehydratase has now been sought in several Voges-Proskauer-positive bacteria. Brief reports have been presented (Wixom, 1962, 1963).

MATERIALS AND METHODS

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Test organisms and maintenance. *Bacillus subtilis*, Marburg type, N.C.T.C. no. 3610, was maintained on slopes of

Oxoid nutrient agar (Oxo Ltd., London) and transferred monthly or as required. The stock cultures of *Staphylococcus aureus*, strain Oxford, N.C.T.C. no. 6571, and *Proteus morganii* (a laboratory strain) were maintained on Oxoid nutrient agar slopes, incubated overnight and subcultured monthly. The strain of *P. morganii* used by Kobayashi & Kalnitsky (1954) is no longer available. All cultures of *B. subtilis* and *S. aureus* were incubated at 37°, and *P. morganii* at 30°. The slopes were stored at 4°.

The sources of the *Acetobacter* and *Acetomonas* cultures are listed in Table 1. Equivalent culture numbers are listed to relate present results with previous nutritional and biochemical studies (Rainbow & Mitson, 1953; Brown & Rainbow, 1956; De Ley, 1959). *Acetobacter suboxydans*, N.C.I.B. no. 6430, is now regarded as *Acetobacter rancens* (Cooksey & Rainbow, 1962; Carr & Shimwell, 1961). *Acetobacter rancens*, NRRL-B-65, is the probable predecessor of A.T.C.C. no. 7839, N.C.I.B. no. 8148 and N.C.I.B. no. 7214 (W. C. Haynes, U.S. Dept. of Agriculture, Peoria, Ill., U.S.A.; personal communication). Although listed in the N.C.I.B. catalogue as equivalent strains, evidently *Acetobacter suboxydans*, A.T.C.C. no. 621, is not identical with N.C.I.B. no. 7069 (Cooksey & Rainbow, 1962). The generic classification of the acetic acid bacteria follows the recommendations of Carr & Shimwell (1961), although other relationships have been proposed (De Ley, 1961). These bacteria were subcultured at monthly intervals on glucose-CaCO₃-yeast-extract slopes (De Ley, 1959) and incubated for 2-3 days. All incubations of the acetic acid bacteria were performed at 30°.

Preparation of inocula. A small loopful of surface growth from a slope was transferred to a test tube containing the simplest defined medium to be described for each organism and incubated at the specified temperature. The log-phase cells of *B. subtilis* and *P. morganii* were then centrifuged, resuspended in the same volume of sterile water and diluted to approx. 1 µg. dry wt. of organisms/ml. The resultant suspension (0.1 ml.) was used to inoculate each 6.0 ml. of medium in the growth experiments. Proportionally larger inocula were used for the growth in the Erlenmeyer flasks.

The inoculum of *S. aureus* requires special comment, as ordinarily this organism is considered to have multiple amino acid requirements. A loopful of organisms was inoculated into a defined medium containing a complete

mixture of amino acids. After growth became visible (by 30-48 hr.), 0.1 ml. of the washed suspension was inoculated into a medium deficient in valine and isoleucine and re-incubated. Growth was again visible by 48 hr. The organisms were centrifuged, and diluted to give the usual suspension containing about 1 µg. dry wt. of organisms/ml.

For the acetic acid bacteria, a loopful of surface growth from a slope was inoculated into 5.0 ml. of medium containing casein hydrolysate, glucose and lactate (described below). After 18-24 hr. incubation with rotation at 30°, 0.1 ml. of the suspension was transferred to 5.0 ml. of a medium containing (NH₄)₂SO₄ and lactate for the *Acetobacter*, or to a medium containing (NH₄)₂SO₄, glucose and lactate for the *Acetomonas*. After reincubation for 40-48 hr., the log-phase organisms were centrifuged, and resuspended in 5.0 ml. of sterile water. The suspension was diluted to approx. 50 µg. dry wt. of organisms/ml. (Klett colorimeter reading 10). The resultant suspension (0.1 ml.) was used to inoculate the test tubes in the determination of the generation time. Eighty times this inoculum size, or, in several cases, larger ratios, were used to inoculate the 400 ml. of media in Erlenmeyer flasks for preparation of cells for enzyme assay.

Media for microbial growth. In general each organism was grown on both a complex medium and one or more chemically defined media. For all defined media, sterile glucose was added separately after autoclaving at 115° for 10 min. *B. subtilis* was grown on nutrient broth (Oxoid) and a glucose-(NH₄)₂SO₄-salts medium (Davis & Mingioli, 1950). The complex medium for *S. aureus* was nutrient broth (Oxoid). The defined medium contained 19 crystalline amino acids (White, 1959), which were essentially in the proportions used by Gladstone (1937), plus glucose, salts and vitamins as described by Lascelles & Woods (1952). Omission of DL-valine (150 mg./l.) and DL-isoleucine (180 mg./l.) provided the mixture containing 17 amino acids referred to below.

P. morganii was grown on the peptone-yeast extract-beef extract-salts medium used by Kobayashi & Kalnitsky (1954) in their studies on acetolactate formation. The defined medium, based on an earlier medium of Pelczar & Porter (1941) as modified by D. D. Woods & C. Mulder (personal communication), contained: KH₂PO₄, 4.5 g./l.; (NH₄)₂SO₄, 0.5 g./l.; NH₄Cl, 0.5 g./l.; FeSO₄·7H₂O, 0.011 g./l.; MgSO₄·7H₂O, 0.040 g./l.; L-cysteine, 60 µM; DL-methionine,

Table 1. *Strains of acetic acid bacteria used*

The source of each organism is indicated by an asterisk.

Name under which organism was received	A.T.C.C. no.	N.C.I.B. no.	Name used in this paper
<i>Acetobacter suboxydans</i> (Kluyver & Leeuw)	—	6430*	<i>Acetobacter rancens</i>
<i>Acetobacter rancens</i> (Beijerinck)	7839*	8148	<i>Acetobacter rancens</i>
<i>Acetobacter kuetzingianus</i> (Hansen) Bergey <i>et al.</i>	6439*	3294	<i>Acetobacter kuetzingianus</i>
<i>Acetobacter acetosus</i> (Henneberg) Bergey <i>et al.</i>	6438*	2224	<i>Acetobacter acetosus</i>
<i>Acetobacter melanogenus</i> (Beijerinck) (M.S. 6.1)	9937*	8084	<i>Acetomonas melanogenus</i>
<i>Acetobacter suboxydans</i> (Kluyver & Leeuw)	621*		<i>Acetomonas suboxydans</i>

0.5 mM; nicotinic acid, 10 μ M; pantothenic acid, 10 μ M. The solution was adjusted to pH 7.4 and sterilized at 115° for 10 min. Sterile glucose (10.0 g./l.) was added separately after autoclaving. For certain experiments the earlier described mixtures of 19 or 17 amino acids were substituted for the above cysteine and methionine.

The basic medium for the acetic acid bacteria contained (g./l. final vol.): glucose, 20.0; lactate, 10.0; vitamin-free casein acid hydrolysate, 6.0; $(\text{NH}_4)_2\text{SO}_4$, 1.0; salts and vitamins (Rainbow & Mitson, 1953; Brown & Rainbow, 1956; De Ley, 1959); Tween 80 (polyethylene sorbitan monoleate; L. Light and Co. Ltd., Colnbrook, Bucks.), 0.10, to minimize cell agglutination. Variation was made by deletion of glucose, casein hydrolysate or both as described by Rainbow & Mitson (1953). Where casein hydrolysate was omitted, the concentration of $(\text{NH}_4)_2\text{SO}_4$ was increased to 3.0 g./l.

Growth experiments and the determination of generation time. Washed suspensions of each organism were used to prepare a graph relating turbidity to dry wt. (16 hr. at 80°). Turbidity measurements were made with the EEL photoelectric colorimeter (Evans Electroselenium Ltd., Halstead, Essex; neutral density filter) or Klett colorimeter (Klett Manufacturing Co., New York, N.Y., U.S.A.). The growth experiments in Table 2 were conducted in duplicate in 6.0 ml. of medium in optically matched 15 cm. $\times$ 18 cm. $\times$ 1.5 cm. diam. inverted T-shaped tubes, which were clamped in a rocking device and shaken in a water bath at 37° at 36 oscillations/min. of 10 cm. excursion. After inoculation, EEL or Klett readings were taken at intervals and converted into units of mg. dry wt. of organisms/ml. Plotting mass against time on semilogarithmic paper gave a straight line during the exponential phase of growth, and allowed calculation of the generation time.

For the acetic acid bacteria, the growth experiments in Table 5 were performed in duplicate 1.5 cm. $\times$ 12.6 cm. test tubes containing 5.0 ml. of medium and rotated on a tissue-culture rotator at 40 rev./min. at a 15° slope in a constant-temperature oven at 30°. The reported generation times are averages of two or more separate experiments.

Preparation of cell-free extracts. Erlenmeyer flasks (1 l.) containing the specified medium (400 ml.) were inoculated and incubated at the earlier stated temperature on a rotary shaker (model G-25, New Brunswick Scientific Co., New Brunswick, N.J., U.S.A.). The organisms were harvested before leaving the exponential phase by centrifuging at 3000 g for 15 min. and washed. Suspensions of *P. morganii* containing 75–100 mg. dry wt. of organisms/ml. of 0.1 M-tris buffer, pH 7.4, were subjected to ultrasonic vibration for 4 min. (25 kcyc./sec., 600 w; ultrasonic generator type E7590B, Mullard Ltd., London, W.C. 1) in a stainless-steel flask cooled by ice-water, and then centrifuged for 15 min. at 18000 g at 3°. *S. aureus*, which was refractory to ultrasonic treatment, was ruptured by three passages of a similar suspension in 0.10 M-tris buffer, pH 7.4, through a French pressure cell at 10000 lb./in.² pressure drop at 3° (American Instrument Co. Inc., Silver Spring, Md., U.S.A.) and then centrifuged. For *B. subtilis* slightly higher dehydratase specific activities were obtained by lysozyme action than by 5 min. ultrasonic treatment or rupture in a French pressure cell. The lytic digestion was performed by adding 0.1 ml. of lysozyme solution (10 mg./ml.; Armour Pharmaceuticals Ltd., Eastbourne, Sussex) per ml. of a suspension containing about 50 mg.

dry wt. of organisms/ml. of 0.1 M-tris buffer, pH 7.4. After incubation for 30 min. at 37°, the suspension was centrifuged for 30 min. at 100000 g. The various *Acetobacter* and *Acetomonas* organisms were ruptured in a 250 w, 10 kcyc. ultrasonic oscillator (Raytheon Manufacturing Co., Waltham, Mass., U.S.A.) for 10 min. at 2–3°. This 10 min. treatment for *A. rancens* and *As. suboxydans* yielded higher specific activity than 5, 20, 30 and 40 min. ultrasonic treatments. Since the keto acid concentration in the various extracts was negligible, a dialysis step was omitted. The various supernatant fluids were stored at –20° for up to 5 days until assayed for enzyme and protein concentration. The percentage loss of initial activity per week at such storage at –20° was an average of 10% for *A. rancens* and 4% for *As. suboxydans*.

Analytical methods. The Voges-Proskauer test for acetoin was performed with the concentrated KOH-creatin reagent (Committee on Bacteriological Technic, Society of American Bacteriologists, 1957). The determination of protein concentration, the synthesis of substrates and criteria of their purity, the assay of the dihydroxy acid dehydratase and the source of the α -oxoisovalerate standard have been described by Wixom *et al.* (1961). The dehydratase assay was based on the determination of the keto acid product by the direct method (addition of dinitrophenylhydrazine and NaOH and reading the extinction at 510 m μ ; Friedemann & Haugen, 1943). Controls without enzyme or substrate were always included in the assay and the values of apparent keto acid (always very small) subtracted from the total keto acid. The dehydratase-assay results are expressed in units of μ moles of keto acid formed/30 min. and as specific activity (units/mg. of protein).

RESULTS

Generation time of micro-organisms. Each organism in Table 2 was grown aerobically in several media at optimum temperature in duplicate tubes. Where the media for the inoculum and growth

Table 2. *Effect of growth media on the generation time of micro-organisms*

Details of the various media and growth conditions are described in the Materials and Methods section.

Medium ...	Generation time (hr.)			
	Valine and isoleucine present		Valine and isoleucine absent	
	Complex broth	Mixture of 19 amino acids	NH ₄ ⁺ ion as main N source	Mixture of 17 amino acids
Organism				
<i>Bacillus subtilis</i>	0.6	—	1.1	—
<i>Staphylococcus aureus</i>	0.6	0.6	—	0.6
<i>Proteus morganii</i>	1.2	1.2	2.1*	1.2

* Methionine plus cysteine also present.

Table 3. *Effect of growth media on the specific activity of the microbial dehydratase*

Nature of the media, growth conditions and methods of rupture are outlined in the Materials and Methods section. For the dehydratase assay, cell-free extracts from exponential-phase cells were incubated in triplicate with 0.10 M-tris-HCl buffer, pH 7.4, 0.02 M-MgCl₂ and 0.02 M-DL- α -dihydroxyisovalerate, pH 7.4, in a final volume of 1.0 ml. for 30 min. at 37°. The range of protein used (0.1–2.9 mg./tube) was varied widely to obtain reliable rates of keto acid formation. After terminating the reaction with trichloroacetic acid and removing protein by centrifugation, a sample was analysed for keto acid concentration.

Medium... Organism	Sp. activity (μ moles of keto acid/30 min./mg. of protein)			
	Valine and isoleucine present		Valine and isoleucine absent	
	Complex broth	Mixture of 19 amino acids	NH ₄ ⁺ ion as main N source	Mixture of 17 amino acids
<i>Bacillus subtilis</i>	0.07	—	0.43	—
<i>Staphylococcus aureus</i>	0.04	0.99	—	2.60
<i>Proteus morganii</i>	0.23	2.96	5.64*	6.73

* Methionine plus cysteine also present.

experiment differed, the transfer was always from a simple medium to a more complex medium. The generation time for *B. subtilis* was greater in a medium devoid of amino acids than in the broth containing preformed amino acids. The strain of *S. aureus* selected from the valine- and isoleucine-deficient inoculum grew at the same rate in nutrient broth, the modified Gladstone (1937) medium containing 19 amino acids, and the same medium without valine and isoleucine.

Growth of *P. morganii* (Table 2) on a defined medium containing L-cysteine, DL-methionine and ammonium sulphate gave a longer generation time than growth on broth. However, substitution of the modified Gladstone mixture of 19 amino acids, or the same mixture without valine and isoleucine in the above simple medium, restored the growth rate to that of the broth. Thus, on the basis of nutritional requirements for growth, valine and isoleucine are non-essential amino acids for these three organisms.

Dehydratase activity of microbial extracts. With *B. subtilis* (Table 3), cell-free extracts of organisms grown on the simple glucose-ammonium sulphate-salts medium had a sixfold higher dehydratase specific activity than those from organisms grown on a complex broth. Extracts of *S. aureus* from the nutrient-broth culture had a very low specific activity. Extracts from organisms grown on the modified Gladstone medium in the presence of 0.67 mM-valine and 0.67 mM-isoleucine had 38% of the specific activity of similar preparations from organisms grown in the absence of these two amino acids. For *P. morganii* the specific activity of the dehydratase from the simple defined medium was 13 times those from broth cultures, i.e. the medium used by Kobayashi & Kalnitsky (1954) for their study of the acetolactate-forming enzyme. Substitution of a

mixture of 17 amino acids for the cystine and methionine in the simple defined medium slightly increased the activity. Like *S. aureus*, *P. morganii* also showed a depression of dehydratase specific activity (44%) when valine and isoleucine were added to the medium containing 17 amino acids.

Voges-Proskauer test. A pink colour in the qualitative acetoin test was observed with *B. subtilis* and *S. aureus*, but not with *P. morganii*, after incubation in the standard medium (Committee on Bacteriological Technic, Society of American Bacteriologists, 1957). The addition of the creatine-potassium hydroxide reagent to cultures (Table 5) at the end of the growth showed a consistent positive pink colour for the four *Acetobacter* species grown on lactate-ammonium sulphate as the sole carbon and nitrogen source, confirming the findings of De Ley (1959). Acetoin was found in several other media, but not for the two *Acetomonas* strains.

Generation time of acetic acid bacteria. Faster and earlier growth was usually observed with the glucose plus lactate medium than the lactate medium for the *Acetobacter* (Table 4). As observed by others, the two *Acetomonas* strains failed to grow during 7 days of incubation with lactate as the sole carbon source. Faster growth was always observed with casein hydrolysate than ammonium salts on either energy source, but more importantly, each organism grew on media devoid of preformed amino acids. The set of growth curves with *Acetobacter rancens*, shown in Fig. 1, is typical for all of the *Acetobacter* with regard to the sequence for initiation of growth and the magnitude of maximum growth. With ammonium sulphate as the nitrogen source, all organisms attained maximum growth before 1 week of incubation under the stated conditions. At the stationary phase of growth, the glucose plus lactate medium

Table 4. *Effect of growth media on the generation time of acetic acid bacteria*

Each organism was grown on media containing glucose plus lactate or lactate only with the two nitrogen sources in duplicate test tubes at 30° with aeration by continuous rotation. Growth was followed turbidimetrically. Results shown are averages from at least two separate experiments.

Medium	Carbon sources	Generation time (hr.)		Ratio (a/b)
		Casein hydrolysate (a)	(NH ₄) ₂ SO ₄ (b)	
<i>A. rancens</i> , N.C.I.B. no. 6430	Glc + Lac	2.8	3.1	0.90
	Lac	2.9	3.8	0.76
<i>A. rancens</i> , N.C.I.B. no. 8148	Glc + Lac	1.7	4.4	0.39
	Lac	2.8	5.2	0.54
<i>A. kuetzingianus</i>	Glc + Lac	3.2	5.5	0.58
	Lac	3.7	5.2	0.71
<i>A. acetosus</i>	Glc + Lac	2.9	3.7	0.78
	Lac	3.5	4.2	0.83
<i>As. melanogenus</i>	Glc + Lac	2.8	8.5	0.33
	Lac	neg.	neg.	—
<i>As. suboxydans</i>	Glc + Lac	2.5	4.2	0.60
	Lac	neg.	neg.	—

Fig. 1. Growth curves of *Acetobacter rancens*, N.C.I.B. no. 6430. The inoculum was 50 μ g. dry wt. of exponential-phase cells grown on medium containing lactate and (NH₄)₂SO₄. The carbon and nitrogen sources of the four media were: glucose, lactate and casein hydrolysate, 3.1 hr. (●); glucose, lactate and (NH₄)₂SO₄, 3.4 hr. (○); lactate and casein hydrolysate, 3.7 hr. (▲); lactate and (NH₄)₂SO₄, 4.1 hr. (△).

was acidic (pH 4.0–5.2) and the lactate medium was alkaline (pH 6.8–8.2). The adequacy of the lactate–ammonium sulphate medium was verified by inoculating *A. rancens* in the usual manner through six successive serial transfers without alteration of the growth patterns.

In contrast with earlier reports (Stokes & Larsen, 1945), *Acetomonas* (*Acetobacter*) *suboxydans* (A.T.C.C. no. 621) grew with ammonium sulphate as the sole nitrogen source (other than vitamins).

Growth on ammonium sulphate commenced later and proceeded at a slower rate than growth with preformed amino acids, but eventually attained a maximum growth that was 80–90% of the maximum turbidity with casein hydrolysate as the nitrogen source. These characteristics were unchanged during six successive serial transfers of *As. suboxydans* on the glucose–lactate–ammonium sulphate medium. However the maximum turbidity of *As. suboxydans* on these two media was one-third to one-fourth that of the *Acetobacter* (Fig. 1). Substitution of L-glutamate (2.22 g./l.) for casein hydrolysate in the medium gave a lower generation time of 2.1 hr. but did not change the maximum turbidity. Improved growth was also obtained with a frequently used glycerol–yeast extract–phosphate medium (King & Cheldelin, 1954) (generation time 1.8 hr. and a maximum turbidity comparable with that in Fig. 1).

Dehydratase activity of extracts of acetic acid bacteria. The specific activity of the dehydratase from cells grown without preformed amino acids was 1.2 to 5.2 times as great as that from cells from a casein hydrolysate medium (Table 5). In addition to the two figures in Table 5, an average specific activity of 0.149 unit/mg. of protein was obtained for *As. suboxydans* grown on the earlier described L-glutamate medium. Similar dehydratase assays for cells grown on glycerol–yeast extract–phosphate medium (King & Cheldelin, 1954) gave an average of 0.133 unit/mg. of protein.

Control mechanism(s) of valine and isoleucine biosynthesis. The data in Table 3 indicate that the dehydratase concentration was decreased when cells of *S. aureus* and *P. morganii* are grown with valine

Table 5. *Effect of growth media on the specific activity of dehydratase in acetic acid bacteria*

Exponential-phase cells from the indicated media were harvested, ruptured by ultrasonic oscillation, centrifuged and assayed for the dihydroxy acid dehydratase. The amount of protein used in the assay varied with the activity of the extract but was usually in the range 0.5–3.0 mg. of protein/tube. Results are averages from at least two separate flasks.

Medium ... Organism	Carbon source	Sp. activity (μ mole of keto acid/30 min./mg. of protein)		Ratio of activities
		Casein hydrolysate	(NH ₄) ₂ SO ₄	
<i>A. rancens</i> , N.C.I.B. no. 6430	Glc + Lac	0.088	0.178	2.0
<i>A. rancens</i> N.C.I.B. no. 8148	Lac	0.115	0.269	2.3
<i>A. kuetzingianus</i>	Lac	0.432	0.526	1.2
<i>A. acetosus</i>	Lac	0.139	0.244	1.8
<i>As. melanogenus</i>	Lac	0.042	0.221	5.2
<i>As. suboxydans</i>	Glc + Lac	0.077	0.205	2.7
	Glc + Lac	0.070	0.139	2.0

Table 6. *Substrate specificity of microbial dehydratase*

Bacterial extracts were incubated with either 0.02 M-DL- α -dihydroxyisovalerate or 0.02 M-DL- α -dihydroxy- β -methyl-*n*-valerate for the substrate as described in Table 2. Results are expressed as rate of product formation.

Substrate ... Organism	Main N source in media	Rate of keto acid formation (μ moles/30 min.)		Ratio of activities
		Dihydroxy-isovalerate	Dihydroxy-methylvalerate	
<i>Bacillus subtilis</i>	(NH ₄) ₂ SO ₄	0.34	0.20	1.7
<i>Staphylococcus aureus</i>	17 amino acids	1.15	0.55	2.1
<i>Proteus morganii</i>	Complex broth	0.63	0.29	2.2
<i>Proteus morganii</i>	(NH ₄) ₂ SO ₄ + Met + CySH	1.07	0.48	2.2
<i>Proteus morganii</i>	19 amino acids	0.58	0.27	2.1
<i>Proteus morganii</i>	17 amino acids	0.81	0.38	2.1
<i>Acetobacter rancens</i>	(NH ₄) ₂ SO ₄	0.55	0.36	1.5
<i>Acetomonas melanogenus</i>	(NH ₄) ₂ SO ₄	0.44	0.26	1.6
<i>Acetomonas suboxydans</i>	(NH ₄) ₂ SO ₄	0.64	0.26	2.4
<i>Acetomonas suboxydans</i>	Glutamate	0.38	0.18	2.1

and isoleucine in the respective media. Other experiments, not shown, indicated that the addition *in vitro* of equimolar concentrations (0.02 M) of valine and isoleucine did not alter the rate of product formation from dihydroxyisovalerate by extracts of *S. aureus* (grown on 17 amino acids), *P. morganii* (grown on 17 amino acids) and *A. rancens* (grown on ammonium salts.) Thus the amino acid end products did not inhibit the activity of the dehydratase, but did decrease the formation of this enzyme in valine biosynthesis in the above organisms.

Substrate specificity of enzyme preparations. Some of the more potent extracts were tested with both the valine intermediate, α -dihydroxyisovalerate, and the isoleucine intermediate, α -dihydroxy- β -methyl-*n*-valerate (Table 6). Both compounds served as substrates. Furthermore, a relatively

constant ratio (1.5–2.4) of the rate of formation of the product from each substrate was found. This ratio has been previously observed with other crude extracts and purified spinach dehydratase preparations (Kanamori & Wixom, 1963; Myers, 1961).

DISCUSSION

Scheme 1 illustrates the position of acetolactate as the branching point for acetoin formation or valine synthesis. In either case acetolactate synthesis proceeds via the intermediate of α -hydroxyethyl thiamine pyrophosphate (Holzer, Fonseca-Wollheim, Kohlhaw & Woenckhaus, 1962; Kram-pitz, Suzuki & Greull, 1962). Furthermore micro-organisms may be placed in one of three groups depending on their nutritional requirements and

metabolic products, which in turn is related to their constitution of acetolactate decarboxylase and dihydroxy acid dehydratase (Table 7). The dehydratase was observed primarily in organisms that can grow on ammonia plus vitamins as the sole

nitrogen source and does not always parallel the distribution of a positive acetoin test. An acetolactate-forming enzyme has been found in all three groups. Since direct assay of the reductoisomerase in crude extracts may be vitiated by competition

Scheme 1. Metabolic paths of α -acetolactate or its homologue in the third group of micro-organisms (Table 7). When $R = CH_3$, acetoin and valine are the end products. If $R = 3CH_2 \cdot CH_3$, then acetylethylcarbinol and isoleucine may be formed. The α -hydroxy- β -oxo acid-forming enzyme(s) has also been called acetolactate-forming enzyme and has a multiple nature in *Aerobacter aerogenes*. OxoGlu, 2-Oxoglutarate; TPP, thiamine pyrophosphate.

Table 7. Relation between nutritional requirements, acetoin formation and the presence of several enzymes in micro-organisms

+, Positive characteristic or presence of the specified enzyme; -, negative observation. Numbers in parentheses refer to pertinent references: (1) Juni (1952a,b); (2) Umbarger & Brown (1958); (3) Lewis & Weinhouse (1958); (4) Myers & Adelberg (1954); (5) Wixom *et al.* (1960); (6) Wixom *et al.* (1961); (7) Kobayashi & Kalnitsky (1954); (8) Dolin & Gunsalus (1951); (9) Moat & Lichstein (1953); (10) Halpern & Umbarger (1959); (11) Wagner, Berquist & Forrest (1959); (12) Radhakrishnan & Snell (1960); (13) De Ley (1959).

Organism	Growth on NH_4^+ ion as main N source	Voges-Proskauer test	Acetolactate decarboxylase	Acetolactate-forming enzyme(s)	Dihydroxy acid dehydratase
Group 1					
<i>Escherichia coli</i>	+	-	-(1)	+(2,3)	+(4)
<i>Saccharomyces cerevisiae</i>	+	+	-(1)	+(3)*	+(5)
<i>Micrococcus lysodeikticus</i>	+	-	-(1)		+(6)
<i>Proteus morganii</i>	-†	-	-(7)	+(7)	+
<i>Acetomonas</i> strains -2	+†	-			+
Group 2					
<i>Streptococcus faecalis</i>	-	+	+(8)	+(8)	-(6)
<i>Lactobacillus arabinosus</i>	-	+	+(9)	+(9)	-(6)
Group 3					
<i>Aerobacter aerogenes</i>	+	+	+(1)	+(1,10)	+(6)
<i>Serratia marcescens</i>	+	+	+(1)	+(1)	+(6)
<i>Bacillus subtilis</i>	+	+	+(1)	+(1)	+
<i>Staphylococcus aureus</i>	-†	+	+(1)	+(1)	+
<i>Neurospora crassa</i>	+	+	+(11,12)	+(12)	+(4)
<i>Acetobacter</i> strains -4	+	+	+(13)	+(13)	+

* See Discussion section.

† See special comment in text regarding growth on valine- and isoleucine-deficient medium.

with acetolactate decarboxylase for their mutual substrate, measurement of the dehydratase is a more reliable indication of the route of valine formation in these Voges-Proskauer-positive organisms. Acetoin production by yeast and other α -carboxylases is a secondary result of pyruvate decarboxylation to acetaldehyde; α -acetolactate is not an intermediate as in the bacterial systems (Juni, 1952*a,b*, 1961). Thus only the third group can carry out both valine synthesis and acetoin formation with acetolactate as the intermediate in both pathways; certain differences in characteristics suggest multiple enzymes for this reaction. Indeed, Halpern & Umbarger (1959) have demonstrated that *A. aerogenes* has two separate acetolactate-forming enzymes with different pH optima and other properties. Since it remains to be determined whether this is valid for the other organisms that produce acetoin and valine, these comments are restricted to the compound of α -acetolactate and not to its mode of formation. The earlier pattern of incorporation of labelled glucose and acetate into valine in *A. aerogenes* (Rafelson, 1955) complements the enzymic studies in this microbe.

The data in Table 6 shows that these crude microbial extracts can also dehydrate $\alpha\beta$ -dihydroxy- β -methyl-*n*-valerate to its keto analogue. Both compounds are known intermediates in isoleucine biosynthesis in *N. crassa*, *E. coli* and *Saccharomyces cerevisiae* (Myers & Adelberg, 1954; Umbarger & Davis, 1962; Wixom *et al.* 1960). Utilizing α -oxobutyrate instead of pyruvate (Scheme 1), the last four reactions in isoleucine formation are catalysed by the same enzymes as valine biogenesis. In this connexion a multiple mutant of *B. subtilis* was found to require valine, isoleucine and phenylalanine for growth (Burkholder & Giles, 1947). The pattern of pyruvate and acetate incorporation into branched fatty acids of *B. subtilis* was consonant with the intermediates of α -acetolactate and α -oxoisovalerate (Kaneda, 1963). Several Japanese investigators demonstrated the accumulation of isoleucine in the presence of added α -aminobutyric acid in *B. subtilis* fermentation (Hayashi, Watanabe, Fujii & Shimura, 1959; Hayashibe & Watanabe, 1962; Shimura & Saito, 1960; Hayashibe & Uemura, 1961*a,b*). For *Serratia marcescens*, added D-threonine stimulated the accumulation of isoleucine (Chibata, Kisumi, Ashikaga & Kato, 1962; Kisumi, 1962; Kisumi, Ashikaga, Kato & Chibata, 1962). Isoleucine accumulation has been observed in *A. aerogenes*, *E. coli* and other species (Hayashibe & Watanabe, 1962). Each of these observations is consistent with the extension of the isoleucine pathway in *E. coli* to *B. subtilis*, *S. marcescens* and *A. aerogenes*.

Valine biosynthesis is regulated in *E. coli* by end-product inhibition and by repression of enzyme

formation, both of which were observed at the acetolactate-formation step (Umbarger & Brown, 1958; Leavitt & Umbarger, 1961). With present knowledge of repression, co-ordinate and multi-valent repression in biosynthetic pathways (Rowbury & Woods, 1961; Moyed & Umbarger, 1962; Freundlich, Burns & Umbarger, 1962), control mechanisms for the middle of the valine and isoleucine pathway should be examined in other organisms. While some figures in Tables 3 and 6 suggest repression, the only valid comparison is between specific activities from the media containing 16 and 19 amino acids for *S. aureus* and *P. morganii*. The presence of 0.67 mM-valine plus 0.67 mM-isoleucine caused 38% and 44% depression of the dehydratase specific activity respectively. Since high concentrations of these amino acids did not affect the action of the dehydratases in cell-free extracts, these preliminary results suggest repression of the formation of the dehydratase.

This investigation has compared earlier nutritional observations and the present dehydratase assay to provide insight on the route of valine biogenesis in diverse micro-organisms (Table 7). The methionine and cysteine in the *P. morganii* medium provided a source of utilizable sulphur, rather than a needed carbon skeleton or nitrogen (D. D. Woods & C. Mulder, personal communication). For *S. aureus*, Gladstone (1937) produced strains which grew on a medium devoid of all amino acids. Consistent reports with ammonium sulphate as the sole nitrogen source other than vitamins may be found for the strains used of *A. rancens*, *A. kuetzingianus*, *A. acetosus* (Brown & Rainbow, 1956; De Ley, 1959) and *As. melanogenus* (Gray & Tatum, 1944; Foda & Vaughn, 1953). *A. rancens*, NRRL-B-65, was also in this category (Brown & Rainbow, 1956), although the data of Foda & Vaughn (1953) indicated a need for preformed amino acids. Other strains may be found in either nutritional group (Rao & Stokes, 1953). In contrast to growth here on ammonium sulphate, Stokes & Larsen (1945) reported that *As. suboxydans*, A.T.C.C. no. 621, required six amino acids, including valine and isoleucine. This difference is related to the inhibition of growth by excess of valine (Kerwar, Cheldelin & Parks, 1963). *As. suboxydans* lacks the tricarboxylic acid cycle and as a consequence has an unusual mode of glutamic acid synthesis (Roehr, 1961; Sekizawa, *et al.* 1962). The presence of the dehydratase continues to be correlated with microbial growth in the absence of valine and isoleucine, and usually with ammonium salts as the sole nitrogen source other than vitamins (Wixom *et al.* 1961). Thus the dehydratase serves as an indicator that the traditional *E. coli* and *N. crassa* pathway of valine and isoleucine biosynthesis can be extended to many other biological systems.

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