BIOORGANIC CHEMISTRY 8, 175–189 (1979)

Stereochemistry of Valine and Isoleucine Biosynthesis

IV. Synthesis, Configuration, and Enzymatic Specificity of α-Acetolactate and α-Aceto-α-hydroxybutyrate[1]

RICHARD K. HILL[2] AND SEIJI SAWADA

Department of Chemistry, University of Georgia, Athens, Georgia 30602

AND

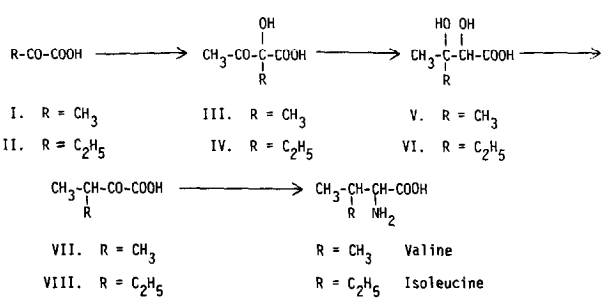
STUART M. ARFIN

Department of Biological Chemistry, California College of Medicine, University of California at Irvine, Irvine, California 92664

Received January 20, 1978

A new synthetic route, involving acetylenic intermediates, has been developed for the preparation of the valine and isoleucine biosynthetic intermediates α-acetolactic acid (**III**) and α-aceto-α-hydroxybutyric acid (**IV**) including the optically active form of these labile acids. The absolute configuration of acetolactate methyl ester **XV** was confirmed as (R)-(−), and the configuration of **XVI** was also established as (R)-(−). Two trideuterio analogs of acetolactate were prepared by this route. The acetolactate anion was found to undergo a rapid room-temperature degenerate rearrangement resulting in racemization and methyl interchange. The isomeroreductase of *Salmonella typhimurium* was found to be specific for the (S) enantiomers of **III** and **IV**, allowing conclusions about the conformation of **IV** during the ethyl migration step in isoleucine biosynthesis. Acetolactate decarboxylase of *Acidobacterium aerogenes* was found to decarboxylate specifically the (S) enantiomers of **III** and **IV**, forming (−)-acetoin from **III** with inversion of configuration.

The biosynthetic pathways to valine and isoleucine in bacteria and yeast, elucidated some years ago (*1*), are shown in Scheme 1. The first common step, in which the carbon



SCHEME 1. Biosynthesis of valine and isoleucine.

[1] For previous papers in this series see Ref. 3 and 4.

[2] To whom correspondence should be addressed.

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atoms which eventually comprise these amino acids are assembled and in which chirality is introduced, is the condensation of an α-ketoacid (**I** or **II**) with α-hydroxyethylthiamine pyrophosphate, mediated by an acetohydroxyacid synthetase, to form α-acetolactate (**III**) and α-aceto-α-hydroxybutyrate (**IV**). These acetohydroxyacids are then converted by an isomeroreductase [or alternatively, as has been demonstrated to be possible in *Mycobacterium tuberculosis* $H_{37}R_v$, by the successive action of a separate acetohydroxyacid isomerase and a reductase (*2*)] to the dihydroxyacids **V** and **VI**. Enzymatic dehydration to **VII** and **VIII** and subsequent transamination of the α-keto acids complete the conversion to valine and isoleucine.

Previous papers in this series have dealt with the configurations of the dihydroxyacids **V** and **VI** (*3*) and with the stereochemistry of the dehydrase step (*4*). The acetohydroxyacids **III** and **IV** are the subject of this paper. Firm evidence that α-acetolactate (**III**) is an obligatory intermediate in valine biosynthesis was provided by Umbarger and Brown (*5*), who showed that a valine auxotroph, an *Escherichia coli* mutant, accumulates **III** when grown on limiting amounts of valine. The conversion of **III** to **V**, **VII**, and valine has been demonstrated in separate experiments (*6*). Several investigators have shown that the biosynthesis of **III** and **IV** is catalyzed by the same enzyme (*7*); thiamine pyrophosphate is required as a cofactor. Acetolactate synthase from *Pseudomonas aeruginosa* has been purified by Arfin and Koziell (*8*), and its cofactor requirements, feedback inhibition, and subunit composition investigated (*8*, *9*).

α-Acetolactic acid (**III**) is chiral, and the α-acetolactate formed on the valine pathway is a single enantiomer. Umbarger and Brown (*5*) found that a decarboxylase from *Acidobacterium aerogenes* which decarboxylates only 50% of synthetic (±)-**III** completely decarboxylated the acetolactate synthesized by *E. coli* extracts. Thus the acetohydroxyacid synthetase of the valine pathway forms **III** of the same optical configuration as that formed and decarboxylated by the classical carboligase of *A. aerogenes*. Juni (*10*) had shown earlier that the decarboxylase is specific for the (+) enantiomer.

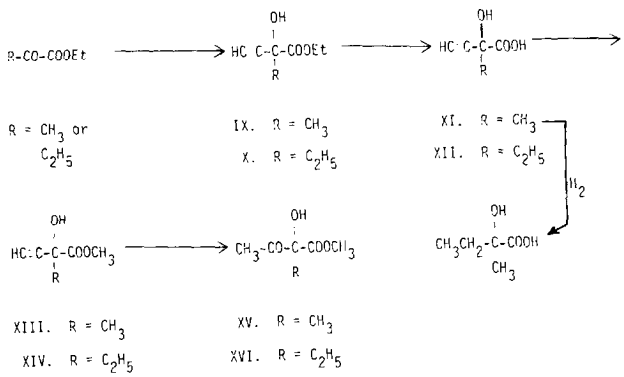
Because of the instabilty of free α-acetolactic acid (**III**) (*11*), it has been necessary to carry out experimental studies on solutions of the anion in aqueous alkaline media; the free acid decarboxylates rapidly on acidification. Consequently the synthetic material used in biosynthetic experiments to date has been the racemic methyl ester **XV** synthesized by the method of Krampitz (*12*); the ester is saponified just before the anion is required. The methyl ester (**XVI**) of **IV** has been synthesized and used in the same way (*7*). The absolute configuration of the natural enantiomers of **III** and **IV** was unknown at the time we undertook this study, although Robins and Crout (*13*) had proved that the ester (−)-**XV**, derived by degradation of the pyrrolizidine alkaloid monocrotaline, has the (R) configuration. During the course of this study Armstrong *et al.* reported that the acetolactate utilized in *Salmonella typhimurium* for valine biosynthesis is the (2S) enantiomer (*14*).

Synthesis and Absolute Configuration

In order to make optically active forms of **III** and **IV** available for biochemical study, we undertook a synthesis via intermediates capable of optical resolution. The instability of the free acids precludes any attempt to resolve the acids themselves, so our efforts

were directed toward the preparation of the optically active methyl esters. Our synthetic route is outlined in Scheme 2.

Following the procedure of Verny and Vesiere (*15*), ethynyl magnesium bromide was added to ethyl pyruvate to yield ethyl 2-hydroxy-2-methyl-3-butynoate (**IX**). The acid **XI** obtained by saponification was resolved with quinine, following a literature procedure (*16*). Dugat *et al.* (*16*) had assigned the (R) absolute configuration to (−)-**XI** by catalytic hydrogenation to (R)-(−)-2-hydroxy-2-methylbutanoic acid (*17*), and we confirmed this assignment by diimide reduction of (−)-(**XI**).



SCHEME 2. Synthesis of optically active methyl α-acetolactate and methyl α-aceto-α-hydroxybutyrate.

After methylation with diazomethane, the triple bond of **XIII** was hydrated using Newman's mercuric resin (*18*) to afford (R)-(−)-methyl acetolactate (**XV**. The spectroscopic properties, optical rotation, and assignment of absolute configuration all agree with those reported by Robins and Crout (*13*). The optical rotation of our synthetic material, $[\alpha]_d^{30}$ −9.6° (ethanol) is in good agreement with the value of −9.9° (ethanol) for the ester obtained by degradation of monocrotaline, showing that the synthetic enantiomer is essentially optically pure. It is of interest that this ester, which is levorotatory in ethanol, is strongly dextrorotatory in water.

The same procedure, with substitution of ethyl α-ketobutyrate for ethyl pyruvate in the first step, led to the optically active enantiomers of methyl α-aceto-α-hydroxybutyrate (**XVI**). Assignment of absolute configuration to this series could not easily be made by chemical correlation but was assured on the basis of the close correspondence in rotations of the intermediates in the two parallel schemes (Table I) and the nearly

TABLE I

OPTICAL ROTATIONS, $[\alpha]_d^{30}$, OF SYNTHETIC INTERMEDIATES IN THE ACETOLACTATE (AL) AND α-ACETO-α-HYDROXYBUTYRATE (AHB) SERIES

Compound	AL series	AHB series
Acetylenic acid forming less soluble quinine salt	(**XI**) − 46.4° (EtOH)	(**XII**) −43.9° (EtOH)
Methyl ester of acetylenic acid	(**XIII**) −27° (EtOH)	(**XIV**) − 25.9° (EtOH)
Acetohydroxy acid methyl ester	(**XV**) −9.6° (EtOH) +27.3° (H_2O)	(**XVI**) −1.8° (EtOH) +45.1° (H_2O)
Acetohydroxy acid anion in dilute NaOH	−41.3°	−73.5°

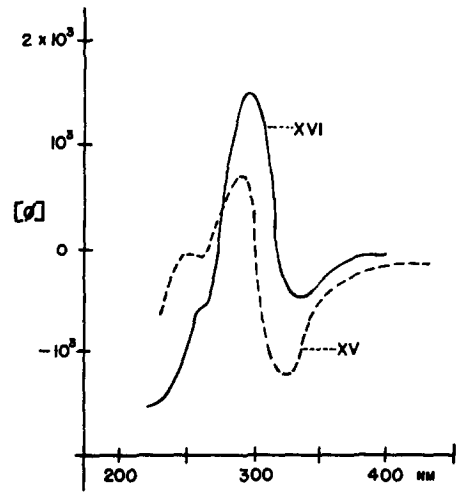


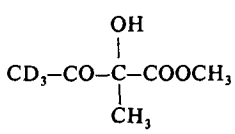
FIG. 1. Optical rotatory dispersion (ORD) spectra of methyl α-acetolactate (**XV**) and methyl α-aceto-α-hydroxybutyrate (**XVI**.

identical ORD spectra of (R)-(−)-**XV** and (R)-(−)-**XVI** (Fig. 1) Again the (2R) enantiomer, which is weakly levorotatory in ethanol, is strongly dextrorotatory in water.

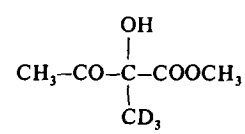
Synthesis of Labeled Samples of Methyl α-Acetolactate

The synthetic route in Scheme 2 lends itself to preparation of specifically deuterated samples of α-acetolactate which would be useful in studying the stereochemistry of the subsequent rearrangement and dehydration steps of valine biosynthesis. Three different methods of introducing deuterium were investigated.

(a) Hydration of the acetylenic ester **XIII** was carried out with a deuterated mercuric resin, prepared by impregnating the ion-exchange resin with a D_2O solution of mercuric sulfate. The nmr spectrum of the methyl α-acetolactate-d_3 formed (**XVII**) showed that the acetyl methyl signal at $\delta 2.16$ had only 10% of the intensity of the methyl ester and C-methyl peaks, and was therefore 90% deuterated.



XVII.



XVIII.

(b) When an attempt was made to exchange the acetyl methyl protons by a dilute solution of NaOD in D_2O, it was found that ester hydrolysis was almost instantaneous, and that a skeletal rearrangement followed proton exchange. This phenomenon is described more fully below.

(c) A synthesis of the lactate methyl-d_3 isomer (**XVIII**) was achieved[3] by carrying out the synthesis of Scheme 2 beginning with ethyl pyruvate-d_3. The protons of pyruvic acid were exchanged by stirring for 4 days at 60°C with a solution of dilute sulfuric acid in D_2O. Analysis of the nmr spectrum of the methyl pyruvate formed with diazomethane showed that the C-methyl group was about 85% deuterated. Carrying this material through the remainder of the synthesis gave methyl α-acetolactate-d_3 (**XVIII**) containing 87% CD_3.

Hydrolysis of Methyl α-Acetolactate

The synthetic methyl esters **XV** and **XVI** must be saponified before use in enzymatic experiments, and because of the possible lability of the acetohydroxyacid salts the hydrolysis was carefully studied by nmr. The nmr spectrum of a solution of **XV** in H_2O

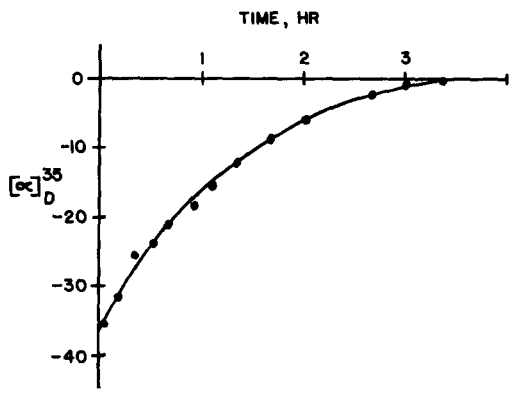


FIG. 2. Racemization of (−)-(**XV**) in dilute alkaline solution.

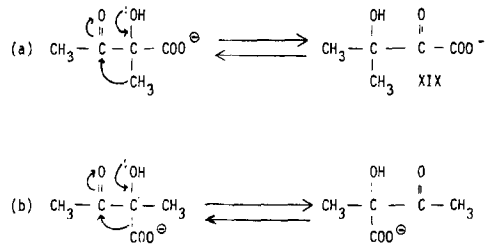
shows three singlets: the α-methyl at δ 1.62, the acetyl methyl at 2.34, and the *O*-methyl at 3.86. After addition of a small amount of NaOH, the *O*-methyl peak disappears almost instantaneously and is replaced by the methyl signal of methanol at δ3.39. The methyl signals of the carboxylate anion so formed, at 1.47 and 2.27, remain constant for at least 3 hr at 35°C, indicating the stability of acetolactate anion during this period; although if the solution is kept at room temperature for several days, these signals decrease, and signals due to acetoin (d, δ1.37 and s, δ2.23) begin to appear. On acidification of the alkaline solution, CO_2 is rapidly liberated, the acetolactate signals disappear, and the nmr spectrum of the remaining solution is that of acetoin plus the residual methanol peak.

When the hydrolysis of a dilute aqueous solution of (−)-**XV** with 2–5% NaOH was followed at 35°C in the polarimeter, however, an unexpected result was observed: the optical rotation decreased rapidly, and the sample was totally racemized within 3.5 hr (Fig. 2). During this period no change in the nmr spectrum of the acetolactate anion was observed, however, so racemization cannot be attributed to decarboxylation or reverse Claisen cleavage. Since **XV** does not possess an epimerizable hydrogen attached to the asymmetric center, the only mechanism for racemization without structural change is some sort of degenerate rearrangement.

[3] For a previous application of acetolactate labeled with ^{14}C and ^{3}H in the α-methyl group, prepared by the Krampitz procedure, see Ref. (*21a*).

Further evidence for such a rearrangement was obtained by following the saponification of **XV** with NaOD–D_2O. Hydrolysis of the methyl ester again occurs immediately on mixing the reagents; the δ2.34 signal disappears rapidly as the acetyl methyl protons are exchanged by deuterium; but unexpectedly the "non-exchangeable" α-methyl protons also exchange with deuterium at about the same rate as racemization, and within 4 hr *both* *C*-methyl signals nearly disappear from the spectrum. The acetyl methyl signal reappears rapidly when water is added to the solution. Moreover, when the α-d_3-acetolactate **XVIII** is saponified with aqueous NaOH, the α-methyl signal at 1.62, absent in the initial spectrum, grows into the nmr spectrum as the solution is allowed to stand at 35°C.

These surprising racemization and deuterium exchange results were discovered independently and simultaneously by Dr. D. H. G. Crout and his colleagues at Exeter; and the findings from the two laboratories agree closely in demonstrating that some



SCHEME 3. Possible mechanisms for α-acetolactate rearrangement.

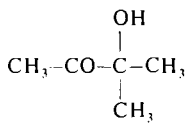
degenerate rearrangement must be occurring which interconverts the two methyls, placing each in turn in the proton-exchangeable position next to the ketone. Crout has shown by ^{14}C-labeling that rearrangement of the anion is essentially complete in 2 hr at 30°C (*19*). It is easy to understand why this degenerate rearrangement was never discovered by previous workers in valine biosynthesis, since it can be revealed only by racemization or isotopic labeling, and the optically active enantiomers have not been available until recently.

Similar results were obtained in the hydrolysis of methyl α-aceto-α-hydroxybutyrate (**XVI**): (a) the methyl ester was instantaneously hydrolyzed on the addition of dilute NaOH; (b) the optical rotation rapidly decreased on standing in alkaline solution; and (c) when ester **XVI** was treated with NaOD–D_2O, not only did the acetyl methyl signal disappear quickly as a result of deuterium exchange; but over several hours the methylene signal also diminished, and the methyl peak of the ethyl group changed from a triplet to a singlet as the methylene protons were also exchanged by deuterium.

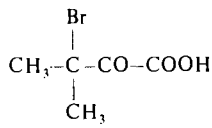
What is the nature of this unexpected rearrangement? At first it appeared likely that an α-ketol rearrangement might be occurring (Scheme 3a), establishing an equilibrium between acetolactate and α-keto-β-hydroxyisovalerate (**XIX**). A number of rapid α-ketol rearrangements have been reported (*20*), and intermediate **XIX** would account both for racemization and for interchange of methyls. Our brief attempts to confirm this mechanism were not successful, however:

(a) Treatment of a close analog, 3-hydroxy-3-methyl-2-butanone (**XX**) with 3% NaOD–D_2O rapidly exchanged the acetyl methyl protons, but the other methyl signal was only slightly diminished, even after 2 days at 70–80°C.

(b) β-Bromo-α-ketoisovaleric acid (**XXI**) was treated with NaOD–D_2O under conditions which should have led to **XIX** (*6a*); had the latter been involved in the equilibrium shown in Scheme 3a, it should have isomerized to α-acetolactate. No α-acetolactate signals were observed in the nmr spectrum, and no exchange of the methyl protons was detected over 4 days at room temperature.



(XX)



(XXI)

These experiments appear to rule out the α-ketol rearrangement of Scheme 3a. In the meantime, moreover, Crout has obtained compelling evidence through ^{14}C- and ^{13}C-labeling studies that the rearrangement involves a novel intramolecular carboxylate migration, as shown in Scheme 3b (14).

This finding of a rapid degenerate isomerization of α-acetolactate means that whenever the anion is generated from optically active or isotopically labeled methyl ester for use in biochemical experiments, it must be used *immediately* after saponification before rearrangement has had time to randomize the skeleton.

Biological Specificity

For determination of enantiomeric specificity, the α-acetolactate and α-aceto-α-hydroxybutyrate enantiomers synthesized in this study were tested against the racemates toward acetohydroxyacid isomeroreductase, the enzyme which catalyzes the conversion of **III** and **IV** to **V** and **VI**. Enzymes from both *S. typhimurium* (*21*) and *E. coli* (*6a*) were available and gave similar results. The synthetic methyl esters **XV** and **XVI** were saponified with the calculated amount of NaOH, and the resulting solutions of the anions used at once. Assays were performed by measuring the rate at which NADPH was oxidized, as described previously by Arfin and Umbarger (*21*). The results are listed in Table 2.

The data show clearly that in both the α-acetolactate and α-aceto-α-hydroxybutyrate series, the (2S) enantiomer is the isomer carried forward in valine and isoleucine bio-

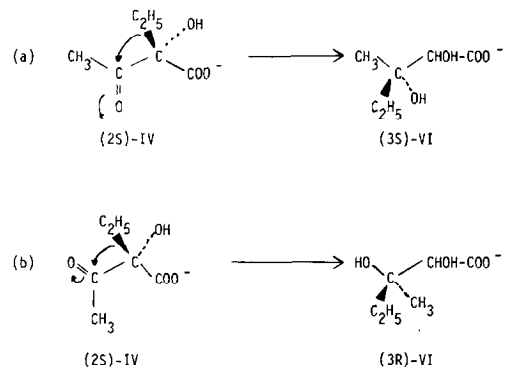
TABLE 2

SPECIFICITY OF ISOMEROREDUCTASE

Substrate (M)	NADPH (μmol oxidized/min/ml)	
	S. typhimurium enzyme	*E. coli* enzyme
(2R)-**XV**, 0.01	0.012	0.010
(2RS)-**XV**, 0.01	0.180	0.240
(2R)-**XV**, 0.001	0.006	0.010
(2RS)-**XV**, 0.001	0.064	0.140
(2R)-**XVI**, 0.001	0.060	0.065
(2S)-**XVI**, 0.001	0.530	0.972
(2RS)-**XVI**, 0.001	0.436	0.763

synthesis and therefore the isomer produced by the acetohydroxyacid synthetase. These results agree with the conclusion of Armstrong *et al.* (*14*) that the isomeroreductase of *S. typhimurium* requires (2S)-acetolactate. The low activity of the (2R) enantiomers, and the fact that the racemic forms show greater than 50% the activity of the (2S) enantiomers, is presumably due to the rapid racemization discussed above.

The knowledge of the absolute configurations of intermediates **IV** and **VI** in the isoleucine pathway now allows some conclusions about the conformation of the transition state during the rearrangement catalyzed by the isomeroreductase. If the reasonable assumption is made that the migration of the ethyl group is intramolecular and suprafacial, then the two basic conformations are possible during the rearrangement (Scheme 4). Rearrangement in conformation (a) would lead to the (3S) configuration in **VI**,



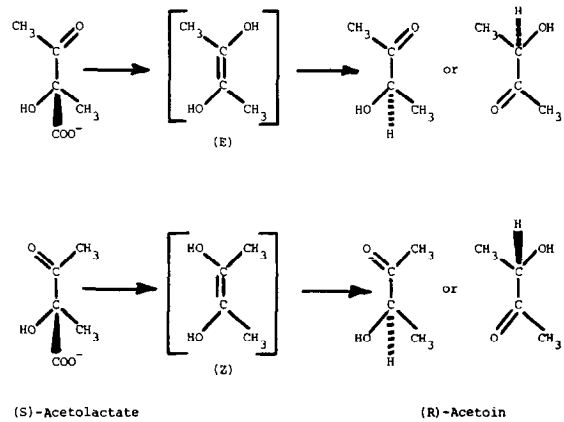
SCHEME 4. Stereochemistry of ethyl migration during isoleucine biosynthesis.

while rearrangement in conformation (b) leads to the (3R) configuration in **VI**. Since the absolute configuration of **VI** has been established unambiguously in two laboratories (*3b*, *22*) as (2R; 3R), conformation (b) must represent the actual alignment of **IV** during the ethyl migration step.

Stereochemistry of Decarboxylation

Many bacteria contain a decarboxylase which will specifically decarboxylate α-acetolactate; the acetolactate decarboxylase from *A. aerogenes* has been particularly well studied (*5*, *10*, *23*), and methods are available for partial purification (*24*). The enzyme decarboxylates α-aceto-α-hydroxybutyrate as well (*23*). It seems likely that the decarboxylation of these β-keto acids proceeds through an enol; but protonation of the enol must be enzyme controlled, since the acetoin formed is optically active (Scheme 5).

Juni had reported (*10*) that a solution in which α-acetolactate was being synthesized from pyruvate became dextrorotatory, and that the acetolactate in this solution was completely decarboxylated by *A. aerogenes*. In order to confirm these qualitative observations we have tested our synthetic samples of acetolactate and acetohydroxybutyrate with the *A. aerogenes* decarboxylase. The results are shown graphically in Figs. 3 and 4. The rates of formation of acetoin and acetylethylcarbinol from **XV** and **XVI** show clearly that in both cases it is the (2S) isomer which is the natural substrate. The (2R) samples begin to decarboxylate appreciably only after an induction period, which must again represent the racemization of the acetohydroxyacid anions.



SCHEME 5. Acetolactate decarboxylation.

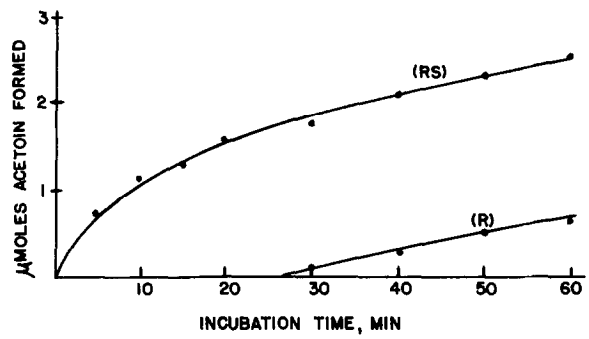


FIG. 3. Rate of decarboxylation of acetolactate by *A. aerogenes*.

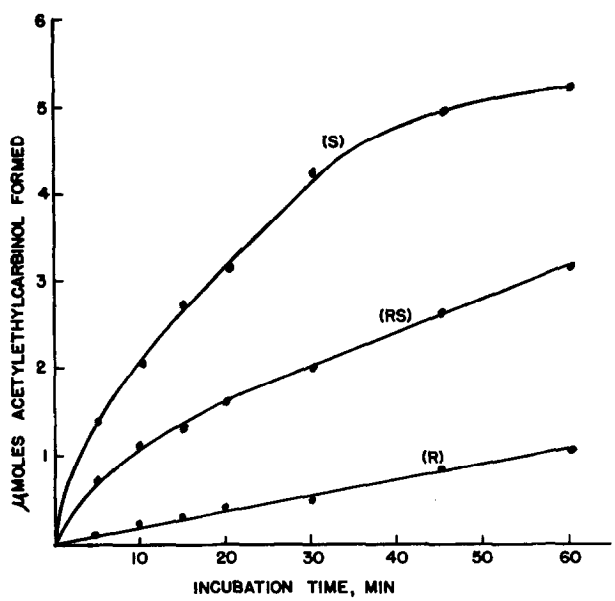


FIG. 4. Rate of decarboxylation of acetohydroxybutyrate by *A. aerogenes*.

The acetoin formed by *A. aerogenes* (and most other bacteria) is levorotatory (*25*); since acetoin has been hydrogenated to 2,3-butanediol of the same sign of rotation (*26*), and the (−) diol has been shown to have the (2R, 3R) configuration (*27*), (−)-acetoin must have the (R) configuration. Formation of (R)-acetoin requires that the intermediate enediol be protonated at the *si* face of one of the trigonal carbons. Consequently if protonation occurs at the same carbon which bore the carboxyl, it must take place with *inversion* of configuration (Scheme 5). If the enediol were held in the (Z) configuration, however, as depicted in Scheme 5, (R)-acetoin might also result from suprafacial protonation of the *other* trigonal carbon. Carbon labeling would be necessary to resolve these alternatives for acetolactate decarboxylation; in the case of α-aceto-α-hydroxybutyrate, of course, the structure of the decarboxylation product would identify the site of protonation. In the cases of enzymatic decarboxylation whose stereochemistry has been examined, both retention and inversion of configuration have been observed (*28*), and no general mechanistic significance has been deduced from the stereochemical course.

EXPERIMENTAL

Infrared spectra were recorded on Perkin–Elmer Models 257 or 621 spectrophotometers. Nuclear magnetic resonance spectra were recorded on a Varian HA-100 instrument. Optical rotations were measured on a Perkin–Elmer Model 141 polarimeter, while ORD spectra were recorded on a Cary 60 recording spectropolarimeter. Melting points were taken in open capillaries and are uncorrected.

α-Acetolactate Series

(±) *Ethyl 2-hydroxy-2-methyl-3-butynoate* (**IX**). The racemic ester was prepared in 16% yield by the addition of ethynyl magnesium bromide to ethyl pyruvate in tetrahydrofuran (THF), using a procedure earlier described (*15*). The ester was collected at 72–75° (17 mm); lit. (*15*) bp 68–70°C (11 mm); nmr ($CDCl_3$): 1.32 (t, 3H), 1.68 (s, 3H). 2.49 (s, 1H), 3.71 (s, 1H), 4.30 (q, 2H).

(±) *2-Hydroxy-2-methyl-3-butynoic acid* (**XI**). Alkaline hydrolysis (4 N NaOH in 9:1 methanol:water, 2 hr, room temperature) of ester **IX** led in 70% yield to acid **XI**, bp 138–145°C (15 mm), mp 108–108.5°C after recrystallization from benzene; lit. (*16*) mp 107–109°C; nmr ($CDCl_3$), 1.75 (s, 3H), 2.54 (s, 1H).

Resolution. The acid was resolved with quinine as described by Dugat *et al.* (*16*). Three recrystallizations gave a salt of mp 193–194°C; lit. (*16*) mp 192°C. Decomposition of the salt with concentrated HCl, followed by recrystallization of the acid from benzene, gave in 15% overall yield the levorotatory enantiomer, mp 130–131°C; $[\alpha]_d^{30}$ −46.4° (*c* 3.57, ethanol), $[\alpha]_d^{30}$ −46.0° (*c* 1.14, acetone); lit. (*16*) mp 132°C, $[\alpha]_d^{30}$ −41° (acetone).

Reduction of acid **XI**. A mixture of 88 mg of acid **XI**, $[\alpha]_d^{30}$ −46.4° (ethanol), 2 ml of 35% hydrogen peroxide, and a trace of mercuric oxide in 10 ml of 50% aqueous methanol was stirred in an ice bath while 8 drops of 95% hydrazine was added. The mixture was stirred for 1 hr, acidified with HCl, and extracted with ether. The extracts were washed with brine, dried over $MgSO_4$, and concentrated. The solid residue was

sublimed at reduced pressure to yield (R)-(−)-2-hydroxy-2-methylbutanoic acid (40 mg), mp 70–72°C, $[\alpha]_d^{30}$ −7.63° (*c* 3.1, $CHCl_3$), $[\alpha]_d^{30}$ − 5.27° (*c* 3, acetone). An authentic sample[4] (*29*) of the optically pure dextrorotatory acid had $[\alpha]_d^{30}$ 7.76° (*c* 1.33, $CHCl_3$), $[\alpha]_d^{30}$ 5.39° (*c* 1.33, acetone). Christensen and Kjaer (*17*) report $[\alpha]_d$ −8.5° ($CHCl_3$), mp 73.5–74.5°.

Methyl 2-hydroxy-2-methyl-3-butynoate, (*R*)-(−)-**XIII**. (R)-(−)-**XIII** was prepared in 90% yield by treatment of **XI**, $[\alpha]_d^{30}$ −45.1° (ethanol) with an ethereal solution of diazomethane. The ester had bp 63–66°C (10 mm), $[\alpha]_d^{30}$ −27.0° (*c* 1.1, ethanol); nmr ($CDCl_3$): 1.67 (s, 3H), 2.50 (s, 1H), 3.67 (s, 1H), 3.83 (s, 3H).

Methyl α-acetolactate, (*R*)-(−)-(**XV**). A solution of 110 mg of ester **XIII**. $[\alpha]_d^{30}$ −27.0° (ethanol), in 5 ml ethanol and 0.5 ml water was treated with 4 g of Newman's Hg-resin (*18*) and allowed to stand 3 hr at 30–40°C. When glc analysis showed disappearance of starting material, 50 ml of ether was added, the mixture dried over $MgSO_4$ and filtered, and the filtrate distilled. Redistillation gave 75 mg (60%) of (R)-(−)-methyl α-acetolactate (**XV**), bp 77–80°C (10 mm). $[\alpha]_d^{30}$ −9.6° (*c* 3.1, ethanol), $[\alpha]_d^{30}$ + 27.3° (*c* 6.4, water); nmr (CCl_4): 1.53 (s, 3H), 2.10 (s, 3H), 3.79 (s, 3H), 3.86 (s, 1H).

Anal. Calcd. for $C_6H_{10}O_4$: C, 49.38; H, 6.89. Found, C, 49.14; H, 6.88.

α-Aceto-α-hydroxybutyrate Series

Ethyl 2-ethyl-2-hydroxy-3-butynoate (**X**). The Grignard reagent was prepared from 40 g (0.36 mol) of ethyl bromide and 7 g (0.3 g-atom) of magnesium turnings in 175 ml of dry THF. This Grignard solution was slowly added to a stirred THF solution into which a stream of acetylene was simultaneously and continuously added. After addition was complete, stirring and acetylene flow were continued for 1 hr at room temperature. The reaction mixture was cooled to 0°C and a solution of 36 g (0.28 mol) of ethyl α-ketobutyrate in 125 ml of THF was added dropwise with stirring. Aqueous ammonium chloride solution (50 ml) was added, the layers separated, and the aqueous layer extracted with several portions of ether. The combined organic extracts were washed with saturated sodium chloride solution, dried over $MgSO_4$, and distilled. Ethyl 2-ethyl-2-hydroxy-3-butynoate (**X**) (9 g, 23%) was collected at 93–96°C (27 mm).

2-Ethyl-2-hydroxy-3-butynoic acid (**XII**). A solution of 8.6 g (42 mmol) of ester **X** and 3.0 g of NaOH in 30 ml of ethanol and 10 ml water was kept overnight at room temperature; glc analysis showed the disappearance of starting material. The reaction mixture was diluted with water, washed with ether, acidified with concentrated HCl, and extracted with ether. After washing with brine and drying over $MgSO_4$, the extracts were concentrated and the residual solid recrystallized from benzene, affording 4.1 g (77%) of acid **XII**, mp 91–91.5°C, bp 95–105°C (0.2 mm).

Anal. Calcd for $C_6H_8O_3$: C, 56.25; H, 6.49. Found: C, 56.47; H, 6.12.

Resolution of **XII**. A mixture of 12 g of the racemic acid **XII** and 30 g of quinine was dissolved in a hot mixture of 20 ml ethanol and 80 ml acetone. After cooling overnight, the precipitate was collected and recrystallized eight times from the same solvent pair. The pure quinine salt, mp 189–190°C, was treated with concentrated HCl and extracted with ether; the extracts furnished the resolved acid on concentration. Sub-

[4] We thank Dr. Norman G. Brink, Merck Sharp and Dohme, for a sample of (+) 2 hydroxy 2 methylbutanoic acid.

limation at reduced pressure afforded 500 mg, mp 117.5–118°, $[\alpha]_d^{30}$ −43.9° (*c* 1.1, ethanol).

The mother liquors from recrystallization of the quinine salt were decomposed with HCl as above. Repeated recrystallization of the recovered acid from benzene–ether gave 200 mg of the enantiomer, mp 117–118°C, $[\alpha]_d^{30}$ +41.8 (*c* 0.91, ethanol).

Methyl 2-ethyl-2-hydroxy-3-butynoate (**XIV**) was prepared from acid **XII** by treatment with ethereal diazomethane, and had bp 68–73°C (11 mm); nmr (CCl_4): 0.96 (t, 3H), 1.89 (d of q, 2H), 2.36 (s, 1H), 3.48 (broad s, 1H), 3.82 (s, 3H). The ester from (−)-(**XII**) had $[\alpha]_d^{30}$ −25.9° (*c* 7.45, ethanol), while that from (+)-(**XII**) had $[\alpha]_d^{30}$ +24.7° (*c* 4.61, ethanol).

Methyl 2-ethyl-2-hydroxy-3-ketobutyrate (**XVI**). A solution of 100 mg of (+)-(**XIV**), $[\alpha]_d^{30}$ +24.7° (ethanol), in 10 ml of 95% ethanol was treated with 4 g of Hg-resin and kept at 30–40°C for 2 hr; glc analysis showed that the starting material had disappeared by this time. The mixture was diluted with 50 ml of ether, dried over $MgSO_4$, filtered, and concentrated. Distillation of the residue gave 80 mg (73%) of ester **XVI**, bp 80–83°C (10 mm), $[\alpha]_d^{30}$ + 1.4° (*c* 0.46, ethanol), $[\alpha]_d^{30}$ −43.5° (*c* 5.13, water); nmr (CCl_4): 0.87 (t, 3H), 1.97 (d of q, 2H), 2.19 (s, 3H), 3.80 (s, 3H), 4.00 (broad s, 1H).

Anal. Calcd for $C_7H_{12}O_6$: C, 52.49; H, 7.56. Found: C, 52.64; H, 7.69.

The enantiomer, prepared in the same way, had $[\alpha]_d^{30}$ −1.8° (EtOH), +45.1° (H_2O).

Preparation of Deuterated Acetolactate Samples

Methyl 2-(trideuterioaceto)lactate (**XVII**). **XVII** was prepared in the same manner as **XV** except that D_2O was substituted for water in the preparation of the mercuric resin and in the hydration; nmr ($CDCl_3$): 1.57 (s, 3H), 3.78 (s, 3H), 2.16 (s, 0.3 H).

Methyl 2-aceto-3,3,3-trideuteriolactate (**XVIII**). A solution of 30 g of pyruvic acid and 20 drops of concd H_2SO_4 in 150 ml of 99.7% D_2O, saturated with NaCl, was stirred 4 days at 50–60°C. Several extractions with ether, followed by drying ($MgSO_4$) and distillation, gave pyruvic acid-d_3, 24 g (80%), bp 55–65°C (15 mm). The acid was treated with a slight excess of ethereal diazomethane, affording methyl trideuteriopyruvate (46%), bp 135–138°C; nmr (CCl_4) 3.80 (s, 3H), 2.39 (broad s, 0.45H).

Addition of the acetylenic Grignard reagent was carried out as described above, to give a 40% yield of methyl 2-hydroxy-2-trideuteriomethyl-3-butynoate, bp 65–75°C (10 mm); nmr (CCl_4): 2.40 (s, 1H), 3.83 (s, 3H). Saponification yielded the acid, mp 107–108°C, which was reesterified with diazomethane. The pure methyl ester showed an nmr signal at 1.62 corresponding to 13% CH_3.

Hydration with mercuric resin afforded methyl 2-aceto-3,3,3-trideuteriolactate (**XVIII**) in 60% yield; nmr (CCl_4): 2.14 (s, 3H), 3.74 (s, 3H), 3.82 (s, 1H), 1.50 (broad s, 0.4 H).

Hydrolysis of **XV**. (a) A solution of 64.3 mg of **XV**, $[\alpha]_d^{30}$ −7.60° (*c* 0.55, EtOH), $[\alpha]_d^{30}$ +19.8° (*c*, 6.43, H_2O) in 1.0 ml of water was made up at 35°C in an nmr tube. Several drops of 4% NaOH were added and the nmr spectrum was monitored at intervals for a period of a week. The changes in the spectrum are described in the text. The optical rotation of the solution was measured approximately every 10 min; the rotation gradually diminished to zero in a little over 3 hr, as shown in Fig. 3.

Three minutes after the NaOH was added, at which time the nmr spectrum showed that ester hydrolysis was complete, an aliquot of the alkaline solution, $[\alpha]_d$ −41°, was acidified in the polarimeter tube with HCl. The rotation immediately became positive, $[\alpha]_d$ ~3°, then quickly diminished to zero as the acid decarboxylated.

(b) When the above experiment was repeated with a solution of NaOD in D_2O, the acetyl methyl nmr signal at 2.34 rapidly vanished. The methyl signal at 1.62 decreased more slowly but had completely disappeared by 4 days. If water was added to this solution the 2.34 signal quickly reappeared.

(c) The deuterated ester **XVIII** was treated with dilute NaOH as described in (a). A signal at 1.62, absent in the initial spectrum, slowly grew into the spectrum as the solution was kept at 35°C for 2 days.

Hydrolysis of **XVI**. (a) A solution of 51.3 mg of **XVI**, $[\alpha]_d^{30}$ −36.4° (*c* 5.13, H_2O), in 1.0 ml of water was treated with several drops of 4% NaOH, and the nmr spectrum and optical rotation were followed with time as described for **XV**. As before, hydrolysis was rapid; a spectrum taken 3 min after mixing showed no methyl ester signal at 3.85. The spectrum of the anion, 0.83 (t, 3H), 1.87 (q, 2H), and 2.30 (s, 3H) remained essentially constant over 2 days if the solution was stored at 5°C. The optical rotation of the alkaline solution, taken a few minutes after mixing, was +56.8°, but this value had fallen to +50° 15 min later and steadily decreased. An aliquot was acidified with HCl after 15 min; the rotation immediately became negative (−13°) and then rapidly fell to zero. Immediately after acidification the nmr spectrum showed signals at 0.90 (t), 1.87 (q), and 2.37 (s). Within 30 min these signals had disappeared and were replaced by signals at 1.04 (t), 0.93 (t), 1.39 (d), and 2.26 (s), indicating the presence of a mixture of the tautomeric acyloins 3-hydroxy-2-pentanone and 2-hydroxy-3-pentanone.

A solution of the antipode of **XVI**, $[\alpha]_d^{30}$ +45.1° (H_2O), became levorotatory (−73.5°) on mixing with 4% NaOH and then immediately dextrorotatory (+15°) on acidification. The rotation then quickly fell to zero.

(b) Hydrolysis was repeated as in (a) with a solution of NaOD in D_2O. The acetyl methyl signal at 2.30 decreased rapidly and had vanished within several hours. Over a longer period the methyl signal at 0.83 became a singlet and the methylene signal at 1.87 slowly vanished.

Isomeroreductase experiments. The enzyme from *S. typhimurium* was purified as described in Ref. (*21a*) through the stage of the 40–60% ammonium sulfate fraction. The *E. coli* isomeroreductase was a 10-fold purified preparation obtained from strain CU-1018 (obtained from Professor H. E. Umbarger), grown under conditions leading to maximal induction of the enzyme. Assays were performed as described in Ref. (*21*).

Decarboxylase experiments. A wild-type strain of *A. aerogenes* grown overnight in L broth (*30*) was used as the source of acetolactate decarboxylase. The enzyme was partially purified as described by Juni (*24*). Incubation mixtures contained 0.1 *M* H_3PO_4-KOH buffer, pH 5.9, 5.5 μmol of acetohydroxyacid and enzyme in a total volume of 1.5 ml, and were incubated at 20°C. Samples (0.1 ml) were withdrawn at intervals and added to 0.9 ml of 0.4 *N* NaOH. Acetoin and acetylethylcarbinol were determined by the Westerfeld procedure as modified by Magee *et al.* (*11e*). Under these conditions there was no nonenzymatic decarboxylation.

ACKNOWLEDGMENTS

This investigation was supported by Research Grant GM-16944 from the National Institutes of Health, to whom we express our appreciation. We thank Dr. David H. G. Crout for friendly exchange of information and for his courtesy in informing us of his results prior to publication.

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