Stereochemistry of the Decarboxylation of α-Acetolactate (2-Hydroxy-2-methyl-3-oxobutanoate) by the Acetolactate Decarboxylase of *Klebsiella aerogenes*

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[3-^{14}C]-α-Acetolactate ([3-^{14}C]-2-hydroxy-2-methyl-3-oxobutanoate) was synthesised and subjected to enzymatic decarboxylation by the acetolactate decarboxylase from *Klebsiella pneumoniae*. Degradation of the acetoin produced showed that the radioactivity was largely confined to the acetyl component. Together with previous results, this proves that during decarboxylation the carboxy group of α-acetolactate is replaced regiospecifically by hydrogen and that this process takes place with overall inversion of configuration.

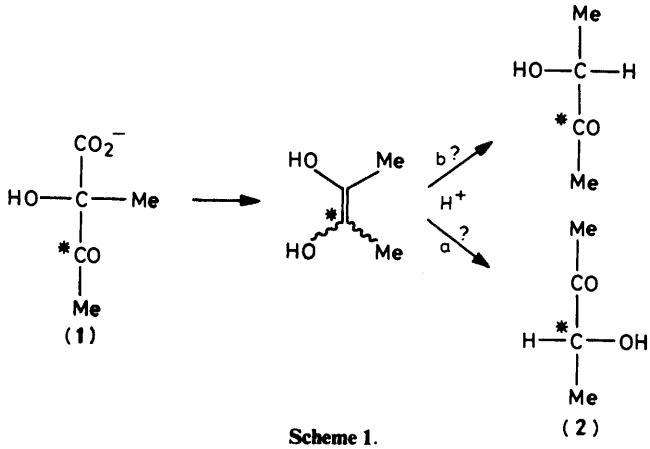
α-Acetolactate (2-hydroxy-2-methyl-3-oxobutanoate) (**1**) is an intermediate in the biosynthesis of valine. Many bacteria secrete an enzyme, acetolactate decarboxylase (2-hydroxy-2-methyl-3-oxobutyrate carboxy-lyase, EC 4.1.1.5), capable of decarboxylating α-acetolactate to acetoin (3-hydroxybutan-2-one) (**2**).[1,2] α-Acetolactate is derived exclusively from pyruvate. The decarboxylation of α-acetolactate classically has been regarded as a mechanism for switching glucose metabolism from acidic to neutral products.[3] Acetoin production, as monitored by the Voges–Proskauer test,[4] is characteristic of the Enterobacteria, and is used as a taxonomic marker for this group.

The acetoin produced by decarboxylation of α-acetolactate (**1**) is optically active. Hill *et al.* have shown that the α-acetolactate decarboxylase of *Aerobacter aerogenes** operates exclusively on (*S*)-α-acetolactate to produce (*R*)-acetoin.[5] However, because this reaction presumably proceeds *via* an enediol intermediate of either C_{2v} (*cis*-enediol) or C_{2h} (*trans*-enediol) symmetry, the available stereochemical information, as pointed out by Hill,[4] was not sufficient to define fully the regio- and stereo-chemical features of this reaction. There are two possible courses: protonation might occur either at the carbon atom that was originally the acetyl carbon atom of α-acetolactate (**1**) (Scheme 1a), or at the carbon atom that was originally the chiral centre (Scheme 1b). This regiochemical ambiguity is unique amongst those decarboxylases, the stereochemistries of which have so far been determined (see below). We have investigated this question by examining the product of decarboxylation of [3-^{14}C]-α-acetolactate.

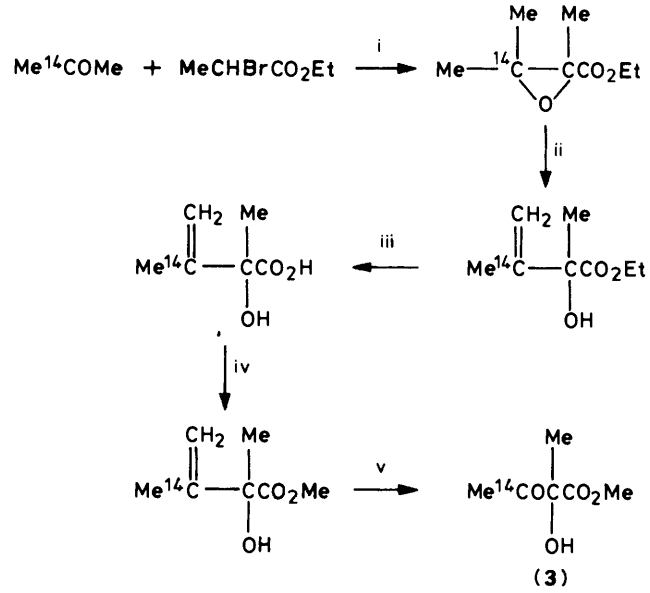
The methyl ester (**3**) of the required substrate was synthesised from [2-^{14}C]acetone as shown in Scheme 2. It was intended to subject this material to enzymatic decarboxylation using the decarboxylase from *Klebsiella aerogenes*, and to determine the location of the isotopic label in the acetoin produced by degradation of either the acetoin or of a suitable derivative.

For such an investigation to be valid, it was necessary to develop a procedure that would ensure that the results would not be influenced by adventitious isomerisations of the acetoin produced, or of the precursor α-acetolactate, or by the non-enzymatic decarboxylation of α-acetolactate.

Because of its lability with respect to decarboxylation, α-acetolactate for enzymatic investigations is prepared by alkaline hydrolysis of the corresponding esters. However, previous investigations had shown that α-acetolactate undergoes a degenerate, base-catalysed tertiary ketol rearrangement with carboxylate group migration (Scheme 3).[7] Since such a rearrangement would vitiate the results of the experiment, the pH limits under which it occurs were investigated. Because of the degeneracy of the rearrangement (Scheme 3), which makes it impossible to follow without using isotopically labelled material, the isomerisation was studied using its

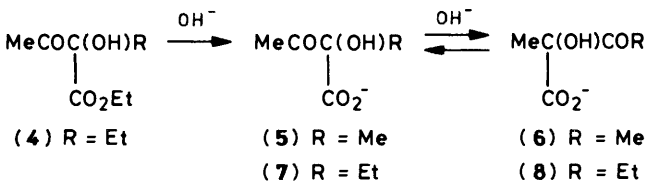


Scheme 1.

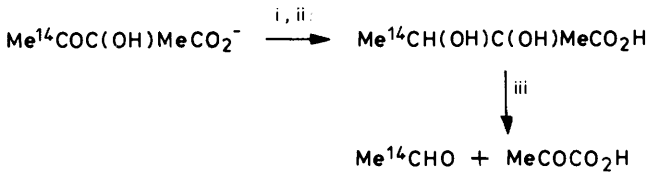


Scheme 2. *Reagents:* i, $LiN(CHMe_2)_2$; ii, TosOH, C_6H_6; iii, $Ba(OH)_2$; iv, CH_2N_2; v, O_3

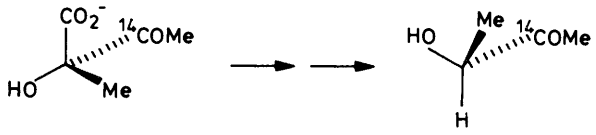
* *Aerobacter aerogenes* has been divided into the non-motile species *Klebsiella aerogenes* and the motile species *Enterobacter aerogenes*.[6]



Scheme 3.



Scheme 4. *Reagents:* i, $NaBH_4$; ii, H^+; iii, $NaIO_4$



Scheme 5.

higher homologue α-acetohydroxybutyrate [2-ethyl-2-hydroxy-3-oxobutanoate (**7**)], the biological precursor of isoleucine. With this compound, the rearrangement (Scheme 3, R = Et) could be studied by n.m.r. at ambient temperature in aqueous solution. The rearrangement was studied, using the corresponding sodium salt, under the following conditions: (*a*) 1M-NaOH (pH 14); (*b*) *ca.* 0.1M-NaOH (pH 12.9); (*c*) saturated calcium hydroxide solution (pH 12.5); and (*d*) phosphate buffer (pH 12.1). At pH 14, n.m.r. signals attributable to the product (**8**) of rearrangement increased in intensity to equal those of the substrate after 3 h, and there was no change over a further 4-h period. At pH 12.9, the signals due to the product (**8**) increased to half the intensity of α-acetohydroxybutyrate after 7 h. At pH 12.5 and pH 12.1, no evidence for rearrangement could be observed over a 7-h period. Accordingly it was concluded that rearrangement does not occur in these systems at a significant rate below pH 12.5.

Following incubation with (*RS*)-[3-^{14}C]-α-acetolactate, it was proposed to remove the (*R*)-isomer and any remaining (*S*)-isomer by passage through anion exchange resin in the hydrogen carbonate form. The necessary proof that this procedure did not cause isomerisation of the acetoin produced has been obtained in a related investigation of the stereochemistry of acetoin formation by the pyruvate decarboxylase of wheat germ.[8]

Methyl [3-^{14}C]-α-acetolactate (**3**) was hydrolysed with the stoicheiometric amount of 0.25M-sodium hydroxide and incubated with a preparation of the acetolactate decarboxylase from *Klebsiella aerogenes*. Unchanged α-acetolactate was removed by passage through anion exchange resin as described above. To aid isolation of the labelled acetoin, an excess of unlabelled, racemic material was added to the eluate, and the acetoin was isolated by continuous ether extraction and purified by conversion into the hydrogen phthalate and recrystallisation of the corresponding dicyclohexylammonium salt to constant activity. These procedures were known not to cause racemisation of the optically active acetoin or of the derivatives.[9]

Two additional experiments were performed as procedural checks. First, a parallel incubation of the [3-^{14}C]-α-acetolactate (**3**) with the complete system, omitting only the enzyme fraction, was carried out to determine the extent of non-enzymatic decarboxylation. Following removal of α-acetolactate from half of the control mixture and isolation of any labelled acetoin present after dilution with unlabelled material, the acetoin was converted into the hydrogen phthalate and the corresponding dicyclohexylammonium salt was recrystallised to constant activity. The latter showed only background levels of radioactivity, confirming the absence of non-enzymatic decarboxylation under the assay conditions.

To show that tertiary ketol rearrangement had not occurred, the α-acetolactate in the remaining half of the control mixture was stabilised by reduction with sodium borohydride. The (2*RS*,3*SR*)-component of the 2,3-dihydroxy-2-methylbutanoic acid formed was diluted with authentic material and converted into the dicyclohexylammonium salt. After recrystallisation to constant activity, the salt was treated with sodium periodate. The cleavage products, acetaldehyde and pyruvate, were isolated as the dimedone derivative and dicyclohexylammonium salts respectively (Scheme 4).[7] These were recrystallised to constant activity. The results (see Experimental section) showed that the radioactivity was entirely confined to the acetaldehyde derivative, thereby proving that tertiary ketol rearrangement had not occurred during the alkaline hydrolysis of the starting ester.

Table. Degradation of acetoin produced from [3-^{14}C]-α-acetolactate by acetolactate decarboxylase

1 2 3 4
$MeCOCH(OH)Me + MeMgI \longrightarrow (Me)_2C(OH)CH(OH)Me$

Derivative	Activity (d.p.m. mmol^{-1})	% Activity
Exp. 1		
2-Methylbutane-2,3-diol (^{14}C-1,-2,-3,-4)	7 180	100
Dimedone derivative of acetaldehyde (^{14}C-3,-4)	1 270	18
Exp. 2		
2-Methylbutane-2,3-diol (^{14}C-1,-2,-3,-4)	133,000	100
2,4-Dinitrophenylhydrazone of acetone (^{14}C-1,-2)	102,000	77
2,4-Dinitrophenylhydrazone of acetaldehyde (^{14}C-3,-4)	19 900	15

The diluted acetoin from the enzymatic decarboxylation was stabilised towards racemisation by treatment of the hydrogen phthalate reisolated from the radiochemically pure dicyclohexylammonium salt, with methylmagnesium iodide. This procedure was known not to cause racemisation of the acetoin.[9] The resulting 2-methylbutane-2,3-diol was treated with periodate. In one experiment the resulting acetaldehyde was isolated as the dimedone derivative and recrystallised to constant activity. (It was not found possible to isolate the acetone efficiently from this experiment.) In a second experiment, the 2,4-dinitrophenylhydrazones of acetone and acetaldehyde were separated and purified by h.p.l.c. and their activities were measured. The results from both degradations were consistent and showed (Table) that close to 80% of the radioactivity was located in the acetone and 20% in the acetaldehyde. It is estimated that these activity determinations are accurate to ± 5% (standard deviation).

These results show that protonation of the presumed enediol intermediate in the enzymatic decarboxylation of α-acetolactate occurs, at least predominantly, at the carbon atom to which the carboxylate group was originally attached. The formation of (*R*)-acetoin shows that, overall, the carboxylate group is replaced by hydrogen with inversion of configuration (Scheme 5).

In view of the precautions taken to avoid chemical artefacts in the experiment, it seems possible that protonation of the enediol intermediate formed might not be fully regiospecific. However, if this were so, the protonation would, nevertheless, need to be fully stereospecific, since (*R*)-acetoin is produced exclusively.[8] Alternatively, the enzymatic decarboxylation may not be fully stereoselective for the (*S*)-isomer of α-acetolactate. Observations by Juni suggested that (*R*)-α-acetolactate was decarboxylated in an enzyme preparation from *Aerobacter aerogenes* at one-twentieth the rate of the (*S*)-isomer.[10] However, if decarboxylation of the (*R*)-isomer is truly enzymatic, the results can only be explained by an unlikely mechanism in which stereospecific protonation of the enediol intermediate occurs at the carbon atom that formerly constituted the acetyl carbon atom of α-acetolactate.

Because (*R*)-acetoin is the exclusive product of the decarboxylation, an explanation likewise cannot lie in the action of an acetoin racemase, the existence of which in *Aerobacter aerogenes* cannot be ruled out completely.[11]

Hill and his colleagues also noted the slow decarboxylation of (*R*)-α-acetolactate after an induction period.[4] This was attributed to prior racemisation of (*R*)-α-acetolactate to the (*S*)-isomer by the base-catalysed carboxylate migration mechanism.[7] The operation of such a mechanism on (*R*)-[3-^{14}C]-α-acetolactate would produce (*S*)-[2-^{14}C]-α-acetolactate, decarboxylation of which with regiospecific protonation of the original α-carbon atom would give acetoin labelled at the carbinol carbon atom. However, as the checks carried out in the current experiments show, such an explanation would appear unlikely.

The present results show that α-acetolactate must be included in the small group of decarboxylases known to operate with overall inversion of configuration, *viz.* 6-phosphogluconate dehydrogenase,[15] sterol 4-carboxylic acid decarboxylase,[16] UDP glucuronate carboxylase (EC 4.1.1.35),[17] and aspartate β-decarboxylase.[13] By contrast, α-decarboxylation of amino acids appears to proceed with retention of configuration in all the cases so far studied.[12,18-23] Other enzymes catalysing the decarboxylation of β-keto acids with overall retention of configuration are known.[24-27] Discussion of enzymatic decarboxylations should also include mention of the biotin-dependent carboxylases which catalyse the reversible carboxylation, with retention of configuration, of various coenzyme A esters.[24,28-30]

The substrates for all of the enzymatic decarboxylations so far investigated are known, or are presumed, to have a β-keto or β-imino function in the reactive, enzyme-bound intermediate. Decarboxylation may be presumed to proceed *via* a conformation in which the C–CO_2H bond is orthogonal to the plane of the adjacent trigonal system. Decarboxylation to an enol or enolate system would be followed by C-protonation. During this process there would appear to be no fundamental stereoelectronic compulsion for attack on one or the other face of the enol or enolate. The observation of an inversion or retention mode may therefore be of greater phylogenetic than mechanistic significance. Thus, the decarboxylation with retention of configuration in the pyridoxal phosphate-dependent α-amino acid decarboxylases accords with the propensity for enzymatic reactions requiring this co-factor to operate exclusively on one face of the Schiff's base system formed by condensation of pyridoxal phosphate with the amino acid. An exception is provided by aspartate β-decarboxylase which, when catalysing decarboxylation of aminomalonic acid, gives a product with retention of configuration[12] but operates to give overall inversion of configuration in the β-decarboxylation of aspartate.[13]

It is noteworthy that those retention-mode enzymes that operate on substrates that are not amino acids[24-27] are all derived from animal sources and operate on substrates which bear a carboxy group in the β-position. The inversion-mode enzymes listed above, with the exception of aspartate β-decarboxylase, catalyse the decarboxylation of substrates lacking a carboxyl group in the β-position. Apart from these features, there appear to be no other common differences between the stereochemical classes of enzyme. For example, in the inversion-mode enzymes, formation of a Schiff's base intermediate is presumably involved in the β-decarboxylation of aspartate,[13] but is not involved in the decarboxylation of the 3-oxo-4-carboxylic acid intermediate in cholesterol biosynthesis.[14]

Experimental

N.m.r. spectra were determined using JEOL MH-100, Hitachi-Perkin-Elmer R600, or JEOL PS-100 spectrometers. Sodium 3-trimethylsilylpropanesulphonate was used as internal standard for spectra recorded in water. U.v. absorbance measurements were made on a Pye Unicam SP 500 series 2 spectrophotometer. G.l.c. analyses were performed using Pye Unicam GCD, 104 or 105 chromatographs fitted with a flame ionisation detector. The systems used were: (1) 1.6 m × 3 mm (i.d.) glass column packed with 15% Carbowax 20M on Chromosorb W (80—100 mesh); (2) 3.4 m × 3 mm (i.d.) glass column packed with 10% SE 30 on 80—100 mesh Chromosorb W. The carrier gas (N_2) flow rate was 30 ml min^{-1}. Bulb-tube distillations were carried out using a bulb-tube oven model GKR 50 (Buchi AG, Flawil, Switzerland). H.p.l.c. separations were carried out using an M 6000A solvent delivery system [Waters Associates (Inst.) Ltd.], in association with Pye LC3 or CE 2012 (Cecil Instruments Ltd.) variable wavelength u.v. detectors. The h.p.l.c. column used was a 25 cm × 10 mm (i.d.) stainless steel column packed with Spherisorb ODS (10 micron). Radioactivity measurements were made using an LKB Rackbeta 1215 liquid scintillation counter on samples dissolved in Packard 229 liquid scintillation cocktail. Low speed and high speed centrifugations were carried out using MSE 18 and MSE 65 centrifuges respectively. *Klebsiella aerogenes* NCTC 8172 was obtained from the Central Public Health Laboratories, London.

Sodium α-Acetohydroxybutyrate [*as* (**7**)].—To a solution of methyl α-acetohydroxybutyrate (30 mg, 0.19 mmol) in water (1 ml) was added dropwise with stirring a 5% excess of sodium hydroxide solution (0.1 M; 2 ml). The solution was stirred for a further 10 min, at which point it was found to be strongly alkaline towards phenolphthalein. The excess of sodium hydroxide was neutralised by the dropwise addition of hydrochloric acid (0.1M) until the purple colouration had faded but had not completely disappeared. The solution was lyophilised to give the sodium α-acetohydroxybutyrate as a colourless gum; δ (D_2O) 2.26 (3 H, s, MeCO), 1.6—2.2 (2 H, m, MeCH_2), and 0.86 (3 H, t, J 7.5 Hz, *Me*CH_2).

Base-catalysed Rearrangement of Sodium α-Acetohydroxybutyrate.—Sodium α-acetohydroxybutyrate (*ca.* 30 mg amounts) was dissolved in the following solutions (0.4 ml): (i) sodium hydroxide (1M; pH 14.0); (ii) sodium hydroxide (*ca.* 0.1M; pH 12.9); (iii) saturated calcium hydroxide (pH 12.5); (iv) disodium hydrogen phosphate–sodium hydroxide buffer, pH 12.1. The ^{1}H n.m.r. spectra of each of these solutions were followed for 7 h to give the results described in the text. The rearrangement product [sodium 2-hydroxy-2-methyl-3-oxopentanoate (**8**)] gave the following n.m.r. signals: δ (D_2O) 2.5—2.8 (2 H, m, MeCH_2), 1.44 [3 H, s, MeC(OH)], and 1.01 (3 H, t, *J* 7.5 Hz, *Me*CH_2).

[3-^{14}C]-2-*Hydroxy*-2,3-*dimethylbut*-3-*enoic Acid.*—This was prepared by adapting the published procedure,[31] as follows. A

flask of 10-ml capacity was flame-dried and allowed to cool under dry nitrogen. After the introduction of a magnetic follower, the flask was sealed with a septum cap. All reactants and reagents were subsequently added by syringe. Di-isopropylamine (424 μl) (freshly distilled and stored over 4A molecular sieves) and hexane (2.5 ml, freshly distilled over lithium aluminium hydride and stored over 3A molecular sieves) were added to the flask and the mixture then cooled to 0 °C. Butyl-lithium in hexane (2.22 ml, 3 mmol) was added and the mixture was cooled to −78 °C. Tetrahydrofuran (2 ml, distilled over calcium hydride–cuprous chloride, freshly distilled before use and stored over 3A molecular sieves) was added, followed by ethyl 2-bromopropionate (0.39 ml, 3 mmol). The faint green solution was stirred for 30 min at −78 °C. A solution of acetone 0.22 ml, 3 mmol) in tetrahydrofuran (1 ml) was loaded into a syringe. A vial containing [2-^{14}C]acetone (0.3 mg, 250 μCi) was cooled to −78 °C and opened. The acetone solution was added, drawn back into the syringe, and added to the reaction mixture. The mixture was stirred at −78 °C for 60 min and allowed to warm to room temperature (10 min). Saturated ammonium chloride (4 ml) was added, the mixture was shaken and separated, the aqueous layer was extracted with ether (6 ml), and the combined organic layers were washed with saturated sodium chloride solution (6 ml) and dried ($MgSO_4$). The solution was filtered, the solvent was cautiously removed under reduced pressure, the residue was transferred to a small flask with the aid of benzene (5 ml), and the solution was concentrated to 3 ml by distillation. In a trial preparation on the same scale, the product was of > 95% purity at this stage by g.l.c. (system No. 2, 130 °C). A solution of toluene-*p*-sulphonic acid monohydrate (19 mg) in benzene (5 ml) was concentrated to 1 ml by distillation. The solution of ethyl [3-^{14}C]-2,3-epoxy-2,3-dimethylbutanoate, above, was added. The mixture was boiled under reflux under nitrogen for 18 h. After this time, no more starting material could be seen by g.l.c. (system No. 2) and the product was of > 95% purity. The solution was washed with sodium carbonate solution (1M; 5 ml) and the alkaline washings were re-extracted with ether (2 × 5 ml). The combined extracts were dried ($MgSO_4$) and evaporated. The residue was boiled under reflux with a solution of barium hydroxide octahydrate (470 mg, 3 mmol) in water (6 ml) for 75 min. The cooled solution was treated with solid carbon dioxide, filtered, acidified to pH1 (HCl) and extracted continuously with ether for 48 h. The ethereal extract was dried ($MgSO_4$) and evaporated and the residue was recrystallised (ether–cyclohexane) to give [3-^{14}C]-2-hydroxy-2,3-dimethylbut-3-enoic acid (263 mg, 141 μCi). Overall chemical yield 67%, radiochemical yield 56%.

Methyl [3-^{14}C]-α-*Acetolactate* (**3**).—To a solution of [3-^{14}C]-2-hydroxy-2,3-dimethylbut-3-enoic acid (10.6 mg, 0.08 mmol, 69.5 μCi mmol^{-1}) in ether (2 ml) was added an excess of an ethereal solution of diazomethane. The ether and the excess of diazomethane were removed under reduced pressure. The product, methyl [3-^{14}C]-2-hydroxy-2,3-dimethylbut-3-enoate was homogeneous by g.l.c. (system No. 2; 125 °C). A solution of the ester in ethyl acetate (10 ml) was ozonised for 10 min, after which time no more starting material was detectable by g.l.c. The solution was shaken vigorously with a suspension of manganese dioxide (30 mg) in water (10 ml). The organic layer was separated, and the aqueous residue was extracted with ether (3 × 30 ml). The combined organic solutions were washed with sodium hydrogen carbonate solution (0.2M, 20 ml) and the washings were re-extracted with ether (2 × 50 ml). The combined organic solutions were dried ($MgSO_4$), and evaporated to give the crude methyl [3-^{14}C]-α-acetolactate (11 mg). This was diluted with inactive material (84 mg) and distilled (bulb-tube, 110 °C, 10 mmHg), to give the ester (**3**) (69 mg, 3.3 μCi mmol^{-1}), > 95% pure by g.l.c. (system No. 2, 125 °C).

Preparation of the Acetolactate Decarboxylase from Klebsiella aerogenes.—The cells were grown in L broth [Tryptone (10 g l^{-1}), yeast extract (5 g l^{-1}), sodium chloride (10 g l^{-1}), and glucose (1 g l^{-1})] at 30 °C. The culture was harvested using an MSE constant flow rotor. The cell paste (7 g) was disrupted in a French press. The broken cells were resuspended in pH 6.2 buffer [piperazine-1,4-diylbisethanesulphonic acid (PIPES) (0.01M), magnesium chloride (0.001M), phenylmethanesulphonyl fluoride (2.10^{-5}M), benzamidine hydrochloride (10^{-5}M), and 2-mercaptoethanol (0.006M)]. The mixture was centrifuged at 10^4 r.p.m. for 10 min and the supernatant was centrifuged at 14.10^3 r.p.m. for 1 h to remove cell debris. The supernatant was heated at 70 °C for 3 min to destroy α-acetohydroxy acid synthetase activity.[32] The precipitated protein was removed by centrifugation at 15.10^3 r.p.m. for 10 min. The supernatant (protein concentration typically 4—8 mg ml^{-1}) was used for decarboxylation of α-acetolactate without further purification. The concentrations of protein in the enzyme preparations were determined by the method of Lowry *et al.*[33]

Assays for α-*Acetolactate Decarboxylase Activity.*—A 1-ml volume of assay solution consisted of pH 6.2 buffer [PIPES (0.01M)], the enzyme preparation (0.1 ml), and magnesium chloride (0.001M), and contained sufficient of the hydrolysate from ethyl α-acetolactate (40 mg) and sodium hydroxide (0.25M; 0.96 ml) to bring the substrate concentration to 2.5 × 10^{-2}M. Acetoin production was monitored by the Westerfeld procedure.[34]

Enzymatic Decarboxylation of [3-^{14}C]-α-*Acetolactate.*—Methyl [3-^{14}C]-α-acetolactate (22 mg, 0.15 mmol; $7.3.10^6$ d.p.m. mmol^{-1}) was dissolved in sodium hydroxide solution (0.25M; 0.87 ml). The solution was left to stand for 20 min, the pH was adjusted to 8.0 with hydrochloric acid (0.5M, 35 μl) and an aliquot (0.82 ml) was added to a mixture of the enzyme preparation from *Klebsiella aerogenes*, above, in 3.75 ml of buffer, pH 6.2. The mixture was incubated at 30 °C for 1.5 h. Assay of a control incubation with inactive α-acetolactate indicated the production of 137 μg ml^{-1} of acetoin. The mixture was applied to a column of Dowex 1-X8 anion exchange resin (10 ml, 20—50 mesh, hydrogen carbonate form) and eluted with water. To the first 40 ml of eluate was added inactive acetoin (250 mg, 2.84 mmol). The solution was saturated with sodium chloride and extracted continuously with ether for 18 h. The ethereal extract was dried ($MgSO_4$) and all but *ca.* 3 ml of the ether was removed by distillation under nitrogen through a 40-cm column packed with glass helices, and from a water bath at 45 °C. The residual solution was mixed with dry pyridine (4 ml) and to the mixture were added phthalic anhydride (420 mg, 2.84 mmol) and 4A molecular sieve pellets (750 mg). The mixture was left for 7 days and was diluted with ether (40 ml). The solution was washed with hydrochloric acid (2M; 40 ml) and the acidic washings were re-extracted with ether (2 × 40 ml). The combined ethereal solutions were dried ($MgSO_4$) and evaporated. The residual hydrogen phthalate, in acetone solution, was treated with dicyclohexylamine (1 equiv.) in acetone. The dicyclohexylammonium salt obtained was recrystallised to constant activity from acetone (133.10^3 d.p.m. mmol^{-1}, 394 mg). A control solution, identical with the incubation mixture but lacking the enzyme preparation (total volume 2 ml), and containing 180 μl of the [3-^{14}C]-α-acetolactate solution, was incubated as in the enzymatic decarboxylation. The resulting solution was divided into two parts. From one portion, the acetoin was isolated in a manner identical with that described above, and was recrystallised to constant activity as the dicyclohexylammonium salt of the hydrogen phthalate. The activity of this salt was 1 d.p.m. mg^{-1},

showing that there was no non-enzymatic decarboxylation under these conditions. To the remaining half of the control mixture, water (5 ml), ethanol (5 ml), and sodium borohydride (30 mg) were added. The solution was left overnight and saturated with sodium chloride. The resulting solution (of pH > 8) was extracted continuously with ether for 24 h. The remaining aqueous solution was acidified (Congo red) with conc. hydrochloric acid and extracted continuously with ether for 60 h. The ethereal extracts were dried ($MgSO_4$) and the ether was evaporated off under reduced pressure. The residual mixture of isomers of 2,3-dihydroxy-2-methylbutanoic acid (**9**) (*ca.* 2 mg) was mixed with the inactive dicyclohexylammonium salt of (2*RS*,3*SR*)-2,3-dihydroxy-2-methylbutanoic acid (2.99 g, 9.5 mmol) and the whole was recrystallised (methanol–ethyl acetate) to constant activity (2 820 d.p.m. $mmol^{-1}$).

Degradation of the Dicyclohexylammonium Salt of [^{14}C]-2,3-*Dihydroxy-2-methylbutanoic Acid.*—To a solution of the dicyclohexylammonium salt of the dihydroxy acid [as (**9**)] (270 mg, 0.86 mmol) in water (12 ml) was added sodium periodate (231 mg, 1.08 mmol). The solution was kept in the dark for 20 min, diluted with water (20 ml) and boiled under reflux in a stream of nitrogen. The exit gases were passed into an aqueous solution of dimedone (400 mg in sodium acetate–hydrochloric acid buffer, pH 4.6; 100 ml). The dimedone derivative of acetaldehyde was recrystallised to constant activity from methanol–water. The remaining solution was cooled, evaporated to dryness, and the residue was dried under reduced pressure over silica gel. The residue was re-extracted with boiling, dry acetone (3 × 10 ml). The acetone extracts were filtered and evaporated. The residual dicyclohexylammonium pyruvate (147 mg) was recrystallised (acetone) to constant activity. The ratio of the activities of the acetaldehyde dimedone derivative and dicyclohexylammonium pyruvate was 129:1.

2-*Methylbutane*-2,3-*diol from the Hydrogen Phthalate of Acetoin.*—The dicyclohexylammonium salt of the hydrogen phthalate of acetoin produced by enzymatic decarboxylation of [3-^{14}C]-α-acetolactate (394 mg, 133.10^3 d.p.m. $mmol^{-1}$) in ethanol–water (1:1; 10 ml) was applied to a column of Dowex 50W-X8 cation exchange resin (10 ml resin; 20—50 mesh, H^+ form) which had previously been equilibrated with ethanol–water (1:1). The column was eluted with the same mixture. The first 50 ml of eluate was extracted with ether (3 × 40 ml). The combined ethereal extracts were dried ($MgSO_4$), and evaporated, finally at 0.1 mmHg for 2 h. The hydrogen phthalate was obtained as a pale yellow oil (218 mg, 98%). This was added in ethereal solution (3 ml) to a solution of methylmagnesium iodide (4 mmol) in ether (6 ml) with stirring. The mixture was boiled under reflux for 1.5 h, cooled and treated with hydrochloric acid (2M; 5 ml). The mixture was shaken, the aqueous layer was saturated with sodium chloride and extracted continuously with ether for 18 h. The ethereal extract was dried ($MgSO_4$) and filtered. The filtrate was stirred vigorously with a few drops of mercury for a few minutes until the brown colouration had disappeared. The mixture was filtered and evaporated. The residue was distilled [bulb-tube, 125 °C, 15 mmHg (lit.,[35] b.p. 93—95 °C, 24 mmHg)] to give the 2-methylbutane-2,3-diol as a colourless oil, pure by g.l.c. (system No. 1, 135 °C) (52 mg, 55%).

Degradation of 2-*Methylbutane*-2,3-*diol.*—(i) The radioactive 2-methylbutane-2,3-diol (2.9 mg, 0.03 mmol) was diluted with inactive 2-methylbutane-2,3-diol (51 mg, 0.49 mmol) and the mixture was diluted to 10 ml (H_2O). Sodium periodate (120 mg, 0.56 mmol) was added and the mixture was kept in the dark for 15 min. Sodium arsenite (65 mg, 0.5 mmol) was added. The mixture was stirred for 15 min, and added to a solution of dimedone (200 mg) in pH 4.6 buffer (50 ml) as above. The dimedone derivative of acetaldehyde (105 mg) was recrystallised to constant activity (ethanol–water) (1 270 d.p.m. $mmol^{-1}$).

(ii) The radioactive 2-methylbutane-2,3-diol (10 mg, 0.096 mmol) was dissolved in water (10 ml) and sodium periodate was added. The mixture was kept for 15 min in the dark, sodium arsenite (14 mg, 0.11 mmol) was added, and the mixture was stirred for 15 min. The mixture was boiled under reflux in a stream of nitrogen. The exit gases were passed into a solution of 2,4-dinitrophenylhydrazine (600 mg) in hydrochloric acid (1.44M). The mixture was extracted with dichloromethane (4 × 20 ml) and the combined organic extracts were washed with water (3 × 20 ml). The organic extracts were dried (Na_2SO_4) and evaporated to give the mixture of 2,4-dinitrophenylhydrazones of acetone and acetaldehyde. Aliquots of this mixture were separated by h.p.l.c. in methanol–water (80:20). Samples of the separated derivatives were rechromatographed to confirm their purity (> 95%). The concentrations of solutions of the derivatives were determined by comparison of their u.v. absorbances against standard solutions. Radioactivity measurements on the solutions were quench-corrected using [^{14}C]hexadecane as the internal standard. Duplicate determinations agreed to within ±3%. The specific activities of the starting 2-methylbutane-2,3-diol and of the degradation products are given in the Table.

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