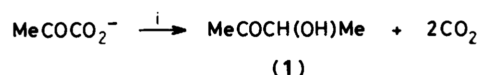
Acetoin Metabolism: Stereochemistry of the Acetoin Produced by the Pyruvate Decarboxylase of Wheat Germ and by the α-Acetolactate Decarboxylase of *Klebsiella aerogenes*

David H. G. Crout,* Jennifer Littlechild, and Stephen M. Morrey
Department of Chemistry, University of Exeter, Exeter EX4 4QD

Acetoin (3-hydroxybutan-2-one) (1) produced from pyruvate and acetaldehyde by the pyruvate decarboxylase of wheat germ consists of a mixture of enantiomers with the (*S*)-(+)-isomer preponderating. With pyruvate alone as substrate, racemic acetoin is produced. Acetoin produced from pyruvate by the sequential action of the acetohydroxy acid synthetase and α-acetolactate decarboxylase of *Klebsiella aerogenes* consists solely of the (*R*)-(−)-isomer.

It is generally accepted that synthetic enzymes operate with a high degree of stereospecificity. The observation that an enzyme operates on its normal substrate with only partial stereospecificity is therefore exceptional. It is even more unusual to observe an enzymatic reaction in which the degree of stereospecificity is partial, constant, and reproducible. A reported example is the reaction catalysed by the pyruvate decarboxylase (2-oxo-acid carboxylase EC 4.1.1.1) of wheat germ[1,2] (Scheme 1) which leads to the production of acetoin. Acetoin (3-hydroxybutan-2-one) (1) and in particular its congener diacetyl (butane-2,3-dione) are of considerable importance as aroma constituents of a number of fermented foods and beverages.



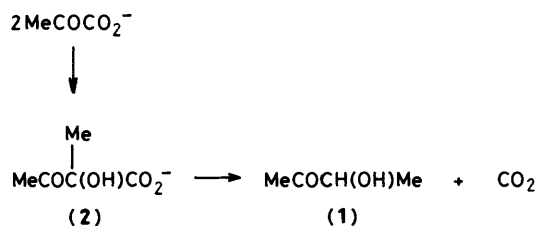
Scheme 1. *Reagent:* i, pyruvate decarboxylase

The principal function of pyruvate decarboxylase is to convert pyruvate into acetaldehyde. Acetoin production is stated to be an adjunct of acetadehyde production with all enzymes capable of decarboxylating pyruvate.[3] Classically, acetoin production has been regarded as a mechanism for switching glucose metabolism from acidic to neutral products.[4]

In 1952, Singer and Pensky reported that the acetoin produced by wheat germ pyruvate decarboxylase, consistently showed an optical rotation of 36 ± 1° which, taking the best available value for the specific optical rotatory power of −84 ± 2° for material isolated from *Aerobacter aerogenes*, indicated a composition for the mixture of 72% of the *S*-(+)-isomer and 28% of the (*R*)-(−)-isomer. This result is quoted as the first example of proven non-stereospecificity in a biosynthetic enzymatic reaction.[2] However, the validity of the result was in doubt, because of the uncertainty in the value for the optical rotatory power of the acetoin taken as the reference standard. There was no evidence that this material was optically pure.

Recently we have synthesized (*R*)- and (*S*)-acetoin of high optical purity and have confirmed specific rotations of −84 ± 3° for the (*R*)-isomer and 82 ± 3° for the (*S*)-isomer.[5] A reinvestigation of the remarkable claim of Singer and Pensky was therefore undertaken.

It was proposed to incubate the enzyme with [^{14}C]pyruvate and acetaldehyde, and to determine the enantiomer composition of the acetoin produced by dilution analysis against a crystalline derivative of optically pure acetoin. In order to confirm the validity of the dilution method, it was first used to assay the enantiomeric composition of the acetoin produced by *Klebsiella aerogenes*. Acetoin is produced in this organism by condensation of pyruvate to α-acetolactate (2) in a reaction catalysed by acetolactate synthase [acetolactate pyruvate-lyase (carboxylating) (EC 4.1.3.18)], followed by decarboxylation of the (*S*)-isomer in a reaction catalysed by acetolactate decarboxylase [2-hydroxy-2-methyl-3-oxobutyrate carboxy-lyase (EC 4.1.1.5)][6] (Scheme 2). The acetoin produced is



Scheme 2.

laevorotatory[7] and consists solely, or predominantly of the *R*-isomer.[8]

For the dilution analysis, the dicyclohexylammonium salt of the hydrogen phthalate of acetoin was used. This could not be obtained directly by optical resolution of the hydrogen phthalate of acetoin because of the formation of a racemic compound.[5] Accordingly, the required hydrogen phthalate was prepared by Lemieux-Johnson oxidation[9] of the optically pure hydrogen phthalate of (*S*)-3-methylbut-3-en-2-ol[5] and purified as the dicyclohexylammonium salt. The hydrogen phthalate was recovered from the salt, converted into the methyl ester and examined by n.m.r. in the presence of a chiral shift reagent.[5] An optical purity of ≥96% was demonstrated.

From *Klebsiella aerogenes* a preparation was obtained which contained high levels of acetolactate synthase and acetolactate decarboxylase activities.[6] [2-^{14}C]Pyruvate was incubated with this preparation and the acetoin produced was isolated and converted into the dicyclohexylammonium salt of the hydrogen phthalate by a procedure which previously had been shown not to cause epimerisation.[10] The salt was recrystallised to constant radioactivity. A small portion was then subjected to dilution analysis against the optically pure salt of (*S*)-acetoin. After 20 recrystallisations, the activity had fallen to 1.6% of the initial value, showing that the acetoin produced by *K. aerogenes* consists of ≥98% of the (*R*)-(−)-isomer.

The acetoin produced from pyruvate by *Aerobacter aerogenes* (= *K. aerogenes*) was known to be laevorotatory,[7] but the reported value of $[\alpha]_D^{25}$ was −94° whereas the true rotation is −84 ± 3°.[5] The discrepancy presumably arose from the

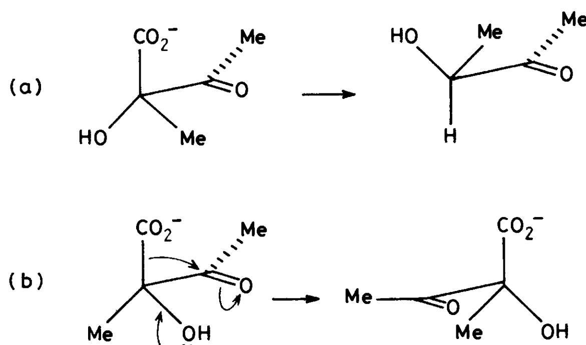
Table. Incubation conditions for acetoin production by the enzyme preparation from *Klebsiella aerogenes* and the pyruvate decarboxylase of wheat germ.

	Assay mixture[a]		
Enzyme preparation (µl)	100	150	100
Sodium pyruvate (0.25M) (µl)	150	100	35
Acetaldehyde (1M) (µl)			50
Dimedone (0.3mM) (µl)	200	200	
^{14}C-Activity (10^6 d.p.m.)	25.8	111	25.8

In addition to the above components, each assay mixture (total volume 1 ml) contained PIPES (1M; 100 µl), $MgSO_4$ (1M; 5 µl), thiamin pyrophosphate (0.019M; 15 µl), and bovine serum albumin (3 mg ml^{-1}; 100 µl).

[a] Assay mixture 1: Enzyme preparation from *K. aerogenes*, Assay mixtures 2 and 3: Enzyme preparation from wheat germ.

presence of optically active impurities in the acetoin preparation from *A. aerogenes*. The present results show that the *A. aerogenes* system produces (*R*)-(−)-acetoin of high optical purity. This result is significant in the light of the reported stereoselectivity of the acetolactate decarboxylase,[11,12] which, although catalysing preferentially the decarboxylation of (*S*)-α-acetolactate, nevertheless catalyses the decarboxylation of the (*R*)-isomer although at a much lower rate and after an induction period. This was attributed to racemisation of the substrate by the carboxylate migration mechansim [Scheme 3(b)].[11] How-



Scheme 3.

ever, this rearrangement only occurs at high pH in non-enzymatic systems,[5] and would certainly not take place at the pH of the enzyme incubation. There remains the intriguing possibility that rearrangement of the (*R*)-isomer is enzyme-catalysed, a supposition made less improbable if it is noted that both decarboxylation [Scheme 3(a)] and rearrangement [Scheme 3(b)] with carboxylate ion migration require labilisation of the C–CO_2^- carbon–carbon bond.

This experiment not only demonstrated the high optical purity of the acetoin produced by *K. aerogenes*, it also demonstrated that the proposed dilution analysis of the acetoin produced by wheat germ would not be vitiated by entrainment of the minor isomer, should it be found that the acetoin produced was indeed a mixture of isomers. This experiment also confirmed the assumption, based on various procedural checks, that the isolation and derivatisation procedure was not attended by epimerisation.

[2-^{14}C]Pyruvate was incubated with an enzyme preparation from wheat germ in the presence of acetaldehyde, as described by Singer and Pensky. Dilution analysis against the dicyclohexylammonium salt of the hydrogen phthalate of (*S*)-acetoin gave, after 15 recrystallisations, material of constant radioactivity which remained constant over a further 2 recrystallisations. The specific activity had fallen to 58% of its initial value, showing that the material isolated consisted of a mixture of 58% *S*-isomer and 42% *R*-isomer with an estimated error of ±3%. Thus, although the ratio was not as high as that found by Singer and Pensky[1a,2] [72% (*S*)-, 38% (*R*)-isomer], an excess of the (*S*)-isomer was indeed produced, supporting their general conclusion.

A further experiment was carried out in which [2-^{14}C]-pyruvate alone was incubated with the enzyme. In this case, dilution analysis gave a dicyclohexylammonium salt of the hydrogen phthalate of acetoin containing 50 ± 2% of the initial activity, constant over a further 3 recrystallisations. In this case however, the production of acetoin was substantially lower than with pyruvate plus acetaldehyde as the substrate.[1a] To assure the validity of this experiment, one further procedural check was necessary since an extra step, passage of the incubation mixture through an anion exchange resin in the acetate form, was involved. This was considered desirable because of the low conversion into acetoin with pyruvate alone as substrate (see Experimental section). The ion exchange treatment was used to remove the excess of labelled pyruvate thus greatly simplifying the radiochemical purification of the labelled acetoin derivatives.

Two checks were conducted. First, measurement of the change in optical activity of acetoin during passage through the column showed that less than 10% epimerisation had occurred. Second, catalysis of exchange of the methine hydrogen of acetoin in D_2O in the presence of the ion exchange resin was studied by n.m.r. After 18 h, no exchange was apparent. Since the acetoin was in contact with the resin for less than 5 min during the isolation procedure, it was concluded that the ion exchange treatment did not cause significant epimerisation.

In discussing his initial observations on the optical activity of the acetoin produced by the pyruvate decarboxylase of wheat germ, Singer[2] considered an explanation involving several active centres, but preferred an explanation based on lack of stereospecificity in the condensation step. However, the wheat germ enzyme is very similar to yeast pyruvate decarboxylase for which Juni[3] and others[13] have adduced strong evidence in favour of a two-site mechanism. Thus, acetaldehyde is a non-competitive inhibitor of decarboxylation, but not of acetoin formation.[3] Increasing concentrations of acetaldehyde progressively retard pyruvate decarboxylation until the ratio of pyruvate decarboxylation to acetoin formation approaches unity.

Our observation of the formation by the wheat germ enzyme of partially racemic acetoin in the presence of acetaldehyde but of racemic acetoin in its absence cannot be explained by Singer's hypothesis but supports the operation of a two-site mechanism for this enzyme also. In the absence of added acetaldehyde, the acetaldehyde required for acetoin synthesis can be provided only by release from 'active acetaldehyde', the product of decarboxylation of the thiamin pyrophosphate–pyruvate adduct. The evidence therefore suggests that condensation of acetoin occurs at this site in a completely non-stereospecific manner. However, in the presence of high concentrations of acetaldehyde, binding would occur at a second site to which 'active acetaldehyde' could be transferred, and at which stereospecific or partially stereospecific condensation to acetoin would occur.

Explanations based on epimerisation following condensation are made unlikely by the inability of pyruvate decarboxylase to catalyse the epimerisation of exogenous acetoin.[1a]

Experimental

All melting points are corrected. Proton n.m.r. spectra were recorded with JEOL JNM-100 or JNM-PS-100 spectrometers

at 100 MHz. Spectra were recorded in deuteriochloroform using $SiMe_4$ as the internal standard. I.r. spectra were recorded on a Perkin-Elmer 398 spectrometer as KBr discs. Radioactivity measurements were carried out in duplicate using an LKB RackBeta 1215 liquid scintillation counter. Sufficient counts were recorded to give a statistical error of $<1\%$. Activities are recorded as disintegrations per minute per milligram (d.p.m. mg^{-1}). Piperazine-1,4-diethanesulphuric acid (PIPES), phenylmethanesulphonylfluoride (PMSF), benzamidine hydrochloride (BAM), and 2-mercaptoethanol for enzyme incubations were obtained from Sigma. [2-^{14}C]pyruvate was obtained from the Radiochemical Centre, Amersham. Fresh wheatgerm was obtained from Lockear and Son, Crewkerne, Somerset. DNase was obtained from Worthington Biochemicals. Ether refers to diethyl ether.

Hydrogen Phthalate of (S)-*Acetoin* (**1**).—To a solution of the hydrogen phthalate of (*S*)-3-methylbut-3-en-2-ol[5] (4.68 g) in dioxane (60 ml) and water (20 ml) was added a solution of osmium tetraoxide (50 mg) in water (5 ml). After 10 min, the addition was begun of finely ground sodium periodate (9.0 g). Addition was continued, with stirring over 45 min. The solution was allowed to stand for a further 2 h during which time a white precipitate was formed. The mixture was diluted with water (50 ml) and extracted with ether (3 × 80 ml). The combined ether extracts were dried ($MgSO_4$) and evaporated. The residue was dissolved in acetone (30 ml) and the solution was treated with a solution of dicyclohexylamine (3.6 g) in acetone (30 ml). The acetone was removed under reduced pressure and the residual salt was thrice recrystallised from acetone to give the dicyclohexylammonium salt of the hydrogen phthalate of acetoin (**1**) (4.6 g, 55%), m.p. 135 °C, $\nu_{max.}$ 1 725 and 1 740 cm^{-1} (CO). (Found: C, 69.1; H, 8.75; N, 3.50. $C_{24}H_{35}NO_5$ requires C, 69.04; H, 8.45; N, 3.36%). The c.d. curve of the salt in methanol showed maxima at 238 ($\Delta\varepsilon = +2.45$) and 278 nm ($\Delta\varepsilon = +1.13$). In methanol–HCl, the free acid showed maxima at 242 ($\Delta\varepsilon = +3.39$) and 227 nm ($\Delta\varepsilon = +0.97$). A sample of the salt (200 mg) in water (10 ml) was applied to a column of Dowex 50W-X8 ion exchange resin (H^+ form; 5 ml) and eluted with water (10 ml). The eluate was extracted with ether (3 × 20 ml) and the combined ether extracts were dried ($MgSO_4$) and evaporated to give the hydrogen phthalate of (*S*)-acetoin as a colourless oil {99 mg, 87%, $[\alpha]_D^{26}$ 27 ± 3° (*c* 1, acetone)}. The acid was methylated (diazomethane) and examined by n.m.r. in the presence of a chiral shift reagent at −20 °C as previously described[5] (molar ratio of shift reagent:ester = 2:1). An enantiomeric purity of >96% was indicated.

Enzyme Preparation from Klebsiella aerogenes.—*Klebsiella aerogenes* NCTC 8172 was grown in 12 l batches in L broth [tryptone (10 g l^{-1}), yeast extract (5 g l^{-1}), sodium chloride (10 g l^{-1}), glucose (1 g l^{-1})] at 30 °C. The culture was harvested in a constant flow MSE rotor. The cell paste (7 g) was disrupted in a French press. The broken cells were resuspended in pH 6.2 buffer [PIPES (0.01M), magnesium chloride (0.001M), PMSF (0.021mM), BAM (0.01mM), 2-mercaptoethanol (0.006M)]. The mixture was centrifuged at 10 000 r.p.m. for 15 min and the supernatant was further centrifuged at 14 000 r.p.m. for 1 h. The supernatant contained both acetohydroxy acid synthetase and α-acetolactate decarboxylase activities and was used for the production of acetoin from radiolabelled pyruvate.

Preparation of the Pyruvate Decarboxylase from Wheat Germ.—Fresh wheat germ (150 g) was ground with glass beads (Sigma Type III, 150—200 μ, acid-washed) and buffer A {2-[4-(2-hydroxyethyl)piperazine-1-yl]ethanesulphonic acid, HEPES, 0.01M}, magnesium chloride (0.001M), 2-mercaptoethanol (0.006M), PMSF (0.021mM) and BAM (0.01mM, total volume 500 ml), in a Waring Blendor for 10 1-min periods. The mixture was made 0.1% with respect to Triton X-100, DNAase (1 mg) was added and the mixture was stirred for 30 min. The mixture was centrifuged at 5 000 r.p.m. for 15 min to remove the glass beads and then centrifuged at 38 000 r.p.m. for 2 h to precipitate the ribosomal fraction. The supernatant fraction, which contained no pyruvate decarboxylase activity, was discarded. The pelleted ribosomal fraction was resuspended in buffer A (20 ml).

Production of (R)-*Acetoin by an Enzyme Preparation from* Klebsiella aerogenes.—Sodium [2-^{14}C]pyruvate was incubated for 3.5 h at 35 °C with the enzyme preparation from *Klebsiella aerogenes*. Further details of the assay conditions are given in the Table. A control assay produced 320 μg of acetoin, as assayed by the Westerfeld procedure.[14] Following incubation, the mixture was diluted with water (30 ml) and unlabelled acetoin (racemic, 250 mg) was added. The mixture was stirred until the acetoin had completely dissolved. The solution was saturated with sodium chloride and extracted continuously with ether for 18 h. The ether extract was dried ($MgSO_4$) and the filtrate was concentrated by distillation under nitrogen through a column of glass helices (40 × 1 cm) from a water-bath at 45 °C. The residue (*ca.* 3 ml) was mixed with pyridine (4 ml) and to the mixture were added phthalic anhydride (250 mg) and 4A molecular sieve pellets (750 mg). The mixture was left at room temperature for 7 days, diluted with ether (40 ml), and the solution extracted with hydrochloric acid (2M; 40 ml). The acidic extract was re-extracted with ether (2 × 40 ml) and the combined ether extracts were dried ($MgSO_4$) and evaporated to give the crude hydrogen phthalate of acetoin (569 mg). The dicyclohexylammonium salt was prepared and recrystallised (acetone) to constant activity (361 mg; 13 100 d.p.m. mg^{-1}). A reverse isotope dilution analysis was performed using the radioactive salt (20.3 mg) and the salt prepared from (*S*)-acetoin (504 mg). After 30 recrystallisations, the activity had fallen to 1.6% of the value calculated for 100% entrainment of the labelled material.

Production of Acetoin from Pyruvate by the Pyruvate Decarboxylase of Wheat Germ.—An incubation mixture was prepared as indicated in the Table (column 2). A control assay produced acetoin (34 μg) under the same conditions (3.5 h incubation at 35 °C). The solution was applied directly to a column of Dowex 1- X8 ion exchange resin (20—50 mesh; acetate form; 10 ml) and the column was eluted with water (30 ml). To the eluate was added unlabelled, inactive acetoin (500 mg) and the mixture was stirred until dissolution was complete. The acetoin was isolated and converted into the hydrogen phthalate [using phthalic anhydride (840 mg)] and thence into the dicyclohexylammonium salt as described above for the acetoin produced by the enzyme preparation from *K. aerogenes*, to give after recrystallisation to constant activity 893 mg of the salt (3 420 d.p.m. mg^{-1}). A reverse isotope dilution analysis was carried out using the labelled salt (21.4 mg) and the unlabelled salt of the derivative of (*S*)-acetoin (508 mg), to give the results described in the text.

Production of Acetoin from Pyruvate Plus Acetaldehyde by the Pyruvate Decarboxylase of Wheat Germ.—An incubation mixture was prepared as indicated in the Table (column 3). A control assay produced acetoin (80 μg) under similar conditions (3.5 h incubation at 35 °C). The labelled acetoin was isolated as described for the isolation of acetoin from the mixture produced by incubation of pyruvate with the enzyme preparation from *K. aerogenes*, above, to give, after similar treatment, the dicyclohexylammonium salt of the hydrogen phthalate of acetoin (349 mg; 3 240 d.p.m. mg^{-1}). A reverse isotope dilution analysis was

carried out using the labelled salt (20.6 mg) and the unlabelled salt (495.7 mg) prepared from (*S*)-acetoin, to give the results described in the text.

Acknowledgements

We thank the S.E.R.C. for financial support.

References

1 (*a*) T. P. Singer and J. Pensky, *Biochim. Biophys. Acta*, 1952, **9**, 316; (*b*) *J. Biol. Chem.*, 1952, **196**, 375.
2 T. P. Singer, *Biochem. Biophys. Acta*, 1952, **8**, 108.
3 E. Juni, *J. Biol. Chem.*, 1961, **236**, 2302.
4 Y. S. Halpern and H. E. Umbarger, *J. Biol. Chem.*, 1959, **234**, 3067.
5 D. H. G. Crout and S. M. Morrey, *J. Chem. Soc., Perkin Trans. 1*, 1983, 2435.
6 E. Juni in 'Methods in Enzymology,' eds. S. P. Colowick and N. O. Kaplan, Academic Press, New York, 1955, vol. 1, p. 471.
7 M. B. Taylor and E. Juni, *Biochim. Biophys. Acta*, 1960, **39**, 448.
8 R. H. Blom, *J. Am. Chem. Soc.*, 1945, **67**, 494.
9 R. Pappo, D. S. Allen, R. U. Lemieux, and W. S. Johnson, *J. Org. Chem.*, 1956, **21**, 478.
10 D. H. G. Crout, J. Littlechild, M. B. Mitchell, and S. M. Morrey, *J. Chem. Soc., Perkin Trans. 1*, 1984, 2271.
11 R. K. Hill, S. Sawada, and S. M. Arfin, *Bioorg. Chem.*, 1979, **8**, 175.
12 E. Juni, *J. Biol. Chem.*, 1952, **195**, 715.
13 J. Ullrich and I. Donner, *Hoppe-Seyler's Z. Physiol. Chem.*, 1970, **351**, 1026.
14 W. W. Westerfeld, *J. Biol. Chem.*, 1945, **161**, 495.

Received 17*th May* 1985; *Paper* 5/831