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Investigation of the Mechanism of the Methylmalonyl-CoA Mutase Reaction with the Substrate Analogue: Ethylmalonyl-CoA

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1. Ethylmalonyl-CoA was found to be a substrate for methylmalonyl-CoA mutase from *Propionibacterium shermanii*, the product being mainly (2*R*)-methylsuccinyl-CoA along with some (2*S*)-diastereoisomer.

2. The relevant ^{1}H-nuclear magnetic resonance signals of methylsuccinic acid and of its dimethyl ester were assigned to the diastereotopic methylene hydrogens using stereospecifically dideuterated specimens of known configuration.

3. [2-$^2H_1$]Ethylmalonyl-CoA was converted by methylmalonyl-CoA mutase in ^2H_2O mainly to (2*R*, 3*S*)-[3-$^2H_1$]methylsuccinyl-CoA. No dideuterated product was observed.

4. Starting from (1*R*)-[1-$^2H_1$]ethanol, (1*S*)-[1-$^2H_1$]ethanol and [$^2H_6$]ethanol the following deuterated specimens of ethylmalonic acid were synthesised and characterised: (3*S*)-[3-$^2H_1$], (3*R*)-[3-$^2H_1$] and [3-$^2H_2$, 4-$^2H_3$], respectively.

5. Conversion of (3*S*)-[3-$^2H_1$]-ethylmalonyl-CoA (70% $^2H_1$ and 2% $^2H_2$ species) on the mutase in water afforded mainly (2*R*)-[2-$^2H_1$]methylsuccinyl-CoA along with some (2*S*)-diastereoisomer. No deuterium loss was observed.

6. Methylmalonyl-CoA mutase converted (3*R*)-[3-$^2H_1$]ethylmalonyl-CoA (81% $^2H_1$ and 2% $^2H_2$ species) to the following methylsuccinyl-CoA species: 33% [3-$^2H_1$], the deuterium being in the *threo* position with respect to the methyl group; 21% [2-$^2H_1$]; 46% unlabelled. The ratio of the species with (2*R*) and (2*S*) configuration was about 60:40.

7. Reaction of [3-$^2H_2$, 4-$^2H_3$]ethylmalonyl-CoA (94.5% [$^2H_5$] species) with the mutase gave the following labelled methylsuccinyl-CoA species: 53.4% [*methyl*-$^2H_3$, 2-$^2H_1$, 3-$^2H_1$], the 3-deuterium being in the *threo* position with respect to the methyl group; 37.6% [*methyl*-$^2H_3$, 2-$^2H_1$]; 5% [*methyl*-$^2H_3$, 2-$^2H_1$, 2-$^2H_1$, 3-$^2H_1$] the 3-deuterium being in *erythro* position with respect to the methyl group; 4% [*methyl*-$^2H_3$, 3-$^2H_1$]. The ratio of the species with (2*R*) and (2*S*) configuration was about 70:30.

8. Implications of these findings for the mechanism of the rearrangements catalysed by coenzyme B_{12} are discussed.

About ten of the known coenzyme-B_{12}-dependent enzymic reactions can be summarised in the following general form (Eqn 1).

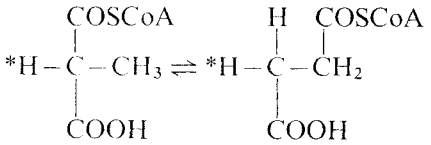
(1)

Abbreviations. NMR, nuclear magnetic resonance; CD, circular dichroism; ORD, optical rotatory dispersion; cobalamin, α-(5,6-dimethylbenzimidazolyl)-cob(III)amide, see *Eur. J. Biochem. 45*, 7–12 (1974).

Enzymes. Methylmalonyl-CoA CoA-carbonyl mutase (EC 5.4.99.2); methylmalonyl-CoA epimerase or racemase (EC 5.1.99.1); methylmalonyl-CoA pyruvate carboxyltransferase (EC 2.1.3.1); L-malate:NAD oxidoreductase (EC 1.1.1.37); L-lactate:NAD oxidoreductase (EC 1.1.1.27); alcohol:NAD oxidoreductase (EC 1.1.1.1); NADH:lipoamide oxidoreductase (EC 1.6.4.3).

One way to inquire into the mechanism of these unusual processes is to focus attention on the substitution events at the migration centres C and C. Although the steric course of the substitution has been examined in a few cases, the results obtained could not be correlated with the mechanism and had no influence upon its most popular formulations (for a recent review see [1]). Thus, despite many common features of these rearrangements, inversion [2–4] (see also Rétey, J., Kunz, F., Stadtman, T. C. and Arigoni, D., unpublished work, cited in [5]), retention [6,7] and racemisation [8,9] have been observed at the substitution centres studied. If we still believe in a common mechanistic pattern, then the conclusion is inescapable that the substitution at C^{α} and C^{β} involves sp^2-hybridised intermediate stages and the formation of the new σ bond can occur, in principle, at both stereoheterotopic faces of the trigonal atom. Which of these two stereochemical possibilities will be realised, depends entirely on the geometry of the individual enzyme protein. In cases in which the sp^2-hybridised intermediate discloses homotopic faces, no differentiation by the enzyme is possible and, provided that free rotation around the $C^{\alpha}-C^{\beta}$ axis exists, racemisation will occur [8,9]. In this way at least indirect information about the nature of the trigonal intermediate can be obtained by stereochemical studies.

The only known case of configurational retention in a rearrangement catalysed by coenzyme B_{12} is the substitution at C-2 of methylmalonyl-CoA (Eqn 2) promoted by the relevant mutase [2]. (The reductive elimination of the 2′-OH group of ribose in the NTP reductase reaction also takes place with retention [10,11], however this reaction does not involve a rearrangement.)



(2)

A further important question in this respect concerns the steric course of the substitution at the other migration centre, that is, at the methyl carbon atom of methylmalonyl-CoA. This problem could be solved in principle by chiral substitution in the methyl group with the isotopes of hydrogen ^1H, ^2H and ^3H [12,13]. Even if methylmalonyl-CoA with a chiral methyl group could be prepared and the tritium and deuterium distribution in succinyl-CoA analysed, an additional difficulty caused by the reversibility of the reaction and the intermolecular scrambling of hydrogen isotopes mediated by coenzyme B_{12} [14] would have to be surmounted.

The discovery [7] that ethylmalonyl-CoA also serves as substrate for methylmalonyl-CoA mutase from *Propionibacterium shermanii* reduces the problem to a relatively easy one, namely, to following the fate of one hydrogen isotope (e.g. ^2H) by reacting stereospecifically labelled ethylmalonyl-CoA substrates with the mutase. A short note on a part of our results has appeared already [7]; here we report our investigations in more detail.

EXPERIMENTAL PROCEDURE

General Methods

Melting points were taken in a vacuum-sealed capillary and are uncorrected. Proton magnetic resonance spectra were recorded with Bruker WH 90 or WH 270 (FT) and in the early stages of this investigation with Varian HR-220 spectrometer. Chemical shifts (δ) are reported in ppm relative to tetramethylsilane (in the case of ^2H_2O solutions the HO^2H signal was arbitrarily taken at $\sigma = 4.75$ ppm), coupling constants in Hz. Mass spectra were measured with a Hitachi-Perkin-Elmer RMU-6A or with a Varian MAT CH-5 spectrograph. The limit of error of the mass spectrometric measurements was estimated to be $\pm$ 1%; it depended however very much on the purity of the sample. Gas chromatography coupled with mass spectrometry of the ethylmalonic and methylsuccinic dimethyl esters was carried out with a LKB 9000 equipment. (Column: QF-1, 2.5-m length, 3-mm diameter, temperature 80 °C, increased by 3 °C per min up to 140 °C; injector temperature 240 °C, ion source: 250 °C, 70 eV.)

Optical rotatory dispersion (ORD) measurements were recorded in a Bellingham and Stanley-Bendix Polarmatic 62 and circular dichroism (CD) curves in a Cary 61 instrument. Optical rotations were measured with a Perkin-Elmer 141 polarimeter. For ultraviolet spectroscopic measurements a Unicam SP-1800 spectrophotometer was used. The microanalysis was carried out with an Elemental Analyzer model 1104 of Carlo Erba.

Materials

NAD, NADH, CoA (Reinheitsgrad II), malate dehydrogenase, lactate dehydrogenase, alcohol dehydrogenase from yeast and diaphorase were commercial products of Boehringer Mannheim GmbH (Mannheim, F.R.G.); [1-$^2H_2$]ethanol, [$^2H_6$]ethanol and deutero-chloroform (C^2HCl_3) were purchased from Merck Sharp & Dohme of Canada Ltd (Montreal, Canada), [*O*-$^2H_1$]ethanol (99.5%) from Ciba-Geigy AG (Basel, Switzerland); 5′-deoxyadenosyl-cobalamin (coenzyme B_{12}) was a gift of Richardson-Merrell S.p.A. (Napoli, Italy); DEAE-cellulose, TEAE-cellulose and cellulose phosphate was obtained from Brown Company (Berlin, New Hampshire, U.S.A.); silica gel plates D-(SILG/UV_{254}) were purchased from Macherey-Nagel

& Co. (Düren, F.R.G.); deuterium oxide (99.93%) was a generous gift from Dr D. Staschewski (Kernforschungszentrum, Karlsruhe) and from the Eidgenössische Institut für Reaktorforschung (Würenlingen, Switzerland). (+)-(2*R*)-Methylsuccinic acid for reference was prepared by resolution of the racemate according to [15] and showed $[\alpha]_D^{20} = +15°$ ($c = 1.454$ in ethanol) or $[\alpha]_D^{20} = +8.71°$ ($c = 1.5496$ in water).

(1*R*)-[1-$^2H_1$]Ethanol (73% $^2H_1$-labelled) has been prepared according to Simon and coworkers [16,17], (1*S*)-[1-$^2H_1$]ethanol (81% $^2H_1$-labelled) by modification of the same method [16,17] starting from [1-$^2H_2$]ethanol. The ethanol specimens were processed to (1*R*)-ethyl tosylate (12.7 g, m.p. 31.5 °C) and to (1*S*)-ethyl tosylate (6.5 g, m.p. 31.5 °C) according to the method of Tipson [18].

Methylmalonyl-CoA mutase was isolated from *Propionibacterium shermanii* (either strain 52 W or St 33) according to Kellermeyer *et al.* [19,20] and modified by Zagalak *et al.* [21]. The specific activity of the preparations used varied between 3 and 14 U/mg.

Syntheses

Threo-*[2,3-$^2H_2$]- and* erythro-*[2,3-$^2H_2$]methylsuccinic Acids.* In separate vessels methylfumaric (1.048 g) and methylmaleic acid (1.008 g) were recrystallised from 10 ml ^2H_2O and dried. The crystals of methylfumaric acid were dissolved in 15 ml and those of the methylmaleic acid in 10 ml ^2H_2O and both solutions were treated with portions of carefully dried (vacuum desiccator, phosphorus pentoxide) potassium azodicarbonate [22] (3 g were used in total for each reaction mixture). The solutions were kept acidic by dropwise addition of 1 M $^2H_2SO_4$. After standing overnight, the acids were extracted with ether and chromatographed on a column (2 × 18 cm) of Dowex 1 × 8 (100–200 mesh, formate form). The materials eluted with 1.1 M aqueous formic acid were purified further by thin-layer chromatography (silica gel plates, chloroform/acetic acid, 5/1, v/v). 10 mg of pure *threo*-[2,3-$^2H_2$]methylsuccinic acid (m.p. 105–107 °C) were obtained from methylfumaric acid and 7 mg of *erythro*-[2,3-$^2H_2$]methylsuccinic acid (m.p. 105–107 °C) from methylmaleic acid. The mass spectrum of the dimethyl ester of the *threo* acid indicated 79% $^2H_2$ and 21% $^2H_1$ labelled molecules (determined from the peaks at $m/e = 129$, 130 and 131); the 220-MHz ^{1}H-NMR spectrum showed, beside the methyl signals, only a rather broad singlet at 2.37 ppm. The corresponding data for the *erythro*-dimethyl ester were: 70% $^2H_2$ and 30% $^2H_1$-labelled molecules and a broad singlet at 2.67 ppm in the 220-MHz ^{1}H-NMR spectrum.

(3S)-[3-$^2H_1$]Ethylmalonic Acid. 1.2 g (52.1 mmol) sodium was dissolved in 25 ml absolute ethanol under nitrogen and 7.61 g (48 mmol) diethyl malonate in 150 ml dry benzene were added. Finally 9 g (45 mmol) (1*R*)-[1-$^2H_1$]ethyl tosylate in 200 ml benzene were introduced into the stirred solution. After refluxing for 17 h ice was added and the usual work-up afforded 9.3 g of a yellowish oil. Chromatography on 500 g silica gel (hexane/ethyl acetate, 8/3) gave 5.7 g (30.2 mmol) (3*S*)-[3-$^2H_1$]ethylmalonic acid diethyl ester (mass spectrum: 70% $^2H_1$, 2% $^2H_2$-labelled and 28% unlabelled molecules as determined on the peaks at $m/e = 188$ and 189). Refluxing the ester with 4.5 g (80 mmol) potassium hydroxide in 40 ml water for 6.5 h (after 5 h 15 ml ethanol were added) and subsequent removal of the solvent in a rotatory evaporator afforded an almost dry slurry. The residue was dissolved in 50 ml 1 M hydrochloric acid and extracted continuously overnight with ether. The crude product (4.4 g) gave three fractions upon recrystallisation from ethyl acetate/hexane of 2.91 g (m.p. 113.1–114.1 °C), 0.500 g (m.p. 110.1–112.1 °C) and 0.264 g (m.p. 95.2–101.3 °C). The NMR integration (90 MHz, in ^2H_2O) agreed with the deuterium content deduced from the mass spectrum of the diethyl ester. Details of the ORD are given in the Table.

(3R)-[3-$^2H_1$]Ethylmalonic Acid. Starting with 0.8 g (34.9 mmol) sodium in 15 ml ethanol, 5.1 g (32.5 mmol) diethylmalonate in 100 ml benzene and 6.2 g (30 mmol) (1*S*)-[1-$^2H_1$]ethyl tosylate in 150 ml benzene the synthesis was carried out in a similar way to that described for the other enantiomer. After purification as above 4.6 g (24.4 mmol) (3*R*)-[3-$^2H_1$]-ethylmalonic acid diethyl ester were obtained (mass spectrum: 81% $^2H_1$, 2% $^2H_2$-labelled and 17% unlabelled molecules as determined on the peaks at $m/e = 188$ and 189). Hydrolysis of the diester and work-up of the product as above afforded two fractions of crystalline (3*R*)-[3-$^2H_1$]ethylmalonic acid: 2.05 g (m.p. 113–114 °C) and 0.393 g (m.p. 110–112 °C). The ^{1}H-NMR spectrum (90 MHz, in ^2H_2O) was in accord with the deuterium content deduced from the mass spectrum of the diethyl ester. Details of the ORD are given in the Table.

[Methyl-$^2H_3$, 3-$^2H_2$]Ethylmalonic Acid. Starting with 2.25 g (98 mmol) sodium in 50 ml ethanol, 11.7 g (74 mmol) diethyl malonate in 250 ml benzene and 14.1 g (69 mmol) [$^2H_5$]ethyl tosylate (96% $^2H_5$ and 4% $^2H_4$-labelled) in 500 ml benzene the synthesis was carried out in a similar way to that described for the labelled (*R*) and (*S*) specimens. 4.95 g (25.7 mmol) purified [*methyl*-$^2H_3$, 3-$^2H_2$]ethylmalonic acid diethyl ester were obtained (mass spectrum: 94.5% $^2H_5$ and 5.5% $^2H_4$-labelled molecules as determined from the peaks at $m/e = 192$ and 193). Hydrolysis of this ester and work-up of the product as above afforded 1.15 g [*methyl*-$^2H_3$, 3-$^2H_2$]ethylmalonic acid (m.p. 110–111 °C).

(3S)-[3-$^2H_1$]Butyric and (3R)-[3-$^2H_1$]butyric Acids. These were prepared by thermal decarboxylation of the corresponding ethylmalonic acids at about 140 °C and direct distillation of the products under normal pressure. Details of the ORD are given in the Table.

S-*Ethylmalonyl*-N-*octanoyl-cysteamine.* This was prepared analogously to *S*-methylmalonyl-*N*-octanoyl-cysteamine [23]. 2.97 g (22.5 mmol) ethylmalonic acid (m.p. 113–113.5 °C) in 25 ml absolute ether and 2.94 g (24.75 mmol) thionyl chloride (freshly distilled) were refluxed for 1.5 h. After removal of the solvent the oily monochloride was kept in a vacuum desiccator (0.5 Torr, 66 Pa) over potassium hydroxide for 1–2 h. In a 100-ml flask with three outlets equipped with thermometer, dropping funnel and magnetic stirrer 2 g *N*-octanoyl-cysteamine and 180 ml pyridine were dissolved in 50 ml dry carbon tetrachloride. While keeping the temperature at −8 °C the ethylmalonic acid monochloride in 20 ml carbon tetrachloride was introduced under stirring. After 1 h reaction time the mixture was allowed to warm to room temperature and 50 ml saturated sodium bicarbonate solution was added. After the work-up described in [23] 2.5 g crude crystalline thioester were obtained. Recrystallisation from ethyl acetate/hexane afforded 1.766 g (5.57 mmol) white crystals (m.p. 69.5–70 °C, found: C, 57.0%; H, 8.61%; N, 4.30%; $C_{15}H_{27}O_4NS$ requires C, 56.8%; H, 8.52%; N, 4.42%).

S-*(3S)-[3-$^2H_1$]Ethylmalonyl*-N-*octanoyl-cysteamine.* This was prepared as described for the unlabelled compound. 2.66 g (20 mmol) (3*S*)-[3-$^2H_1$]-ethylmalonic acid gave 0.586 g (1.84 mmol) recrystallised product (m.p. 69.5–70.5 °C, mass spectrum 74% $^2H_1$, 4% $^2H_2$-labelled and 22% unlabelled molecules as determined on the peaks at *m/e* 273, 274 and 275).

S-*(3R)-[3-$^2H_1$]Ethylmalonyl*-N-*octanoyl-cysteamine.* This was prepared as described for the unlabelled compound. 2.050 g (15.4 mmol) (3*R*)-[3-$^2H_1$]-ethylmalonic acid afforded only 233 mg (0.73 mmol) recrystallised product (m.p. 69–70 °C, mass spectrum: 90% $^2H_1$-labelled and 10% unlabelled species), however 1.5 g of unreacted acid could be recovered and converted once more to give additionally 0.500 mg (1.572 mmol) of the thioester of the same quality.

S-*[methyl-$^2H_3$, 3-$^2H_2$]Ethylmalonyl*-N-*octanoyl-cysteamine.* This was prepared as described for the unlabelled compound. 1.115 g (8.4 mmol) [*methyl*-$^2H_3$, 3-$^2H_2$]ethylmalonic acid afforded three crops of crystals: 71 mg (m.p. 71–73 °C), 103 mg (m.p. 70–72 °C) and 135 mg (m.p. 68–70 °C); the total 0.96 mmol. The mass spectrum showed an M^+ peak at *m/e* 322, the $M^{\pm}$ peak of this compound is not indicative of the deuterium content as revealed by the ^{1}H-NMR and the mass spectrum of other derivatives of this pentadeuterated acid.

ENZYMIC CONVERSIONS OF ETHYLMALONYL-CoA SPECIES

Experiment 1

Ethylmalonyl-CoA was prepared as follows: to 201 mg CoA in 2 ml water at pH 7, a solution of 317 mg (1 mmol) *S*-ethylmalonyl-*N*-octanoyl-cysteamine in 2 ml water (pH 9) was added at 0 °C and under argon. The pH was brought to 9 with 5 M potassium hydroxide and the solution allowed to stand at room temperature for 1 h. After a few minutes a white turbidity appeared and developed to a thick precipitate. The latter (*n*-octanoyl-cysteamine) was removed by continuous extraction with ether (2 h). Subsequently the pH was brought to 3 by addition of Dowex-50 (H^+ form) and the extraction continued until all the white precipitate had been removed (about 3 h). Photometric determination of the thioester content with the 'hydroxamic acid method' [24] showed 204 µmol ethylmalonyl-CoA.

The enzymic reaction mixture was prepared by mixing the above ethylmalonyl-CoA preparation (4 ml) with 4 ml 0.5 M Tris-HCl buffer (pH 7.4), 10 ml of water, 100 U of methylmalonyl-CoA mutase (spec. act. 13 U/mg) in 1 ml and 1 mg of 5′-deoxyadenosyl-cobalamin.

After incubation in the dark at 30 °C for 5.5 h the mixture was cooled to 0 °C and treated with 0.5 ml 60% perchloric acid. The denatured protein was removed by centrifugation and the pH adjusted with 5 M potassium hydroxide to about 11–12. After standing overnight at 30 °C the cooled reaction mixture was acidified with sulphuric acid (3 M) to pH 1, saturated with ammonium sulphate and continuously extracted with ether.

16 mg crude product were obtained which showed both ethylmalonic and methylsuccinic acid on a thin-layer plate (silica gel, chloroform/acetic acid, 5/1, v/v). Gas chromatography coupled with mass spectrometry on the dimethyl esters revealed that the mixture consisted of 13% methylsuccinic acid and 87% ethylmalonic acid. Preparative thin-layer chromatography followed by chromatography on Dowex 1×8 (100–200 mesh, formate form) afforded 1.3 mg pure methylsuccinic acid; m.p. 105–107 °C, circular dichroism: $[\theta]_{206}^{21°} = +960°$ ($c = 0.258$ mg/ml in water) as compared with $[\theta]_{205}^{21°} = +1740°$ ($c = 0.249$ mg/ml in water) measured simultaneously on a reference (2*R*)-methylsuccinic acid which showed 88% of the rotation recorded as the highest literature value [25, 26].

Experiment 2

Starting from 300 mg CoA and 481 mg (1.526 mmol) *S*-ethylmalonyl-*N*-octanoyl-cysteamine, ethylmalonyl-CoA was prepared as described above, except that throughout ^2H_2O (≈ 99.93% ^2H content) was used instead of water.

The enzymic reaction mixture was prepared from 202 μmol of the above CoA ester (10 ml), 4 ml 1 M Tris/^{2}HCl buffer in ^2H_2O and 12 ml ^2H_2O. After adjusting the pH to about 7.4 (pH-meter reading) by addition of portions of solid anhydrous potassium carbonate 707 U of methylmalonyl-CoA mutase (spec. act. 13 U/mg, prepared by precipitating the mutase with ammonium sulphate, carefully washing the centrifuged protein pellet with 1–2 ml ^2H_2O and dissolving the washed pellet in 10 ml ^2H_2O) and 1 mg of 5′-deoxyadenosyl-cobalamin were added and the mixture incubated at 30 °C in the dark for 6.5 h. Hydrolysis and work-up as described above gave 4 mg ethylmalonic acid along with 11 mg pure methylsuccinic acid (m.p. 103–105 °C; mass spectrum: 95% $^2H_1$-labelled and 5% unlabelled molecules as determined from the peaks at m/e = 129 and 130 on the dimethyl ester, 220-MHz ^{1}H-NMR spectrum of the dimethyl ester in C^2HCl_3 (Fig. 4); broad signals at δ = 2.37 and 2.86 ppm, absence of the signal at 2.67 ppm, optical rotatory dispersion; positive Cotton effect, no quantitative evaluation was possible).

Experiment 3a and 3b

(3*S*)-[3-$^2H_1$]Ethylmalonyl-CoA was prepared starting from 214 mg CoA and 353.2 mg (1110 μmol) *S*-(3*S*)-[3-$^2H_1$]ethylmalonyl-*N*-octanoyl-cysteamine. The labelled CoA ester (124 μmol according to the 'hydroxamic acid test') in 8.7 ml aqueous solution was incubated with a mixture prepared from 4 ml 1 M Tris/HCl buffer (pH 7.5), 800 U of methylmalonyl-CoA mutase (spec. act. 9 U/mg) in 14 ml, 0.5 mg 5′-deoxyadenosyl-cobalamin and 10 ml water. After 14 h at 30 °C in the dark usual hydrolysis and work-up gave 12.4 mg methylsuccinic acid (m.p. 103–105 °C, mass spectrum of dimethyl ester: 74% $^2H_1$-, 8% $^2H_2$-labelled and 18% unlabelled molecules as determined from m/e = 129, 130 and 131; 220-MHz ^{1}H-NMR spectrum of dimethyl ester in C^2HCl_3: the signal at 2.86 ppm integrated to 0.27 proton indicating 73% deuterium in the tertiary position; the signals at 2.39 and 2.67 ppm appeared as an AB system.

This experiment (3b) has been repeated with a mutase preparation isolated from *Propionibacterium shermanii* St 33 and showing slightly different properties from that of the enzyme from 52 W strain. The specific activity of this preparation was 7 U/mg and 445 U were used in total to obtain, by the procedure described above, 3.6 mg methylsuccinic acid; m.p. 99–102 °C; mass spectrum of the dimethyl ester: 72% $^2H_1$, 9% $^2H_2$-labelled and 19% unlabelled molecules; the 270-MHz ^{1}H-NMR spectra both of the dimethyl ester (in C^2HCl_3) and of the free acid (in ^2H_2O) have been recorded. The latter was estimated to be more exact and indicated 70 ± 2% $^2H_1$-labelled and 30 ± 2% unlabelled molecules (see Fig. 5); ORD and CD revealed a positive Cotton effect, $[\theta]^{23}_{204}$ = + 1000° (c = 0.108 mg/ml in water) as compared with $[\theta]^{23}_{205}$ = + 1640° (c = 0.260 mg/ml in water) measured simultaneously on a reference (2*R*)-methylsuccinic acid which showed 88% of the rotation recorded as the highest literature value [25, 26].

Experiment 4a and 4b

(3*R*)-[3-$^2H_1$]Ethylmalonyl-CoA was prepared starting from 208.5 mg CoA and 225 mg (710 μmol) *S*-(3*R*)-[3-$^2H_1$]ethylmalonyl-*N*-octanoyl-cysteamine. The labelled CoA ester (84 μmol according to the 'hydroxamic acid test') in 7 ml aqueous solution was incubated with a mixture prepared from 4 ml 1 M Tris/HCl buffer (pH 7.5), 800 U methylmalonyl-CoA mutase (spec. act. 9 U/mg) in 13 ml, 0.5 mg 5′-deoxyadenosyl-cobalamin and 10 ml water. After 12 h at 30 °C in the dark the usual hydrolysis and work-up gave 7.3 mg methylsuccinic acid; m.p. 102–103 °C, mass spectrum of dimethyl ester: 28% $^2H_1$-labelled and 72% unlabelled molecules; 220-MHz ^{1}H-NMR spectrum of dimethyl ester in C^2HCl_3: integration of the signals at 2.37, 2.67 and 2.96 ppm gave ≈ 14% $^2H_1$ in position 2 and ≈ 16% $^2H_1$ in the *threo*-3 position with respect to the methyl group. This experiment was also repeated with a mutase preparation from *Propionibacterium shermanii* St 33. The specific activity of this preparation was 9.1 U/mg and 693 U were used in total to obtain, after the procedure described above, 2.5 mg methylsuccinic acid; m.p. 101–103 °C; mass spectrum: 45% $^2H_1$, 5.8% $^2H_2$-labelled and 49.2% unlabelled molecules as determined at m/e = 114, 115 and 116 on the free acid; the 270-MHz ^{1}H NMR spectra both of the dimethyl ester (in C^2HCl_3) and of the free acid (in ^2H_2O) have been recorded. The latter was estimated to be more exact and is depicted in Fig. 6. ORD and CD revealed a positive Cotton effect, $[\theta]^{23}_{205}$ = + 291° (c = 0.118 mg/ml in water) as compared with $[\theta]^{23}_{205}$ = + 1640° (c = 0.260 mg/ml in water) measured simultaneously on a reference (2*R*)-methylsuccinic acid which showed 88% of the rotation recorded as highest literature value [25, 26].

Experiment 5a and 5b

[*methyl*-$^2H_3$, 3-$^2H_2$]Ethylmalonyl-CoA was prepared starting from 214.5 mg CoA and 319 mg (990 μmol) *S*-[*methyl*-$^2H_3$, 3-$^2H_2$]ethylmalonyl-*N*-octanoyl-cysteamine. The labelled CoA ester (128 μmol according to the 'hydroxamic acid test') in 13 ml aqueous solution was mixed with 4 ml 1 M Tris/HCl buffer (pH 7.5), 814 U of methylmalonyl-CoA mutase (spec. act. 3.1 U/mg) from *P. shermanii* St 33, 1mg 5′-deoxyadenosyl-cobalamine and 10 ml water. After incubation at 33 °C in the dark for 12.75 h the usual

hydrolysis and work-up gave 10.6 mg methylsuccinic acid; m.p. 102 – 105 °C; mass spectrum of the dimethyl ester: 64% $^2H_5$ and 36% $^2H_4$-labelled molecules as determined on the peaks at m/e = 131, 132, 133 and 134; the 270-MHz ^{1}H-NMR spectra both of the dimethyl ester (in C^2HCl_3) and of the free acid (in ^2H_2O) have been recorded. The latter was estimated to be more exact and is depicted in Fig. 7. ORD and CD revealed a positive Cotton effect, $[\theta]^{23}_{205} = +784°$ (c = 0.320 mg/ml in water) as compared with $[\theta]^{23}_{205} = +1640°$ (c = 0.260 mg/ml in water) measured simultaneously on a reference (2*R*)-methylsuccinic acid which showed 88% of the rotation recorded as highest literature value [25, 26].

This experiment was also repeated (5b) again using the mutase preparation from the St 33 strain (spec. act. 3 U/mg), but in 0.3 M potassium phosphate buffer (pH 7) instead of in Tris/HCl buffer. The conversion of 68 µmol CoA ester was catalysed by 770 U of the enzyme for 7 h. 0.9 mg methylsuccinic acid was obtained, the 270-MHz ^{1}H-NMR spectrum of which was very similar to the one recorded on the product of Experiment 5a (see Fig. 7). This time the unrearranged [*methyl*-$^2H_3$, 3-$^2H_2$]ethylmalonic acid has been also isolated and its ^{1}H-NMR spectrum recorded. The spectrum showed essentially one singlet, corresponding to the α-H atom of ethylmalonic acid. No signals for the ethyl protons could be detected.

RESULTS

Assignment of the Nuclear Magnetic Resonance Signals of Methylsuccinic Acid

The 1H-NMR spectra of methylsuccinic acid and of its dimethyl ester at 220 and 270 MHz show between 2 and 3 ppm three separated groups of signals (Fig. 1) constituting and ABX system, X being further affected by the geminal methyl group. *Threo* and *erythro* [2,3-$^2H_2$]methylsuccinic acids were synthesised. (The terms '*threo*' and '*erythro*' may refer either to the steric relationship between the vicinal deuteriums or between the methyl group and the protium at C-3; both relationships are either '*threo*' or '*erythro*' in the molecules in question, see Fig. 2.) By their NMR spectra the diastereotopic methylene protons could be correlated with the AB part of the ABX system. In the spectrum of the dimethyl ester the quartet at 2.37 ppm could be assigned to the 3-*threo* methylene proton ('*threo*' with respect to the vicinal methyl group) and the quartet at 2.67 ppm to the 3-*erythro* methylene proton. The NMR spectrum of the free methylsuccinic acid in ^2H_2O was very much dependent on the p^{2}H; however, the quartet due to 3-*threo* proton was always at higher field than the quartet due to the 3-*erythro* proton, which was in agreement with the spectrum of the dimethyl ester.

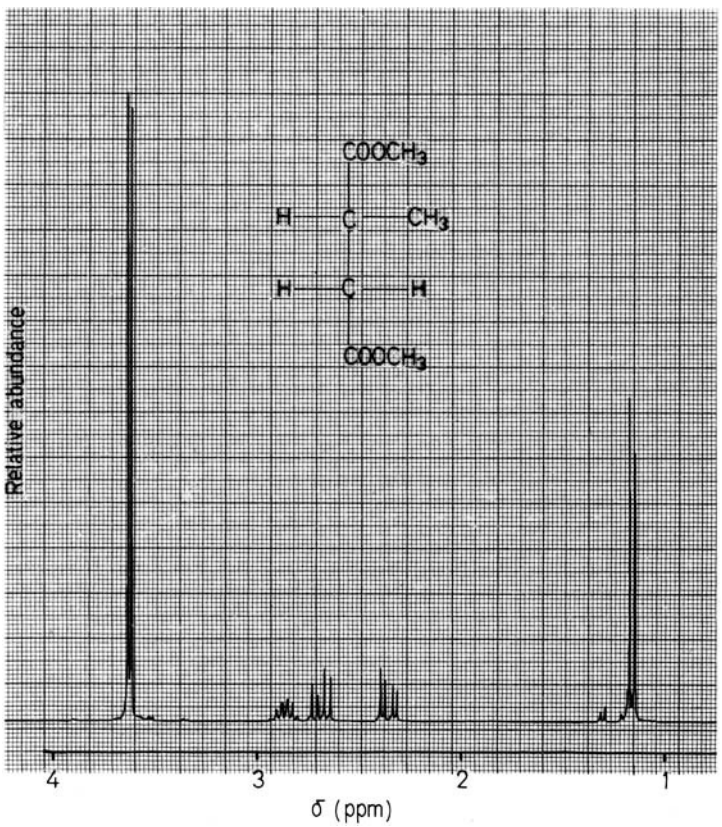


Fig. 1. *The ^{1}H-NMR spectrum of methylsuccinic acid dimethyl ester in C^2HCl_3 at 270 MHz*

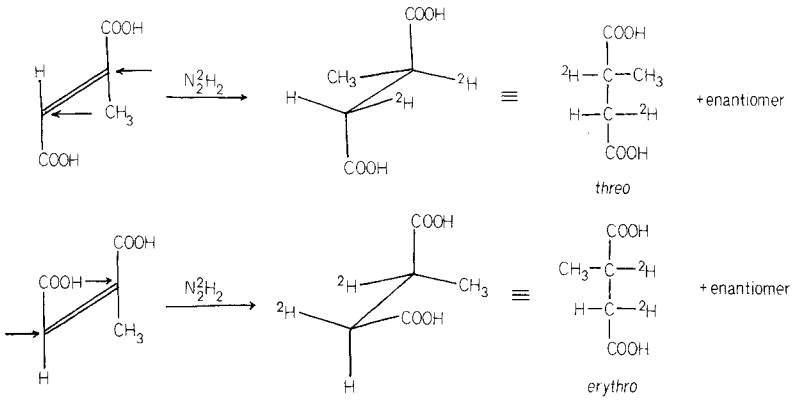


Fig. 2. *Steric course of the deuteration of methylfumaric and methylmaleic acids with deuterodiimide, $N_2^2H_2$*

Table 1. *Optical rotatory dispersion of stereospecifically deuterated ethylmalonic and butyric acids*
The values are corrected for 100% deuterium content. Concentrations (c) are g/100 ml in water

Wavelength	[α] for			
	(3*S*)-[3-$^2H_1$]-ethylmalonic acid $c = 4.16$	(3*R*)-[3-$^2H_1$]-ethylmalonic acid $c = 3.685$	(3*S*)-[3-$^2H_1$]-butyric acid $c = 4.385$	(3*R*)-[3-$^2H_1$]-butyric acid $c = 4.83$
nm	degrees			
250	−6.6	–	−8.35	–
256	−5.46	+5.39	−6.95	–
263	−4.67	+4.69	−5.93	–
270	−4.01	+4.09	−5.11	–
278	−3.57	+3.58	−4.5	–
286	−3.05	+3.14	−4.01	–
294	−2.74	+2.82	−3.52	+3.525
303	−2.41	+2.48	−3.14	+3.095
313	−2.19	+2.22	−2.755	+2.72
323	−1.98	+2.02	−2.48	+2.41
333	−1.80	+1.79	−2.19	+2.105

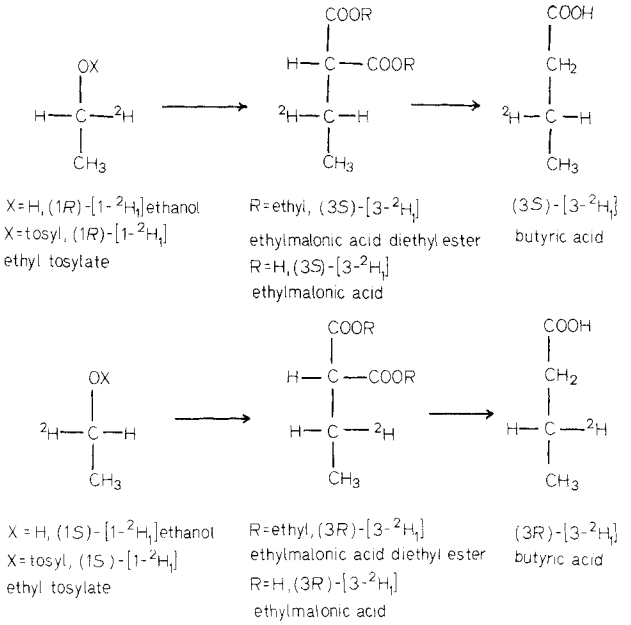


Fig. 3. *Synthesis of stereospecifically deuterated ethylmalonic and butyric acids from (1R)-[1-$^2H_1$]ethanol and (1S)-[1-$^2H_1$]ethanol*

Synthesis of Chiral [3-$^2H_1$]Ethylmalonic Acids

Starting from enzymically prepared (1*R*)-[1-$^2H_1$]-ethanol and (1*S*)-[1-$^2H_1$]ethanol [16,17], the absolute configuration of which was known [27,28], (3*S*)-[3-$^2H_1$]ethylmalonic and (3*R*)-[3-$^2H_1$]-ethylmalonic acids were synthesised using the corresponding *p*-toluenesulphonates as alkylating agents in a malonic ester synthesis (Fig. 3). No deuterium loss occurred in any of the steps as revealed by mass and NMR spectrometric analysis of the products. Evidence for the inversion at the chiral CH^2H centre, expected to take place during the S_N2-type substitution, has been provided by ORD data recorded both on the chiral [3-$^2H_1$]ethylmalonic and on the derived [3-$^2H_1$]butyric acids (Table 1) [29].

Starting from [$^2H_6$]ethanol, [*methyl*-$^2H_3$, 3-$^2H_2$]-ethylmalonic acid was synthesised in a similar way. All specimens of ethylmalonic acid were transformed into the half thioester: *S*-ethylmalonyl-*N*-octanoyl-cysteamine which served as starting material for the preparation of the corresponding ethylmalonyl-CoA species by transesterification [23].

The Fate of the Hydrogen Atoms of Ethylmalonyl-CoA during the Rearrangement

In a first experiment (Expt 1) it was shown that ethylmalonyl-CoA serves as a substrate for methylmalonyl-CoA mutase from *P. shermanii*. Although no spectroscopic assay could be carried out, one can estimate from the substrate/product ratio, the reaction time and the number of enzyme units used that ethylmalonyl-CoA reacts about 1000–10000 times more slowly than the natural substrate. CD examination of the derived methylsuccinic acid revealed that the product consisted of about 75% (2*R*) and 25% (2*S*)-2-methylsuccinyl-CoA. [2-$^2H_1$]Ethylmalonyl-CoA was

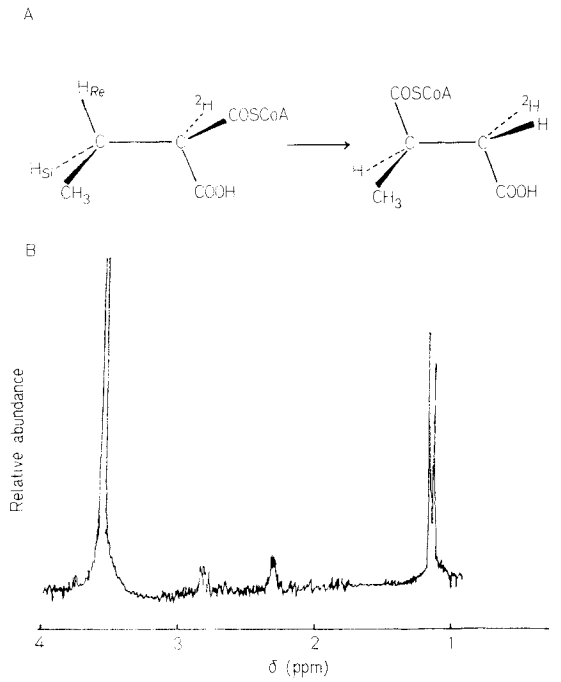


Fig. 4. *The conversion of [2-^{2}H]ethylmalonyl-CoA on methylmalonyl-CoA mutase.* (A) The structure of substrate and main product. (B) The ^{1}H-NMR spectrum of the produced methylsuccinic acid as dimethyl ester in C^2HCl_3 at 220 MHz

prepared by exchanging the acidic α-hydrogen atom in ^2H_2O. Conducting the mutase reaction in ^2H_2O ensured that no back-exchange occurred during the procedure (Expt 2). For analysis, the produced 2-methylsuccinyl-CoA was hydrolysed and the methylsuccinic acid purified. The mass and NMR spectrometric analysis, carried out on the dimethyl ester, revealed that approximately one deuterium was incorporated into the 3-*erythro* position of methylsuccinic acid (Fig. 4). No quantitative chiroptical measurements were carried out on this sample, but the molecules with (2*R*) configuration prevailed. *Examination of* the product of the mutase reaction with (3*S*)-[3-$^2H_1$]ethylmalonyl-CoA as substrate revealed that most, if not all, of the deuterium was retained at the C atom to which it had been attached in the original substrate (Expt 3a and 3b). Therefore stereospecific migration of hydrogen from the 3-*pro-R* position must have taken place. No loss of deuterium was detected on the basis of the mass and NMR spectra (Fig. 5). ORD and CD measurements indicated 76–77% (2*R*) and 23–24% (2*S*)-methylsuccinic acid neglecting the chiroptical contribution of deuterium.

Enzymic conversion of (3*R*)-[3-$^2H_1$]ethylmalonyl-CoA (81% $^2H_1$ and 2% $^2H_2$-labelled species, Expt 4a and 4b) gave a product mixture, the NMR examination of which gave unexpected results (Fig. 6). The percentage of unlabelled molecules increased from 17% in the substrate to 70% (Expt 4a), or 49% (Expt 4b), in the product. Furthermore, a considerable part of the substrate molecules (14% or 21% respectively) declined deuterium migration and in only 33% (16% in Expt 4a) of the cases was the deuterium found in the *threo*-3 position as expected. ORD and CD measurements showed a low optical purity of the product of Expt 4b, 58.5% being in the (2*R*) and 41.5% in the (2*S*) configuration.

Finally Experiments 5a and 5b were carried out with [*methyl*-$^2H_3$, 3-$^2H_2$]ethylmalonyl-CoA as substrate. In this case no choice was given between protium and deuterium migration, since only about 2% of the molecules carried one protium in one of the two geminal methylene positions. Both the mass and NMR spectra indicated 36–40% $^2H_4$-labelled molecules in the product, confirming that a considerable part of the migrating deuterium is lost during the rearrangement. The NMR spectra gave detailed information about the distribution of deuterium in the methylsuccinic acid obtained in this experiment (see Fig. 7) and this will be discussed in detail in the next section. According to ORD and CD measurements, this methylsuccinic acid consisted of 69% (2*R*) and 31% (2*S*) species.

In order to rule out Tris molecules as a source of protium, Experiment 5b has been conducted with [*methyl*-$^2H_3$, 3-$^2H_2$]ethylmalonyl-CoA in phosphate buffer. According to the ^{1}H-NMR spectrum of the produced methylsuccinic acid again about 30–40% of the migrating deuterium was lost, whereas no loss of deuterium in the ethylmalonic acid recovered from the unreacted [*methyl*-$^2H_3$, 3-$^2H_2$]ethylmalonyl-CoA could be detected.

DISCUSSION

Interpretation of the ^{1}H-Nuclear Magnetic Resonance Spectra

The use of ethylmalonyl-CoA as substrate analogue allows not only determination of the steric course at both migration centres of the coenzyme-B_{12}-catalysed rearrangement but also provides a convenient method for analysing the deuterium distribution in the product methylsuccinate by means of ^{1}H-NMR spectroscopy. None of these advantages exists when the natural substrate, methylmalonyl-CoA, is used. The assignment of the NMR signals of methylsuccinic acid and of its dimethyl ester relies on the fact that the hydrogenation or deuteration of double bonds by diimide takes place strictly in a *syn* fashion [30, 31] and, therefore, the configuration of the [2,3-^{2}H]-methylsuccinic acid derived from methylfumaric and methylmaleic acids is *threo* and *erythro*, respectively (Fig. 2). A comparison of the ^{1}H-NMR spectra of free methylsuccinic acid at pH 3.5 and 7 gives supportive evidence for the experimental assignment of the signals to the 3-methylene protons. Application of the Karplus

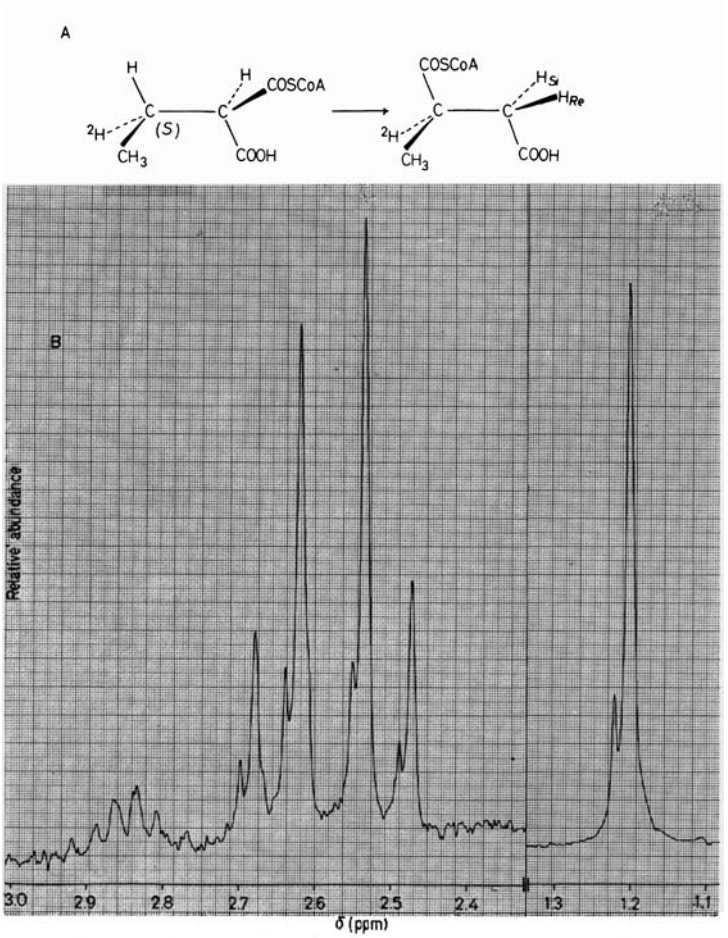


Fig. 5. *The conversion of (3S)-[3-$^2H_1$]ethylmalonyl-CoA on methylmalonyl-CoA mutase.* (A) The structure of substrate and main product. (B) The ^{1}H-NMR spectrum of the produced methylsuccinic acid in deuterium oxide at 270 MHz. In the methyl region the amplitude is decreased to one fourth

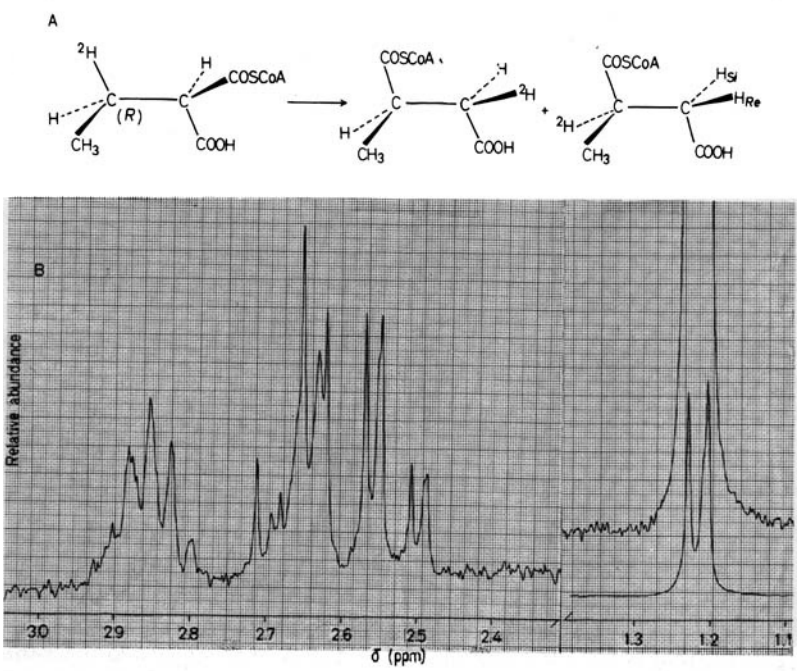


Fig. 6. *The conversion of (3R)-[3-$^2H_1$]ethylmalonyl-CoA on methylmalonyl-CoA mutase.* (A) The structure of substrate and main products. (B) The ^{1}H-NMR spectrum of the produced methylsuccinic acid in deuterium oxide at 270 MHz. In the methyl region the amplitude is decreased to one fourth

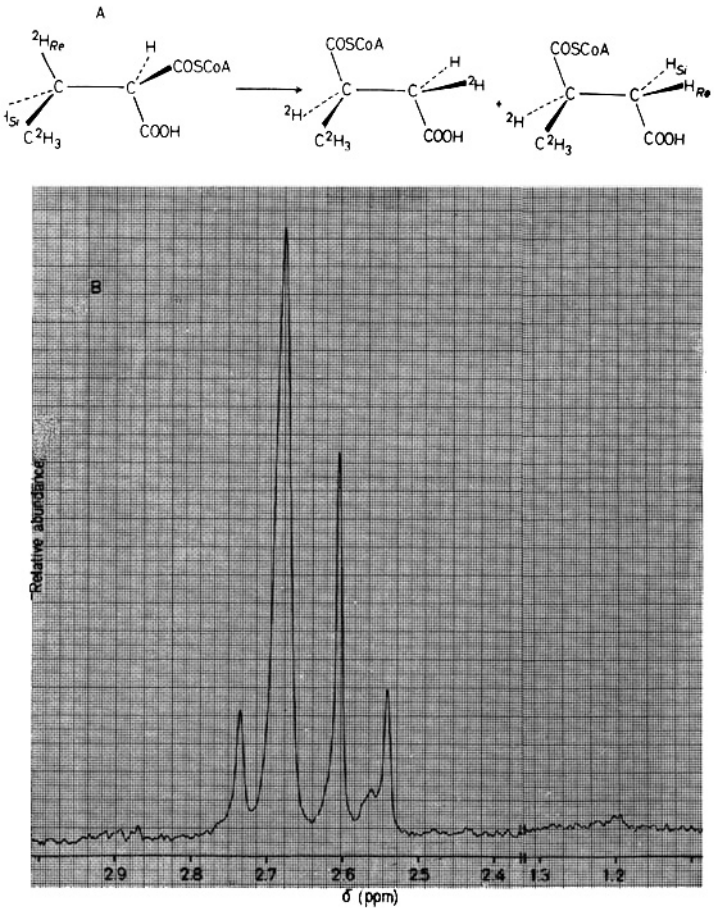


Fig. 7. *The conversion of [3-$^2H_2$, 4-$^2H_3$]ethylmalonyl-CoA on methylmalonyl-CoA mutase.* (A) The structure of substrate and main products. (B) The ^{1}H-NMR spectrum of the produced methylsuccinic acid in deuterium oxide at 270 MHz

relationship [32] to the observed coupling constants suggests that at pH 7 the *anti*-periplanar conformation and at pH 3.5 the *syn*-clinal conformation predominate, which is also plausible on electrostatic grounds. That neutralization of methylsuccinic acid is accompanied by profound conformational changes is also indicated by the reversal of the sign of the optical rotation when going from acidic to basic pH values [33]. A more detailed analysis of the conformational equilibrium of methylsuccinic acid will appear elsewhere. The above analysis of the ^{1}H-NMR spectra complemented by mass spectrometric and chiroptical measurements allowed the exact determination of deuterium in each labelled specimen (Fig. 5–7).

Steric Course of the Rearrangement

The complete elucidation of the steric course at two migration centres requires determination of configuration at four independent chiral (or prochiral) carbon atoms. We did this at three centres and made an assumption for the fourth, that is for the absolute configuration at C-2 of the reacting ethylmalonyl-CoA. It is known from previous work [6, 34] that only (2*R*)-methylmalonyl-CoA serves as substrate for the mutase, although it has never been excluded that the (2*S*) diastereoisomer also reacts at a very slow rate. Since there is no precedence in a vast number of known cases that an enzyme, when reacting with a homologous substrate, reverses its stereochemical requirements, the assumption seems to us justified that (2*R*) and not (2*S*)-ethylmalonyl-CoA is the substrate of the mutase. Indeed, the opposite situation would also be difficult to imagine in terms of active-site geometry. Since C-2 and C-3 of the substrates constitute the axis along which the migration takes place, it is logical that their location must be fixed at the active site. This in turn determines the orientation of the three remaining substituents (H, COO^- and COS-CoA) at C-2. For changing the configuration from (2*R*) to (2*S*) two of these three substituents, unequal both with respect to their chemical nature and to their role in the rearrangement, should be interchanged (see Fig. 8). It is unlikely that the active site would tolerate such a drastic distortion of the complementary binding pattern.

It has been suggested to us that (2*S*)-ethylmalonyl-CoA could have reacted at a comparable rate to the (2*R*) diastereoisomer and that this could explain the observed stereochemical inhomogeneities. Although

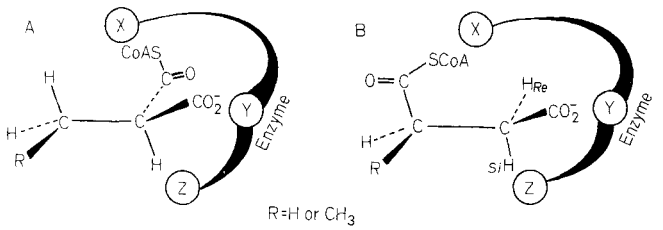


Fig. 8. *Hypothetical arrangement of (2R)-methylmalonyl-Co or (2R)-ethylmalonyl-CoA (A) and succinyl-Co or (2R)-methylsuccinyl-CoA (B) at the active site of methylmalonyl-CoA mutase.* X and Y are specific binding sites for the CoA moiety and the carboxylate group, respectively

such an interpretation is not rigorously ruled out, it is, for the reasons mentioned above, very unlikely.

The absolute configuration of the genuine chiral centre (C-2) of the product has been determined experimentally, but showed no homogeneous stereochemistry. This is not so astonishing if the natural substrate succinyl-CoA is taken as the starting point (Fig. 8B). Substitution of the 2-H_{Re} atom (for nomenclature of enantiotopic of diastereotopic groups see [35, 36]) of this substrate by a methyl group leads to (2*R*)-methylsuccinyl-CoA, that of the 2-H_{Si} atom to (2*S*)-methylsuccinyl-CoA. The active site tolerates both but prefers the (2*R*) configuration. The extent of this preference for the reverse reaction has, however, not yet been determined.

Examination of the rearrangement with deuterated substrates revealed that the situation is even more complicated. C-2 of succinyl-CoA originates from the methyl group of methylmalonyl-CoA and carries two of the three hydrogen atoms of this methyl group. The fate of these three stereohomotopic hydrogen atoms is undetermined at the start, but becomes determined and different for each in the course of the rearrangement. On the other hand, the methylene group of ethylmalonyl-CoA carries a methyl group and two diastereotopic hydrogen atoms. One of these hydrogen atoms (but not the methyl group!) will be abstracted by the enzyme and it is *a priori* unlikely that both hydrogens have the same chance for migration. Indeed, the *pro-R* hydrogen was transferred almost exclusively as revealed by Experiment 3a and 3b, in which the ethylmalonyl-CoA was labelled with deuterium in the 3-*pro-S* position. The results of Experiment 4 with (3*R*)-[3-$^2H_1$]ethylmalonyl-CoA were, however, surprising in two respects. First, they showed that the high degree of stereospecificity for 3-H_{Re} migration observed in Experiment 3 is at least partially due to a kinetic deuterium isotope effect. If the isotope effect works against enzymic preference for 3-H_{Re} migration, then in about one quarter of the cases (21% out of 81% labelled molecules) the hydrogen in the 3-*pro-S* position will migrate and deuterium remains on the carbon to which it has been attached in the substrate. A further corollary of the reluctance of deuterium against migration is the rather low optical purity of the product.

Is there some interdependence between the configuration at C-2 of the methylsuccinate product and the original steric position of the migrating hydrogen atom? Inspection of the chiroptical data of the methylsuccinic acids from Experiment 3b and of 4 strongly suggests such an interdependence. The (2*R*) component of methylsuccinate from Experiment 3b with predominant, if not exclusive, 3-*pro-R*-H migration amounts to about 77%, whereas the (2*R*) component of the product from Experiment 4b with at least 21% 3-*pro-S*-H migration is reduced to 58.5%. This suggests that the ethylmalonyl-CoA molecules, in which 3-*pro-R* hydrogen migrates, afford mainly but not exclusively (2*R*)-methylsuccinyl-CoA species, whereas molecules in which the 3-*pro-S* hydrogen migrates give mainly (2*S*)-methylsuccinyl-CoA species.

From the NMR spectrum of the product of Experiment 4a and 4b (Fig. 6) one can conclude that all deuterium at C-3 is in the *threo* position (with respect to the methyl group), whereas it is not known whether all molecules carrying deuterium in this position are (2*R*). If a smaller portion of them were (2*S*), then maintaining the relative configuration at C-2 and C-3 would be noteworthy and potentially significant for the mechanism. This and the observed loss of a substantial portion of the migrating deuterium will be discussed in the last sections.

In order to force deuterium migration, an ethylmalonyl-CoA species with fully deuterated ethyl group was converted on the mutase (Experiment 5a and 5b). NMR integration of the produced methylsuccinate (Fig. 7) indicates only traces of protium at position 2 and in the methyl group. Although about 60% of the molecules carry deuterium in the 3-*threo* position as expected, the small broad peak at 2.56 ppm indicates the presence of a few species ($\approx$ 5%) carrying deuterium in the 3-*erythro* position. This is the only case in which a slight scrambling of the migrating hydrogen isotope between the 3-*threo* and *erythro* positions has been observed.

The Loss of Migrating Deuterium

In the course of the mutase reaction with ethylmalonyl-CoA all of the non-migrating hydrogen atoms of the substrate are retained in the product. A substantial loss of the migrating hydrogen (made visible by deuterium labelling) is a completely new facet of the rearrangement. The high loss of deuterium ($\approx$ 40%) starting from [$^2H_5$]ethylmalonyl-CoA as substrate (Experiment 5) cannot be explained simply by a kinetic isotope effect since only 1% protium was present at each of the five ethyl positions of the substrate. The following questions must be raised. (a)

Why no loss of migrating deuterium has been observed with methylmalonyl-CoA as substrate? (b) To which ingredient is the migrating deuterium lost and where does the protium in the 3-*threo* position of the product come from?

A critical survey of the literature reveals that no experimental evidence has ever been given for complete retention of the migrating deuterium. The first experiment with [*methyl*-$^2H_3$]methylmalonyl-CoA had been carried out by Erfle *et al.* [37]. In their first paper they cite the data from two experiments, in which, during the conversion of [*methyl*-$^2H_3$]methylmalonyl-CoA to succinyl-CoA, mediated by a mutase preparation from liver, 16.7% and 6% deuterium are lost respectively. In their second paper [38] the first experiment is not cited. The explanation they give for deuterium loss does not take into account enzymic stereospecificity and cannot therefore be valid. (The migrating deuterium occupies a sterically distinct position in succinyl-CoA and will migrate back in the reverse reaction to become one of the deuterium atoms of the methyl group. Therefore it will never occupy the exchangeable α position in methylmalonyl-CoA.) Furthermore these authors did not check whether loss of deuterium also occurred in the unreacted methylmalonyl-CoA.

An approximately 1:1 mixture of [*methyl*-$^2H_3$]-methylmalonyl-CoA and the unlabelled compound was converted to succinyl-CoA on methylmalonyl-CoA mutase from *P. shermanii* by a second group of workers [14]. They analysed the deuterium content both in the product and in the unreacted substrate by mass spectrometry and made calculations for isotope effects on the basis of assumed mechanistic models. However, they start from the assumption that no migrating deuterium is lost and rely on the work of Erfle *et al.* [37, 38]. Sprecher *et al.* [6] labelled methylmalonyl-CoA in the (non-migrating) position 2 with deuterium and converted it with a crude mitochondrial preparation to succinyl-CoA. 70% of the succinate was monodeuterated, however, 13% was dideuterated and 15% was unlabelled [6]. Similar results were obtained when [2-^{2}H]methylmalonyl-CoA was converted on highly purified methylmalonyl-CoA mutase from *P. shermanii* (Rétey, J., unpublished results, cited by Arigoni and Eliel [39]). No explanation can be given for the formation of dideuterated and unlabelled species in these experiments but the action of a contaminating enzyme is not ruled out. One candidate for such a contaminating enzyme would be CoA-transferase which might catalyse the transfer of CoA between the two carboxylate groups of succinate. However, this would only explain the formation of dideuterated and eventually trideuterated molecules but not that of the unlabelled ones. It is noteworthy that in several experiments (Rétey, J., unpublished) the percentage of dideuterated molecules matches that of the unlabelled ones so that the degree of deuteration always amounts to one. In summary, no definitive answer can be given to the first question (a) raised in the beginning of this section.

What about the second question (b)? Here we must rely on the results of the present work. Since in Experiment 2, which was conducted in ^2H_2O, only one deuterium was incorporated into the 3-*erythro* position of methylsuccinyl-CoA and according to the mass spectrum no dideuterated species were formed, exchange between solvent and migrating hydrogen isotope is ruled out. Exchange of the migrating hydrogen with solvent protons could also be imagined by lack of stereospecificity of the mutase; e.g. instead of the hydrogen atom which migrated in the forward reaction (3-H_{Re} in methylsuccinyl-CoA, see Fig. 8B) 3-H_{Si} could migrate in the backward reaction. If deuterium is in the 3-*pro*-*R* position, H_{Si} migration would be favoured by a kinetic isotope effect. However, in Experiment 5b NMR analysis of the unreacted ethylmalonic acid revealed that no protium was incorporated into the ethyl group, while the produced methylsuccinic acid exchanged 40% of the migrating deuterium.

Moreover, neither the enzyme nor the coenzyme B_{12} can be exchange partners, because they were present in catalytic amounts only, while the loss of migrating deuterium amounted to 40% of the converted substrate. Finally, Tris buffer as a source of protons has also been ruled out by conducting the reaction in phosphate buffer. Retention of all deuterium in the unreacted ethylmalonate from Experiment 5b testifies that deuterium loss must have occurred either during or after the enzymic rearrangement. Since no deuterium loss was observed from any other position of methylsuccinyl-CoA the post-rearrangement exchange can also be abandoned. The only organic substance present in stoichiometric amounts is the coenzyme A moiety of the substrate. We suspect that this might be the exchange partner for the migrating deuterium and the 5′-methylene group of coenzyme B_{12} might mediate this exchange. The elucidation of this mysterious loss of migrating hydrogen needs further experimental work.

The Mechanism of the Rearrangement of Ethylmalonyl-CoA

There is general agreement that the first important step in the coenzyme-B_{12}-catalysed rearrangements is the enzyme-mediated cleavage of the cobalt-carbon bond of the coenzyme (Fig. 9). However, unanimity ceases at this point, since this cleavage can take place either in a homolytic or in a heterolytic fashion. Both experimental evidence and chemical intuition favours homolytic cleavage but there are still defenders of the heterolytic fission [40]. More or less direct evidence

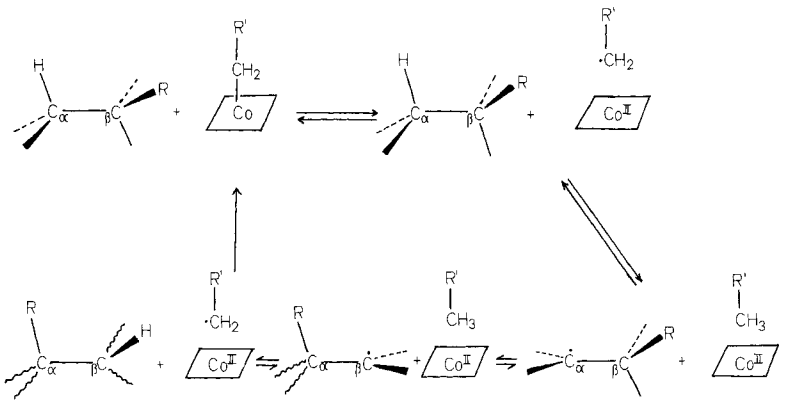


Fig. 9. *A general mechanism postulated for enzymic rearrangements catalysed by coenzyme* B_{12}

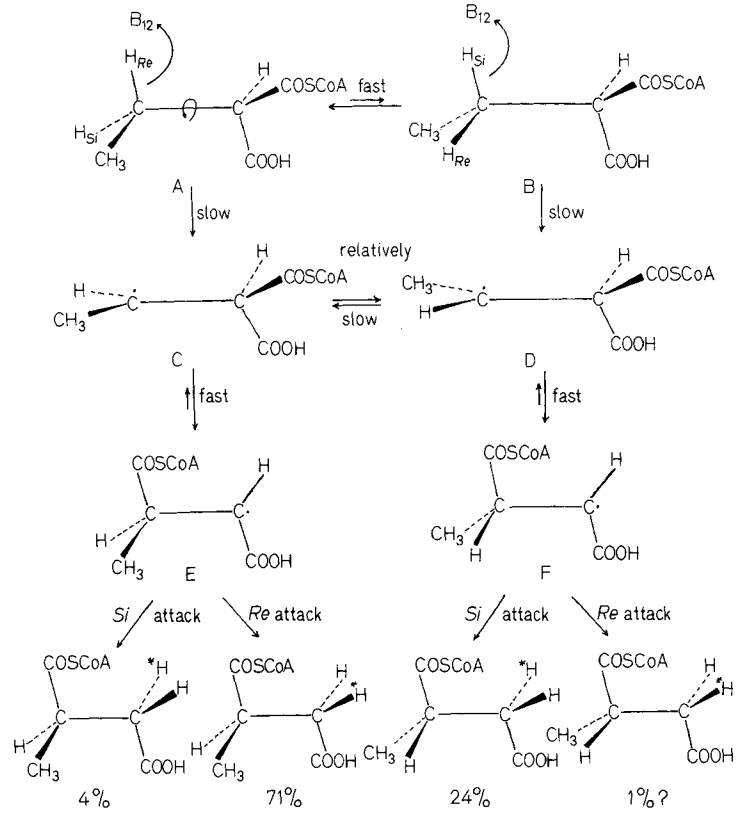


Fig. 10. *Steric course of the rearrangement of ethylmalonyl-CoA on methylmalonyl-CoA mutase from* Propionibacterium shermanii *as revealed by the use of stereospecifically deuterated substrates*

for homolytic cleavage has been provided by electron paramagnetic resonance and ultraviolet/visible spectroscopy on reaction mixtures with ethanolamine ammonia lyase [41–43] and dioldehydrase [44,45]. The type of heterolytic cleavage envisaged in a recent paper [40] is in contradiction with the experimentally proven scrambling of the diastereotopic hydrogen atoms at the cobalt-bound methylene group [46–49], because in the postulated intermediate (4′, 5′-anhydroadenosine) the two hydrogens at C-5′ are still diastereotopic and therefore distinct. A methylene radical intermediate, however, would not only be compatible with the loss of identity of the 5′-hydrogen atoms, but would also be reactive enough to abstract a hydrogen radical from a non-activated position of the substrate, which is the next postulated step in the mechanism (Fig. 9). This hydrogen transfer is further in agreement with the observed exchange of the migrating hydrogen with the 5′-hydrogen atoms of the coenzyme [46–49]. As a next step the rearrangement of the substrate radical to the product radical is envisaged. Model experiments from our laboratory [50,51]

suggest a crucial role of the cobalt atom in the rearrangement which does not necessarily mean that σ bonds between substrate and cobalt must be formed as postulated by some authors [52]. The role of the cobalt in the rearrangement is rather to stabilise the transition state in which the migrating group is half way between the migration termini. The rearranged radical then abstracts one of the hydrogen atoms of the 5′-deoxyadenosine methyl group and finally the σ bond between the C-5′ atom and the central cobalt is restored. In our opinion, the results described in the present work are compatible with and supportive for the general mechanism sketched in Fig. 9.

Most of the stereochemical details observed during the work with stereospecifically deuterated ethylmalonyl-CoA species can be accommodated in the general mechanism as shown in Fig. 10. When (2*R*)-ethylmalonyl-CoA occupies the substrate binding site the additional methyl group has to adopt one of the positions originally tailored for a hydrogen atom of the natural substrate. Since only hydrogen is meaningful in the transferable position (pointing upwards in A or B of Fig. 9), there are two possible conformers, A and B, of the enzyme-bound ethylmalonyl-CoA. The experimentally observed predominance of H_{Re} migration suggests that conformation A is the thermodynamically preferred one. However, substantial H_{Si} migration takes place when H_{Re} is replaced by deuterium. This indicates further that hydrogen abstraction is a slower step than the interconversion between conformations A and B. Substrate radicals C and D are in a rapid cobalt-catalysed equilibrium with the product radicals E and F, respectively, but, in order to allow for the observed optical impurity of the product, some leaking must be postulated through a relatively slow interconversion between C and D. Normally, 20–25% of C leaks to D, however, when deuterium substitution renders the last hydrogen transfer step slower, more leakage is possible as suggested by the diminished optical purity of the heavily deuterated methylsuccinate from Experiment 5a. The back-transfer of hydrogen to species E and F is stereoselectively governed by the already established configuration at C-2. Thus *Re* attack is preferred on species E and *Si* attack on species F as inferred from the localisation by NMR of the migrating hydrogen in the 3-*threo* position. However, *Si* attack on E and *Re* attack on F also occurs to a minor extent as revealed by the NMR data from Experiment 5 (Fig. 7).

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REFERENCES

1. Abeles, R. H. & Dolphin, D. (1976) *Acc. Chem. Res. 9*, 114–120.
2. Sprecher, M., Switzer, R. L. & Sprinson, D. B. (1966) *J. Biol. Chem. 241*, 864–867.
3. Rétey, J., Umani-Ronchi, A. & Arigoni, D. (1966) *Experientia (Basel) 22*, 502.
4. Zagalak, B., Frey, P. A., Karabatsos, G. L. & Abeles, R. H. (1966) *J. Biol. Chem. 241*, 3028–3035.
5. Friedrich, W. (1975) *Vitamin B_{12} und verwandte Corrinoide* (Ammon, R. & Dirscherl, W., eds) 3rd edn, vol. III.2, pp. 225, Georg Thieme Verlag, Stuttgart.
6. Sprecher, M., Clark, M. S. & Sprinson, D. B. (1966) *J. Biol. Chem. 241*, 872–877.
7. Rétey, J. & Zagalak, B. (1973) *Angew. Chem. 85*, 721–722; *Angew. Chem. Int. Ed. 12*, 671–672.
8. Rétey, J., Suckling, C. J., Arigoni, D. & Babior, B. M. (1974) *J. Biol. Chem. 249*, 6359–6360.
9. Bonnetti, V. (1974) Doctoral Thesis, No. 5366, ETH Zürich.
10. Batterham, T. J., Ghambeer, R. K., Blakley, R. L. & Brownson, C. (1967) *Biochemistry, 6*, 1203–1208.
11. Griffin, C. E., Hamilton, F. D., Hopper, S. P. & Abrams, R. (1968) *Arch. Biochem. Biophys. 126*, 905–911.
12. Cornforth, J. W., Redmond, J. W., Eggerer, H., Buckel, W. & Gutschow, Ch. (1969) *Nature (Lond.) 221*, 1212–1213.
13. Lüthy, J., Rétey, J. & Arigoni, D. (1969) *Nature (Lond.) 221*, 1213–1215.
14. Miller, W. W. & Richards, J. H. (1969) *J. Am. Chem. Soc. 91*, 1498–1507.
15. Ladenburg, A. (1895) *Ber. Deutsch. Chem. Ges. 28*, 1170–1171.
16. Günther, H., Biller, F., Kellner, M. & Simon, H. (1973) *Angew. Chem. 85*, 141–142; *Angew. Chem. Int. Ed. 12*, 146–147.
17. Günther, H., Alizade, M. A., Kellner, M., Biller, F. & Simon, H. (1973) *Z. Naturforsch. 28*, 241–246.
18. Tipson, R. S. (1944) *J. Org. Chem. 9*, 235–241.
19. Kellermeyer, R. W., Allen, S. H. G., Stjernholm, R. & Wood, H. G. (1964) *J. Biol. Chem. 239*, 2562–2569.
20. Kellermeyer, R. W. & Wood, H. G. (1969) *Methods Enzymol. 13*, 207–215.
21. Zagalak, B., Rétey, J. & Sund, H. (1974) *Eur. J. Biochem. 44*, 529–535.
22. Thiele, J. (1892) *Liebigs Ann. Chem. 271*, 127–136.
23. Overath, P., Stadtman, E. R., Kellerman, G. M. & Lynen, F. (1962) *Biochem. Z. 336*, 77–98.
24. Stadtman, E. R. (1957) *Methods Enzymol. 3*, 228–231.
25. Rossi, R., Diversi, P. & Ingrosso, G. (1968) *Gaz. Chim. Ital. 98*, 1391–1399.
26. Berner, E. & Leonardsen, R. (1939) *Liebigs Ann. Chem. 538*, 1–43.
27. Lemieux, R. U. & Howard, J. (1963) *Can. J. Chem. 41*, 308–316.
28. Weber, H., Seibl, J. & Arigoni, D. (1966) *Helv. Chim. Acta, 49*, 741–748.

29. Bücklers, L., Umani-Ronchi, A., Rétey, J. & Arigoni, D. (1970) *Experientia (Basel) 26*, 931–933.
30. Corey, E. J., Mock, W. L. & Pasto, D. J. (1961) *J. Am. Chem. Soc. 83*, 2957–2958.
31. Hünig, S., Müller, H. R. & Thier, W. (1961) *Tetrahedron Lett.*, 353–357.
32. Karplus, M. (1963) *J. Am. Chem. Soc. 85*, 2870–2871.
33. Fredga, A., Jennings, J. P., Klyne, W., Scopes, P. M., Sjøberg, B. & Sjøberg, S. (1965) *J. Chem. Soc.*, 2928–2933.
34. Rétey, J. & Lynen, F. (1965) *Biochem. Z. 340*, 256–271.
35. Prelog, V. & Helmchen, G. (1972) *Helv. Chim. Acta, 55*, 2581–2598.
36. Hirschmann, H. & Hanson, K. R. (1977) *Tetrahedron, 33*, 891–897.
37. Erfle, J. D., Clark, J. M., Jr & Johnson, B. C. (1964) *Ann. N.Y. Acad. Sci. 112*, 684–694.
38. Erfle, J. D., Clark, J. M., Jr, Nystrom, R. F. & Johnson, B. C. (1964) *J. Biol. Chem. 239*, 1920–1924.
39. Arigoni, D. & Eliel, E. L. (1969) *Topics in Stereochemistry, 4*, 127–243.
40. Katz, R. N., Vickrey, T. M. & Schrauzer, G. N. (1976) *Angew. Chem. 88*, 584–585; *Angew. Chem. Int. Ed. 15*, 542–543.
41. Babior, B. M., Moss, T. H. & Gould, D. C. (1972) *J. Biol. Chem. 247*, 4389–4392.
42. Babior, B. M., Moss, T. H., Orme-Johnson, W. H. & Beinert, H. (1974) *J. Biol. Chem. 249*, 4537–4544.
43. Joblin, K. N., Johnson, A. W., Lappert, M. F., Holloway, M. R. & White, H. A. (1975) *FEBS Lett. 53*, 193–198.
44. Finlay, T. H., Valinsky, J., Mildvan, A. S. & Abeles, R. H. (1973) *J. Biol. Chem. 248*, 1285–1290.
45. Cockle, S. A., Hill, H. A. O., Williams, R. J. P., Davies, S. P. & Foster, M. A. (1972) *J. Am. Chem. Soc. 94*, 275–277.
46. Essenberg, M. K., Frey, P. A. & Abeles, R. H. (1971) *J. Am. Chem. Soc. 93*, 1242–1250.
47. Frey, P. A. & Abeles, R. H. (1966) *J. Biol. Chem. 241*, 2732–2733.
48. Rétey, J. & Arigoni, D. (1966) *Experientia (Basel) 22*, 783–784.
49. Cardinale, G. J. & Abeles, R. H. (1967) *Biochim. Biophys. Acta, 132*, 517–518.
50. Bidlingmaier, G., Flohr, H., Kempe, U. M., Krebs, T. & Rétey, J. (1975) *Angew. Chem. 87*, 877–878; *Angew. Chem. Int. Ed. 14*, 822–823.
51. Flohr, H., Pannhorst, W. & Rétey, J. (1976) *Angew. Chem. 88*, 613–614; *Angew. Chem. Int. Ed. 15*, 561–562.
52. Dowd, P. & Shapiro, M. (1976) *J. Am. Chem. Soc. 98*, 3724–3725.

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