Stereochemistry of the Methyl Group in (*R*)-3-Methylitaconate Derived by Rearrangement of 2-Methylideneglutarate Catalysed by a Coenzyme B_{12}-Dependent Mutase

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Dedicated to Professor *Albert Eschenmoser* on the occasion of his 75th birthday

2-Methylideneglutarate mutase is an adenosylcobalamin (coenzyme B_{12})-dependent enzyme that catalyses the equilibration of 2-methylideneglutarate with (*R*)-3-methylitaconate. This reaction is believed to occur *via* protein-bound free radicals derived from substrate and product. The stereochemistry of the formation of the methyl group of 3-methylitaconate has been probed using a 'chiral methyl group'. The methyl group in 3-([$^{2}H_{1}$,^{3}H]methyl)itaconate derived from either (*R*)- or (*S*)-2-methylidene[3-$^{2}H_{1}$,3-$^{3}H_{1}$]glutarate was a 50:50 mixture of (*R*)- and (*S*)-forms. It is concluded that the barrier to rotation about the C–C bond between the methylene radical centre and adjacent C-atom in the product-related radical [$^{\bullet}CH_2CH(^{-}O_2CC{=}CH_2)CO_2^{-}$] is relatively low, and that the interaction of the radical with cob(II)alamin is minimal. Hence, cob(II)alamin is a spectator of the molecular rearrangement of the substrate radical to product radical.

1. Introduction. – The enzyme 2-methylideneglutarate mutase isolated from the anaerobe *Eubacterium* (*Clostridium*) *barkeri* catalyses the equilibration of 2-methylideneglutarate **1**[2]) with (*R*)-3-methylitaconate **2** ($K_{eq} = 0.06$) [1]. This process is a carbon-skeleton rearrangement dependent upon coenzyme B_{12} (adenosylcobalamin), and is believed to occur *via* protein-bound free radicals structurally related to the substrate and product [2]. Homolysis of the Co–C bond of coenzyme B_{12} generates cob(II)alamin and the 5′-deoxyadenosyl radical, which abstracts a H-atom from C(4) of 2-methylideneglutarate or the Me group of (*R*)-3-methylitaconate. The resulting substrate 2-methylideneglutarate or product 3-methylitaconate radical interconvert, possibly *via* a cyclopropylmethyl radical ('addition-elimination mechanism'; *Scheme 1* path *a*) or *via* acrylate and the 2-acrylate radical ('fragmentation-recombination

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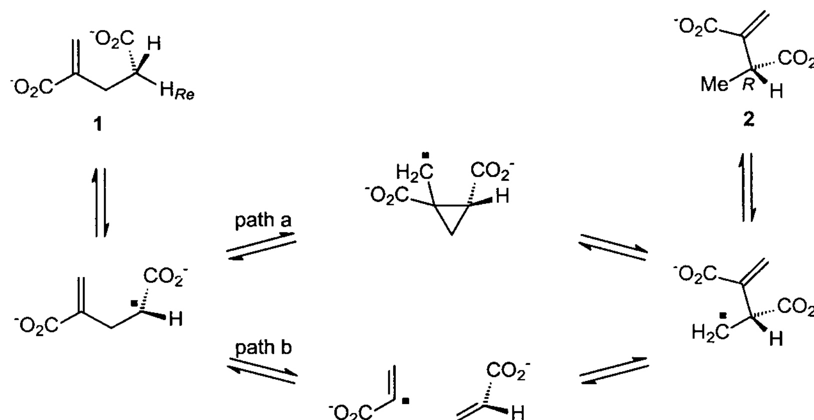
2) Abbreviations and nomenclature: 2-methylideneglutarate: 2-methylidenepentanedioic acid; 2-methylidene[3-$^{2}H_{1}$,3-$^{3}H_{1}$]glutarate: 2-methylidenepentanedioic acid labelled at C(3), 3-methylitaconate: 2-methyl-3-methylidenebutanedioic acid; 3-([$^{2}H_{1}$,$^{3}H_{1}$]methyl)itaconate: methyl-labelled 3-methylitaconate; triflic anhydride (Tf_2O): trifluoromethanesulfonic anhydride; isocitrate dehydrogenase: ICDH.

Scheme 1. *Possible Pathways for 2-Methylideneglutarate Mutase* (path *a*, addition-elimination; path *b*, fragmentation-recombination)



mechanism'; *Scheme 1* path *b*) [3]. The product is formed by transferring a H-atom from the Me group of 5′-deoxyadenosine to the 3-methylitaconate radical.

An issue of critical importance for the mechanistic interpretation is the degree of interaction of cob(II)alamin with the putative intermediate radicals, which could range from essentially no interaction to the formation of organocorrinoid intermediates with a conventional Co–C σ-bond [4]. EPR and X-ray crystallographic data for several coenzyme B_{12}-dependent systems indicate that intermediate free radicals are bound some distance from cob(II)alamin (*e.g.*, 6.5 Å in glutamate mutase [5]). It is therefore expected that the barrier to rotation about the σ-bond between the methylidene C-atom bearing the radical and the adjacent C-atom in the product-related radical for all coenzyme B_{12}-dependent C-skeleton rearrangements will be relatively low (*n.b.*, there is essentially free rotation in the ethyl radical [6]) and hardly perturbed by the Co-atom of cob(II)alamin.

The degree of interaction of a methylidene group with an adjacent centre can be investigated by means of a 'chiral methyl' probe [7][8], *i.e.*, a Me group substituted with deuterium and tritium. If this group is *monochiral* ((*R*) or (*S*))[3]), and if there is a significant primary kinetic isotope effect in any reaction abstracting a H-atom from the Me group, then the chirality information in the Me group may be transferred to a product. In the classical studies of chiral Me groups, the methylidene group was a carbanion centre derived from a chiral Me group of a particular chirality [7][8]. As the methylidene carbanion could engage in a resonance interaction with an adjacent C=O group, the barrier to rotation about the C–C bond between the two centres was relatively high. The particular chirality of the Me group and the direction of capture of the carbanion intermediate therefore determined the stereochemical outcome of the reaction with respect to the reacting methylidene centre. If the methylidene group is a

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[3]) This term, which replaces 'homochiral', 'enantiopure' *etc.* as the designation of a compound consisting of a single enantiomer, was proposed by *Cornforth* [9]. It was also proposed that *isochiral* should replace the term 'racemic'.

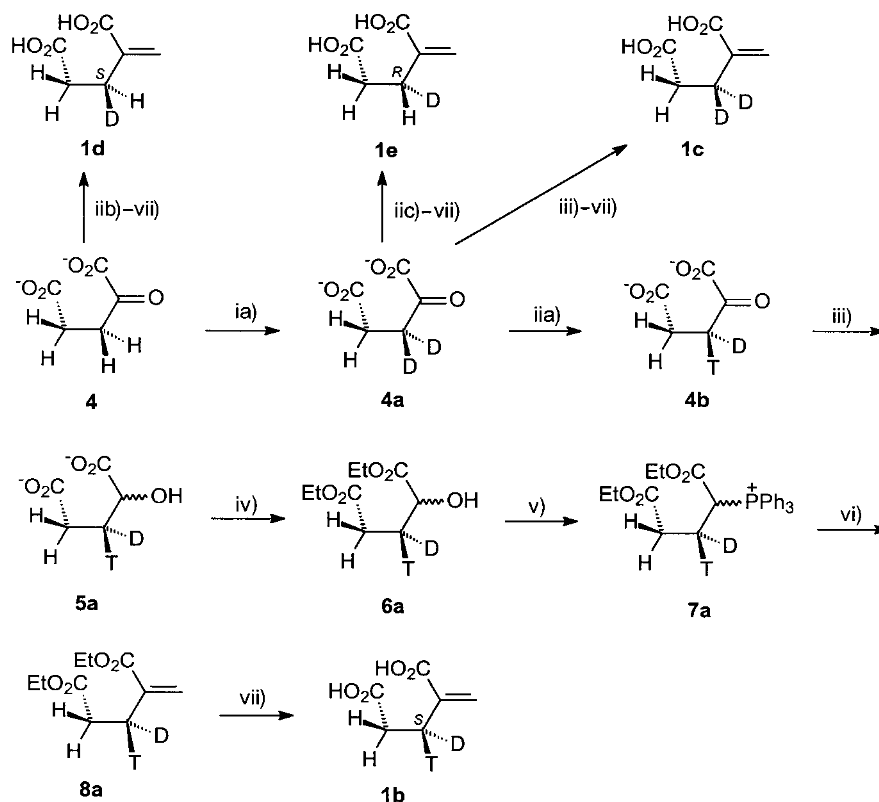
radical centre, and the adjacent centre is sp^3-hybridised, then the barrier to rotation about the C–C bond connecting the two centres is expected to be relatively low (see above). Hence, a chiral Me group generated from this centre is likely to be a 50:50 mixture of (*R*)- and (*S*)-forms. This outcome has already been shown for the conversion of either (*R*)- or (*S*)-[2-$^2H_1$,2-$^3H_1$]ethanolamine to [2-$^2H_1$,2-$^3H_1$]acetaldehyde and ammonia catalysed by the coenzyme B_{12}-dependent enzyme ethanolamine ammonia lyase [10]. The interpretation of this result was that the intermediate radical ($^{\bullet}CH_2CH(NH_2)OH$) undergoes rotation around its C–C bond faster than H-atom transfer from 5′-deoxyadenosine. The use of chiral Me as a mechanistic probe has been reviewed [11][12].

In this paper, we describe the use of the chiral Me group to probe the mechanism of the 2-methylideneglutarate mutase reaction. We have investigated the energy barrier to rotation about the σ-bond between the methylidene C-atom bearing the radical and the adjacent C-atom in the 3-methylitaconate radical. The results indicate that the barrier is sufficiently low that 2-methylideneglutarate stereospecifically labelled with deuterium and tritium at C(3), **1a** or **1b**, leads to 3-([$^2H_1$,$^3H_1$]methyl)itaconate containing a 50:50 mixture of (*R*)- and (*S*)-([$^2H_1$,$^3H_1$]methyl) groups (as in **2a** and **2b**, resp.). A previous attempt to determine the chirality of the chiral Me group of 3-([$^2H_1$,$^3H_1$]methyl)itaconate derived from both (*R*)-**1a** and (*S*)-2-methylidene[3-$^2H_1$,3-$^3H_1$]glutarate **1b** was vitiated by the use of impure 2-methylideneglutarate mutase and problems with the conversion of the 3-([$^2H_1$,$^3H_1$]methyl)itaconate (**2a**/**2b**) to 'chiral acetic acid' (**3a** or **3b**) [13]. We describe experiments with pure recombinant 2-methylideneglutarate mutase and a protocol for the conversion of the 3-([$^2H_1$,$^3H_1$]methyl)itaconate (**2a**/**2b**) to chiral acetic acid (**3a**/**3b**), which minimises exchange processes in any of the intermediates containing a chiral methyl group.

2. Results and Discussion. – 2.1. *Synthesis of (*R*)- and (*S*)-2-Methylidene[3-$^2H_1$,3-$^3H_1$]glutarates* (**1a** and **1b**, resp.). The synthesis was based on the stereospecific exchange of the *pro-S* H-atom at C(3) of 2-oxoglutarate (**4**) catalysed by isocitrate dehydrogenase [14]. The complete routes from 2-oxoglutarate (**4**) are shown in *Schemes 2* and *3*, and were initially developed by converting **4** to unlabelled 2-methylideneglutarate and specifically deuteriated 2-methylideneglutarates **1c**–**1e**. Heating 2-oxoglutarate (**4**) in D_2O gave 2-oxo[3-$^2H_2$]glutarate **4a**, which was converted into (*S*)-2-oxo[3-$^2H_1$,3-$^3H_1$]glutarate (**4b**) using tritiated water with isocitrate dehydrogenase. It was necessary to trap the isotopic labels immediately, and this was done by the rapid reduction of the (*S*)-2-oxo[3-$^2H_1$,3-$^3H_1$]glutarate **4b** with $NaBH_4$. The resulting 2-hydroxy[3-$^2H_1$,3-$^3H_1$]glutarate (**5a**) was converted to diethyl 2-hydroxy[3-$^2H_1$,3-$^3H_1$]glutarate **6a** using $Et_3O^+BF_4^-$ in water. The OH group of the diethyl ester **6a** was activated as its trifluoromethanesulfonate, which was reacted with Ph_3P. Condensation of the derived phosphorane **7a** with formaldehyde gave diethyl (*S*)-2-methylidene[3-$^2H_1$,3-$^3H_1$]glutarate (**8a**), which was hydrolysed to (*S*)-2-methylidene[3-$^2H_1$,3-$^3H_1$]glutarate (**1b**) (*Scheme 2*).

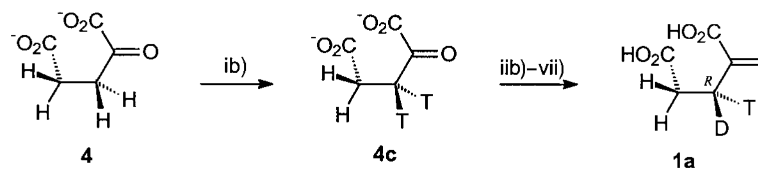
Initial tritiation of 2-oxoglutarate (**4**) at C(3) by heating with tritiated water to give **4c**, followed by washing out tritium at the *pro-S* position by treatment with isocitrate dehydrogenase in D_2O, gave (*R*)-2-oxo[3-$^2H_1$,3-$^3H_1$]glutarate (**4d**). This was converted *via* intermediates **5b**–**8b** to (*R*)-2-methylidene[3-$^2H_1$,3-$^3H_1$]glutarate **1a** (*Scheme 3*) by

Scheme 2. *Synthesis of (3*S*)-2-Methylidene[3-$^2H_1$,3-$^3H_1$]glutaric Acid* (**1b**), *2-Methylidene[3,3-$^2H_2$]glutaric Acid* (**1c**), *(3*S*)-2-Methylidene[3-$^2H_1$]glutaric Acid* (**1d**), *and (3*R*)-2-Methylidene[3-$^2H_1$]glutaric Acid* (**1e**)



ia) D_2O. ib) Tritiated water. iia) Tritiated water, isocitrate dehydrogenase (ICDH), NADPH. iib) D_2O, ICDH, NADPH. iic) H_2O, ICDH, NADPH, iii) $NaBH_4$. iv) $Et_3O^+BF_4^-$, $NaHCO_3$. v) Tf_2O, py then Ph_3P. vi) CH_2O, NaOEt. vii) NaOH. viii) $^{14}CH_2O$, NaOEt.

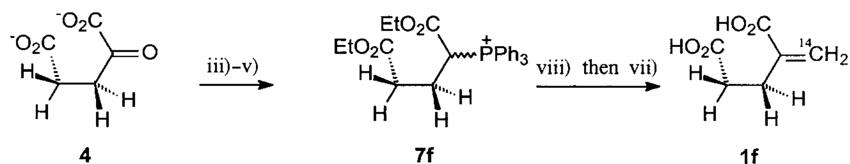
Scheme 3. *Synthesis of (3*R*)-2-Methylidene[3-$^2H_1$,3-$^3H_1$]glutaric acid* (**1a**) (for reagents, see *Scheme 2*)



following the route described above for (*S*)-2-methylidene[3-$^2H_1$,3-$^3H_1$]glutarate (**1b**) (*Scheme 2*).

The analysis of the tritiated samples was performed by measuring the $^3H/^{14}C$ ratio. For this purpose 2-([^{14}C]methylidene)glutaric acid (**1f**) was synthesised from [1,3-bis(ethoxycarbonyl)propyl]triphenylphosphonium triflate (**7f**) and [^{14}C]formaldehyde (*Scheme 4*), and mixed with **1a** and **1b**.

Scheme 4. *Synthesis of 2-([^{14}C]Methylidene)glutaric acid* (**1f**) (for reagents, see *Scheme 2*)



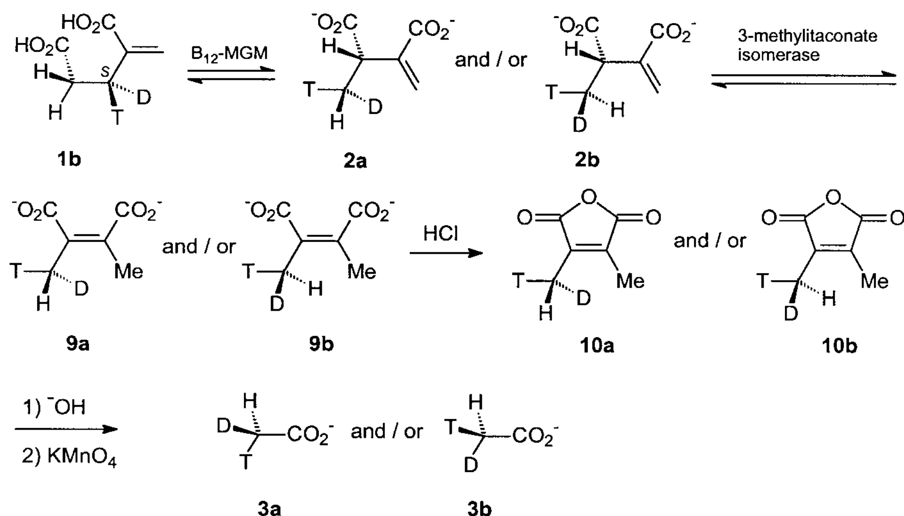
2.2. *Control Experiments Pertaining to Syntheses and Degradations.* The integrity of the isotopic labels throughout the synthetic route was confirmed by converting 2-oxo[3,3-$^2H_2$]glutarate (**4a**) to 2-methylidene[3,3-$^2H_2$]glutarate (**1c**) *via* **5c**–**8c** (*Scheme 2*). Syntheses were also performed in which 2-oxoglutarate (**4**) was subjected to exchange in D_2O catalysed by isocitrate dehydrogenase to give (*S*)-2-oxo[3-$^2H_1$]glutarate (**4e**), and 2-oxo[3,3-$^2H_2$]glutarate was exchanged in H_2O to give (*R*)-2-oxo[3-$^2H_1$]glutarate (**4f**). These 2-oxoglutarates were converted to the corresponding 2-methylidene[3-$^2H_1$]glutarates ((3*S*)- and (3*R*)-2-methylidene[3-$^2H_1$]glutarates (**1d** and **1e**, resp.)) *via* intermediates **5d**–**8d** and **5e**–**8e**, respectively (*Scheme 2*). Analysis of the three deuteriated 2-methylideneglutarates **1c**–**1e** by ^{1}H-NMR showed no loss of deuterium compared to the starting 2-oxoglutarate (*i.e.*, that which was the immediate precursor of 2-hydroxyglutarate). The ^{1}H- and ^{2}H-NMR analyses of the intermediate 2-hydroxy[3-$^2H_1$]glutarate (**5e**) from reduction of (*R*)-2-oxo[3-$^2H_1$]glutarate (**4f**) indicated that it was a 50:50 mixture of the (2*R*,3*R*)- and (2*S*,3*R*)-diastereoisomers, as *two* resonances were observed for H–C(3) and ^{2}H–C(3). Similarly, the 2-hydroxy[3-$^2H_1$]glutarate (**5d**) from reduction of (*S*)-2-oxo[3-$^2H_1$]glutarate (**4e**) was a 50:50 mixture of the (2*R*,3*S*)- and (2*S*,3*S*)-diastereoisomers.

The determination of the sense of chirality of the chiral Me group in a sample of 3-([$^2H_1$,^{3}H]methyl)itaconate was accomplished *via* 2,3-dimethylmaleate derived enzymatically from the 3-methylitaconate (see below). Acidification of the 2,3-dimethylmaleate brought about a rapid conversion to 2,3-dimethylmaleic anhydride [15]. The anhydride was immediately extracted with CH_2Cl_2 and saponified to 2,3-dimethylmaleate, which was oxidised to chiral acetic acid using alkaline permanganate. Since this oxidation may proceed at least in part *via* pyruvate, some loss of tritium due to keto-enol tautomerism could occur. On the other hand, if some Me groups are oxidised, it is expected that Me containing only protium atoms will be oxidised faster than a chiral Me group due to a primary kinetic isotope effect. Since the tritium label and the internal counting standard ^{14}C are located on different Me groups, the effect of the processes described would be to cause the ^{3}H/^{14}C ratio to rise relative to that in the 2,3-dimethylmaleate. The actual yield of acetate in the oxidative degradation, which was developed with unlabelled 2,3-dimethylmaleate, was *ca.* 80% after isolation by ion-exchange chromatography. The missing 20% of acetate could be due to over-oxidation of Me groups or loss during workup. The almost constant ^{3}H/^{14}C ratios observed before and after oxidation ((*R*)-series 2.56 to 2.55, (*S*)-series 2.70 to 2.69) indicates either that no loss of tritium occurred, or that the loss was counterbalanced by the preferential oxidation of the ^{14}C-labelled Me groups containing merely protium. In a control experiment, the oxidation of unlabelled 2,3-dimethylmaleate was performed in the presence of tritiated water. A complete exchange would have led to a calculated

activity of 3.6×10^5 dpm in the acetate, whereas only 2 dpm were found. It can therefore be concluded that, during permanganate oxidation, any chirality of the Me groups in 2,3-dimethylmaleate was *completely* recovered in acetate.

2.3. *Conversion of 3-([$^2H_1$,$^3H_1$]Methyl)itaconates* (**2a** and/or **2b**) *to Chiral Acetates* (**3a** and/or **3b**) via *2,3-Dimethylmaleate* (**9a** and/or **9b**). To prevent racemisation at the chiral Me group of 3-methylitaconates derived from (*R*)- and (*S*)-2-methylidene-[$^2H_1$,$^3H_1$]glutarate, it was necessary to inhibit the reversion to 2-methylideneglutarate. 3-Methylitaconate was removed as it was formed from 2-methylideneglutarate by including 3-methylitaconate isomerase in the reaction mixture. This brought about the thermodynamically favourable conversion of 3-methylitaconate to 2,3-dimethylmaleate *in situ* ($K_{eq} = 7$) [16]. The conversion of a labelled 2-methylideneglurarate (**1a** or **1b**) to labelled 2,3-dimethylmaleate (**9a** and/or **9b**) was allowed to proceed to not more than 5%. The labelled 2,3-dimethylmaleate was then trapped by acidification of the reaction mixture, which induced rapid formation of its anhydride (**10a** and/or **10b**) [15]. After hydrolysis of the anhydride back to 2,3-dimethylmaleate (**9a** and/or **9b**), permanganate oxidation gave chiral acetic acid (**3a** and/or **3b**), which was subjected to a standard analysis [7] to measure its chirality (see *Sect. 2.4*). The complete sequence from (*S*)-2-methylidene[$^2H_1$,$^3H_1$]glutarate (**1b**) to chiral acetic acid (**3a** or **3b**) is shown in *Scheme 5*.

Scheme 5. *Conversion of (3S)-2-Methylidene[3-$^2H_1$,3-$^3H_1$]glutaric Acid* (**1b**) *to Chiral Acetic Acid* (B_{12}-MGM = coenzyme B_{12}-dependent 2-methylideneglutarate mutase)



2.4. *Examination of the Acetates for Chirality.* This was achieved with the standard method based on the use of malate synthase and fumarase [7]. Radiochemical analysis (*Table*) showed that upon incubation of the labelled malate derived *via* labelled acetate from (*R*)-2-methylidene[3-$^2H_1$,3-$^3H_1$]glutarate (**1a**) with fumarase, (50 ± 2)% of its tritium content was lost. Hence, the chiral Me group of the 3-methylitaconate produced from (*R*)-2-methylidene[3-$^2H_1$,3-$^3H_1$]glutarate was a 50:50 mixture of (*R*)- and (*S*)-

Table. *Specific Activities of Isotopically Labelled Compounds*

Labelled compound		(*R*)-Series		(*S*)-Series	
		total ^{3}H/dpm	^{3}H/^{14}C ratio [dpm/dpm]	total ^{3}H/dpm	^{3}H/^{14}C ratio [dpm/dpm]
2-Methylideneglutarate		1.6×10^6	2.56	1.7×10^6	2.70
Acetate		5.3×10^4	2.55	5.9×10^4	2.69
Malate	$t = 0$ min	4271	2.14 (100%)	4810	2.23 (100%)
Malate/fumarate	$t = 60$	2558	1.03 (48.1%)	2459	1.08 (48.4%)
Malate/fumarate	$t = 90$	2089	1.12 (52.3%)	2048	1.10 (49.3%)
Malate/fumarate	$t = 120$	2734	1.07 (50.0%)	2261	1.08 (48.4%)
Malate/fumarate	Mean value 60–120 min		1.07 (50 ± 2%)		1.09 (49 ± 2%)

forms. The labelled malate derived from (*S*)-2-methylidene[3-$^2H_1$,3-$^3H_1$]glutarate (**1b**) with fumarase lost (49 ± 2)% of its tritium. Hence, the chiral Me of the 3-methylitaconate from (*S*)-2-methylidene[3-$^2H_1$,3-$^3H_1$]glutarate was also a 50:50 mixture of (*R*)- and (*S*)-forms.

Arigoni and *Mazacek* demonstrated that the malate derived from monochiral (*R*)-acetate, of much higher specific activity than that used in the present work, retained 82% of its tritium content after exchange catalysed by fumarase, whereas that derived from monochiral (*S*)-acetate retained 20% [17]. Therefore, the isochiral [9] (racemic) mixture should give 51% retention, whereas [^{3}H]acetate leads to a malate specimen, which upon incubation with fumarase, loses exactly 50% of its tritium content. The results described here, however, are not accurate enough (see above) to distinguish between these possibilities.

2.5. *Mechanistic Implications.* We have found that the chiral Me group of 3-methylitaconate derived from 2-methylideneglutarate in the isomerisation catalysed by 2-methylideneglutarate mutase is essentially a 50:50 mixture of opposite chiralities. This implies that the radical centre in the 3-methylitaconate radical is rotating with a relatively low energy barrier and does not interact significantly with the Co-atom in cob(II)alamin. This result is consistent with the notion that the rearrangement of 2-methylideneglutarate to 3-methylitaconate occurs *without* the participation of cob(II)alamin. The addition-elimination and fragmentation-recombination mechanisms for this enzyme [3], as well as a route involving 'partial protonation' of the substrate methylidene group [18], are compatible with this finding.

3. Conclusion. – This is the first time that a chiral Me experiment has been performed for a coenzyme B_{12}-dependent enzyme using homogenous protein. The experiments described generated isochiral [9] acetic acid, which we have interpreted as evidence for the intermediacy of the 3-methylitaconate radical ($^{\bullet}CH_2CH(^{-}O_2CC{=}CH_2)CO_2^-$) in the equilibration of 2-methylideneglutarate with (*R*)-3-methylitaconate catalysed by coenzyme B_{12}-dependent 2-methylideneglutarate mutase. The results suggest that a protein-mediated rearrangement of the 2-methylideneglutarate radical to the 3-methylitaconate radical occurs (*cf. Scheme 1*), with cob(II)alamin as a spectator. In our experiments, it was necessary to exclude the possibility that isochiral acetic acid was formed *via* reversal of the formation of 3-methylitaconate. We believe

that this was avoided by trapping the 3-methylitaconate by its conversion into 2,3-dimethylmaleate catalysed by methylitaconate isomerase.

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Experimental Part

General. Commercial reagents were utilised without further purification. CH_2Cl_2 and pyridine were distilled from CaH_2. EtOH was distilled from Mg/I_2. Petrol was the fraction boiling in the range 40–60°. Deuteriated solvents were from *Aldrich Chemical Company*, Gillingham, Dorset, UK. Tritiated water (300 mCi/ml) was a gift from Dr. *D. Moltzan* (Fachbereich Chemie, Philipps-Universität Marburg). [^{14}C]Formaldehyde (0.1 mCi/ml) was from *American Radiolabeled Chemicals Inc.* (Avonmouth, Bristol, UK). Acetyl-CoA synthase from yeast, L-lactate dehydrogenase from rabbit muscle and fumarase from pig heart were from *Roche*, D-Mannheim. Maleate synthase, isocitrate dehydrogenase and coenzyme B_{12} were from *Sigma*, Poole, Dorset, UK. TLC: aluminium sheets pre-coated with silica gel (*Kieselgel 60 F_{254}*, 0.2 mm, *Merck*, D-Darmstadt). Silica gel ('flash', *Kieselgel 60*) was used for column chromatography (CC). M.p.: determined in capillaries using a *Gallenkamp* apparatus and are uncorrected. ^{1}H-, ^{2}H-, and ^{13}C-NMR: recorded at the frequency stated; chemical shifts (δ) in ppm, with residual protons in the deuteriated solvents as an internal standard.

1. Syntheses of Deuteriated Compounds. – 1.1. *2-Oxo[3,3-$^2H_2$]glutarate Disodium Salt* (**4a**). 2-Oxoglutaric acid disodium salt (2.0 g, 10.5 mmol) was dissolved in D_2O (25 ml) and heated for 3 h at 80°. The solvent was removed by freeze-drying to give **4a** (2.0 g, 10.5 mmol, 100%). White solid. ^{1}H-NMR (200 MHz, D_2O, pD 7): 2.54 (*s*, 2 H). ^{13}C-NMR (50 MHz, D_2O): 207.10; 183.07; 171.41; 36.70 (^1J(C,D) = 15); 32.09.

1.2. *2-Hydroxy[3,3-$^2H_2$]glutaric Acid* (**5c**). $NaBH_4$ (20 mg, 0.52 mmol) was added to an aq. 0.5M soln. of 2-oxo[3,3-$^2H_2$]glutarate (2.0 ml, 1 mmol, pH 7.5) and allowed to react for 5 min. 2M HCl (0.1 ml) was added and the resulting solution was freeze-dried to give a white powder containing **5c**. ^{1}H-NMR (200 MHz, D_2O): 2.39 (*d*, J = 16.7, 1 H); 2.44 (*d*, J = 16.7, 1 H); 4.18 (*s*, 1 H). ^{2}H-NMR (500 MHz, H_2O): 1.81 (*s*, 1 ^{2}H); 2.20 (*s*, 1 ^{2}H). ^{13}C-NMR (125 MHz, D_2O): 181.9; 180.3; 71.1; 32.5; 29.8 (^1J(C,D) = 19.9).

1.3. *Diethyl 2-Hydroxy[3,3-$^2H_2$]glutarate* (**6c**). *Method a*. Compound **5c** (0.79 g, 5.3 mol) was dissolved in 0.23M NaOH (65 ml) and the soln. was stirred at r.t. overnight. Solid $NaHCO_3$ (22.5 g, 0.27 mol) and a soln. of $Et_3O^+BF_4^-$ (42 g, 0.22 mol) in MeCN (10 ml) were added simultaneously over a period of 10 min, the pH being kept between 7 and 8. The resulting suspension was allowed to stir at r.t. for 20 min. The mixture was diluted with H_2O (50 ml) and decanted. The aq. soln. was extracted with CH_2Cl_2, and the white precipitate was washed with CH_2Cl_2. The combined org. extracts were washed with brine and dried ($MgSO_4$). The solvent was removed and the resulting yellow oil was purified by chromatography (silica gel; 20% AcOEt in petrol) to afford **6c** (0.80 g, 3.9 mmol, 73%). Colorless oil.

Method b. Compound **5c** (400 mg, 2.7 mmol) was dissolved in abs. EtOH (150 ml). TsOH (20 mg, 0.1 mmol) was added and the reaction was boiled at reflux for 48 h. The EtOH was removed and the residue was partitioned between CH_2Cl_2 and 10% $NaHCO_3$. The org. phase was washed with brine and dried (Na_2SO_4). The solvent was removed and the resulting pale yellow oil was purified by chromatography (silica gel; 20% AcOEt in petrol) to afford **6c** (475 mg, 2.3 mmol, 86%). Colorless oil. ^{1}H-NMR (200 MHz, $CDCl_3$): 1.23 (*t*, J = 7, 3 H); 1.28 (*t*, J = 7, 3 H); 2.36 (*d*, J = 11, 1 H); 2.42 (*d*, J = 11, 1 H); 3.01 (*d*, OH); 4.00–4.23 (*m*, 5 H). ^{13}C-NMR (50 MHz, $CDCl_3$): 174.6; 173.1; 69.4; 61.9; 60.5; 29.5; 28.8 (^1J(C,D) = 19); 14.1; 14.0.

1.4. *[1,3-Bis(ethoxycarbonyl)[2,2-$^2H_2$]propyl]triphenylphosphonium Triflate* (**7c**). Triflic anhydride (0.66 ml, 4.0 mmol) was added over a period of 5 min to a chilled (−5°) soln. of **6c** (0.80 g, 3.9 mmol) and pyridine (0.32 ml, 4.0 mmol) in CH_2Cl_2 (5.6 ml) under N_2. The mixture was stirred for 30 min at −5°, Ph_3P (2.4 g, 9.1 mmol) in CH_2Cl_2 (3.2 ml) was added and the resulting mixture stirred overnight at r.t. The solvent was removed and the residue was purified by chromatography on silica using CH_2Cl_2 as eluent to remove the excess Ph_3P and then AcOEt to recover **7c** (1.74 g, 2.9 mmol 74%). The slightly yellow solid was recrystallized with AcOEt to give **7c**. Colorless crystals. M.p. 120–123°. ^{1}H-NMR (200 MHz, $CDCl_3$): 1.02 (*t*, J = 7.1, 3 H); 1.23 (*t*, J = 7.1, 3 H); 2.59 (*d*, J = 17, 1 H); 2.72 (*d*, J = 17, 1 H); 3.90 (*q*, J = 7.1, 2 H); 4.01 (*q*, J = 7.1, 2 H); 5.2 (*d*, ^2J(P,H) = 14.2, 1 H); 7.60–7.90 (*m*, 15 H). ^{13}C-NMR (50 MHz, $CDCl_3$): 172.1; 167.3; 135.6; 134.4 (^3J(P,C) = 9); 134.1 (^2J(P,C) = 13); 115.2 (^1J(P,C) = 86); 62.2 (^1J(P,C) = 120); 40.5, 31.5, 31.2; 23.9 (^1J(C,D) = 19); 14.1; 13.6. CI-MS: 451, 363, 279, 185, 145, 107, 79.

1.5. *Diethyl 2-Methylidene[3,3-$^2H_2$]glutarate* (**8c**). Paraformaldehyde (57 mg, 1.9 mmol) was added to a soln. of **7c** (800 mg, 1.33 mmol) in dry EtOH (3.0 ml). To this mixture was added dropwise 1M NaOEt (2.0 ml,

2 mmol) and the mixture was allowed to stir overnight. The solvent was removed and the residue was purified by chromatography (silica gel; 10% AcOEt in petrol) to give **8c** (250 mg, 1.23 mmol, 92%). Colorless oil. ^{1}H-NMR (500 MHz, $CDCl_3$): 1.22 (*t*, $J = 7$, 3 H); 1.26 (*t*, $J = 7$, 3 H); 2.47 (*s*, 2 H); 4.10 (*q*, $J = 7$, 2 H); 4.17 (*q*, $J = 7$, 2 H); 5.52 (*d*, $^2J = 0.7$, 1 H); 6.12 (*d*, $^2J = 0.7$, 1 H). ^{13}C-NMR (125 MHz, $CDCl_3$): 172.7; 166.7; 139.2; 125.5; 60.7; 60.4; 33.1 (^1J(C,D) = 18); 27.3; 14.2 (2 ×).

1.6. *2-Methylidene[3,3-$^2H_2$]glutaric Acid* (**1c**). 5M NaOH (1.0 ml) was added to **8c** (250 mg, 1.22 mmol) in MeOH (1.4 ml) and the mixture was stirred for 16 h at r.t. The MeOH was removed, the residual aq. soln. was acidified with 2M HCl and the resulting mixture was extracted with Et_2O (3 ×). The combined Et_2O extracts were washed with H_2O and brine, and dried ($MgSO_4$). The solvent was removed to afford a white solid. The crude product was recrystallized from MeCN to give **1c** (117 mg, 0.80 mmol, 65%). White crystals. M.p. 128 – 130° ([19]: 129 – 130°). ^{1}H-NMR (500 MHz, D_2O): 2.46 (*s*, 2 H); 5.59 (*s*, 1 H); 6.08 (*s*, 1 H). ^{13}C-NMR (125 MHz, D_2O): 174.09; 168.20; 140.52; 126.05; 33.21; 27.62 (^1J(C,D) = 19.6). EI-MS: 146, 128, 101, 98, 87, 83, 71, 60, 55.

1.7. *(3*R*)-2-Hydroxy[3-$^2H_1$]glutaric Acid* (**5e**). A soln. containing 0.5M **4a** (2.0 ml, 1.0 mmol, pH 7.5), 20 mM *Tris* · HCl pH 7.4, 0.3 mM $MgCl_2$, 1.5 mM NADPH and 200 U isocitrate dehydrogenase was incubated at 37° for 2 h. $NaBH_4$ (20 mg, 0.52 mmol) was added to the mixture and allowed to react for 5 min. 2M HCl (100 µl) was added and the resulting soln. was freeze-dried to give a white powder containing **5e**. ^{1}H-NMR (200 MHz, D_2O): 1.85 – 1.95 (*m*, 0.5 H); 1.96 – 2.05 (*m*, 0.5 H); 2.22 – 2.44 (*m*, 2 H); 4.06 (*d*, $J = 3.8$, 1 H). ^{2}H-NMR (500 MHz, H_2O): 1.82 (*s*, 0.5 ^{2}H); 2.21 (*s*, 0.5 ^{2}H). ^{13}C-NMR (125 MHz, D_2O): 181.90; 180.35; 71.14; 32.48; 29.78 (^1J(C,D) = 19.9).

1.8. *Diethyl (3*R*)-2-Hydroxy[3-$^2H_1$]glutarate* (**6e**). Compound **5e** (149 mg, 1.0 mmol) was reacted with an excess of $Et_3O^+BF_4^-$ in the manner described for the preparation of **6c** (see *1.3*, method *a*) to afford, after workup, **6e** (132 mg, 0.65 mmol, 65%). Colorless oil. ^{1}H-NMR (200 MHz, $CDCl_3$): 1.23 (*t*, $J = 7.0$, 3 H); 1.28 (*t*, $J = 7.0$, 3 H); 1.81 – 2.03 (*m*, 0.5 H); 2.05 – 2.24 (*m*, 0.5 H); 2.32 – 2.58 (*m*, 2 H); 2.87 (br., OH); 4.02 – 4.24 (*m*, 5 H). ^{13}C-NMR (50 MHz, $CDCl_3$): 174.6; 173.2; 69.4; 61.8; 60.5; 29.7; 29.3 (^1J(C,D) = 20); 14.1; 14.0.

1.9. *(3*R*)-[1,3-Bis(ethoxycarbonyl)[2-$^2H_1$]propyl]phosphonium Triflate* (**7e**). Compound **6e** (132 mg, 0.65 mmol) was treated with triflic anhydride in the manner described for **7c** (see *1.4*), followed by reaction of the intermediate triflate with PPh_3. After workup, **7e** (290 mg, 0.48 mmol, 73%) was obtained. Colorless crystals. M.p. 120 – 123°. ^{1}H-NMR (200 MHz, $CDCl_3$): 1.02 (*t*, $J = 7.1$, 3 H); 1.23 (*t*, $J = 7.1$, 3 H); 2.00 – 2.20 (*m*, 0.5 H); 2.21 – 2.40 (*m*, 0.5 H); 2.62 – 2.82 (*m*, 2 H); 3.97 (*q*, $J = 7.1$, 2 H); 4.12 (*q*, $J = 7.1$, 2 H); 5.13 (*m*, 1 H); 7.60 – 7.90 (*m*, 15 H). ^{13}C-NMR (50 MHz, $CDCl_3$): 172.1; 167.3; 135.6; 134.4 (^3J(P,C) = 9); 134.1 (^2J(P,C) = 13); 115.2 (^1J(P,C) = 86); 62.2 (^1J(P,C) = 120); 40.5; 31.5; 31.2; 23.9 (^1J(C,D) = 19); 14.1; 13.6.

1.10. *Diethyl (3*R*)-2-Methylidene[3-$^2H_1$]glutarate* (**8e**). Paraformaldehyde was reacted with **7e** (290 mg, 0.48 mmol) in the manner described for the preparation of **8c** (see *1.5*) to give, after workup, **8e** (74 mg, 0.37 mmol, 76%). Colorless oil. ^{1}H-NMR (500 MHz): 1.22 (*t*, $J = 7$, 3 H); 1.26 (*t*, $J = 7$, 3 H); 2.31 – 2.42 (*m*, 2 H); 2.42 – 2.51 (*m*, 1 H); 4.10 (*q*, $J = 7$, 2 H); 4.17 (*q*, $J = 7$, 2 H); 5.52 (*s*, 1 H); 6.12 (*s*, 1 H). ^{13}C-NMR (125 MHz, CD_3CN): 173.5; 167.6; 140.7; 125.9; 61.7; 61.2; 33.8; 27.9 (^1J(C,D) = 17); 14.7; 14.6.

1.11. *(3*R*)-2-Methylidene[3-$^2H_1$]glutaric Acid* (**1e**). NaOH (5M, 0.30 ml) was added to **8e** (74 mg, 0.37 mmol) in MeOH (0.4 ml) and the mixture was stirred for 16 h at r.t. After removing the MeOH, the residual aq. soln. was acidified with 2M HCl and extracted with Et_2O (3 ×). The combined Et_2O extracts were washed with H_2O and brine, and dried ($MgSO_4$). The Et_2O was removed to afford **1e** (51 mg, 0.35 mmol, 94%). White solid. M.p. 129 – 130°. ^{1}H-NMR (500 MHz, (D_6)acetone): 2.38 – 2.46 (*m*, 3 H); 5.59 (*s*, 1 H); 6.08 (*s*, 1 H). ^{13}C-NMR (125 MHz, (D_6)acetone): 174.09; 168.22; 140.52; 126.06; 33.22; 27.61 (^1J(C,D) = 19.6).

1.12. *(3*S*)-2-Hydroxy[3-$^2H_1$]glutaric Acid* (**5d**). A soln. containing 0.5M 2-oxoglutarate (**4**, 2.0 ml, 1.0 mmol, pD 7.5) in D_2O, 20 mM *Tris* · HCl pD 7.4 in D_2O, 0.3 mM $MgCl_2$ in D_2O, 1.5 mM NADPH in D_2O and 200 U isocitrate dehydrogenase was incubated at 37° for 2 h. $NaBH_4$ (10 mg, 0.52 mmol, 2.1 equiv.) was added to the mixture and allowed to react for 5 min. 2M HCl (100 µl) was added and the resulting soln. was freeze-dried to give a white powder containing **5d**. ^{1}H-NMR (500 MHz, D_2O): 1.85 – 1.95 (*m*, 0.5 H); 1.96 – 2.05 (*m*, 0.5 H); 2.22 – 2.44 (*m*, 2 H); 4.06 (*d*, $J = 3.8$, 1 H). ^{2}H-NMR (76 MHz, H_2O): 1.82 (*s*, 0.5 ^{2}H); 2.21 (*s*, 0.5 ^{2}H). ^{13}C-NMR (125 MHz, D_2O): 181.90; 180.35; 71.14; 32.48; 29.78 (^1J(C,D) = 19.9).

1.13. *Diethyl (3*S*)-2-Hydroxy[3-$^2H_1$]glutarate* (**6d**). Compound **5d** (149 mg, 1.0 mmol) was reacted with an excess of $Et_3O^+BF_4^-$ in the manner described for the preparation of **6c** (see *1.3*, method *a*) to afford, after workup, **6e** (135 mg, 0.66 mmol, 66%). Colorless oil. ^{1}H-NMR (200 MHz, $CDCl_3$): 1.23 (*t*, $J = 7.0$, 3 H); 1.28 (*t*, $J = 7.0$, 3 H); 1.81 – 2.03 (*m*, 0.5 H); 2.05 – 2.24 (*m*, 0.5 H); 2.32 – 2.58 (*m*, 2 H); 2.87 (br., OH); 4.02 – 4.24 (*m*, 5 H). ^{13}C-NMR (50 MHz, $CDCl_3$): 174.6; 173.2; 69.4; 61.8; 60.5; 29.7; 29.3 (^1J(C,D) = 20); 14.1; 14.0.

1.14. *(3*S*)-[1,3-Bis(ethoxycarbonyl)[2-$^2H_1$]propyl]triphenylphosphonium Triflate* (**7d**). Compound **6d** (135 mg, 0.66 mmol) was treated with triflic anhydride in the manner described for **7c** (see *1.4*), followed by reaction of the intermediate triflate with PPh_3. After workup, compound **7e** (300 mg, 0.49 mmol, 74%) was obtained. Colorless crystals. M.p. 120–123°. ^{1}H-NMR (200 MHz, $CDCl_3$): 1.02 (*t*, *J* = 7.1, 3 H); 1.23 (*t*, *J* = 7.1, 3 H); 2.00–2.20 (*m*, 0.5 H); 2.21–2.40 (*m*, 0.5 H); 2.62–2.82 (*m*, 2 H); 3.97 (*q*, *J* = 7.1, 2 H); 4.12 (*q*, *J* = 7.1, 2 H); 5.13 (*m*, 1 H); 7.60–7.90 (*m*, 15 H). ^{13}C-NMR (50 MHz, $CDCl_3$): 172.1; 167.3; 135.6; 134.4 (^3J(P,C) = 9); 134.1 (^2J(P,C) = 13); 115.2 (^1J(P,C) = 86); 62.2 (^1J(P,C) = 120); 40.5; 31.5; 31.2; 23.9 (^1J(C,D) = 19); 14.1; 13.6.

1.15. *Diethyl (3*S*)-2-Methylidene[3-$^2H_1$]glutarate* (**8d**). Paraformaldehyde was reacted with **7d** (300 mg, 0.49 mmol) in the manner described for the preparation of **8c** (see *1.5*) to give, after workup, **8d** (72 mg, 0.36 mmol, 74%). Colorless oil. ^{1}H-NMR (200 MHz, $CDCl_3$): 1.22 (*t*, *J* = 7, 3 H); 1.26 (*t*, *J* = 7, 3 H); 2.44–2.63 (*m*, 3 H); 4.10 (*q*, *J* = 7, 2 H); 4.17 (*q*, *J* = 7, 2 H); 5.52 (*s*, 1 H); 6.12 (*s*, 1 H). ^{13}C-NMR (50 MHz, $CDCl_3$): 172.7; 166.7; 139.2; 125.5; 60.7; 60.4; 33.1; 27.3 (^1J(C,D) = 18); 14.2 (2 ×).

1.16. *(3*S*)-2-Methylidene[3-$^2H_1$]glutaric Acid* (**1d**). NaOH (5M, 0.30 ml) was added to **8d** (72 mg, 0.36 mmol) in MeOH (0.4 ml) and the mixture was stirred for 16 h at r.t. After removing the MeOH, the residual aq. soln. was acidified with 2M HCl and extracted with Et_2O (3 ×). The combined Et_2O extracts were washed with H_2O and brine, and dried ($MgSO_4$). The Et_2O was removed to afford **1d** (49 mg, 0.33 mmol, 93%). White solid. M.p. 128–130°. ^{1}H-NMR (500 MHz, (D_6)acetone): 2.38–2.46 (*m*, 3 H); 5.59 (*s*, 1 H); 6.08 (*s*, 1 H). ^{13}C-NMR (125 MHz, (D_6)acetone): 174.09; 168.22; 140.52; 126.06; 33.22; 27.61 (^1J(C,D) = 20).

2. Synthesis of Radiolabelled Compounds. – 2.1 The synthesis of each tritiated compound was performed by a similar procedure and using the same quantities as described for the corresponding deuteriated compound, except that tritiated water (25 μl, 300 mCi/ml) was introduced in the exchange step catalysed by isocitrate dehydrogenase (see *1.7*) for the series leading to *(3*S*)-2-methylidene[3-$^2H_1$,3-$^3H_1$]glutaric acid* (**1b**), and 2-oxoglutarate (190 mg, 1.0 mmol) heated in 2 ml water containing tritiated water (25 μl, 300 mCi/ml) was converted to *2-oxo-[3,3-$^3H_2$]glutarate disodium salt* (**4c**) for the series leading to *(3*R*)-2-methylidene[3-$^2H_1$,3-$^3H_1$]glutaric acid* (**1a**). In this way, the following radiolabelled compounds were obtained from **4a**: *(3*S*)-2-hydroxy[3-$^2H_1$,3-$^3H_1$]glutaric acid* (**5a**), *diethyl (3*S*)-2-hydroxy[3-$^2H_1$,3-$^3H_1$]glutarate* (**6a**); *(3*S*)-[1,3-bis(ethoxycarbonyl)[3-$^2H_1$,3-$^3H_1$]propyl]triphenylphosphonium triflate* (**7a**); *diethyl (3*S*)-2-methylidene[3-$^2H_1$,3-$^3H_1$]glutarate* (**8a**); *(3*S*)-2-methylidene[3-$^2H_1$,3-$^3H_1$]glutaric acid* (**1b**). The following radiolabelled compounds were obtained from **4c**: *(3*R*)-2-hydroxy[3-$^2H_1$,3-$^3H_1$]glutaric acid* (**5b**); *diethyl (3*R*)-2-hydroxy[3-$^2H_1$,3-$^3H_1$]glutarate* (**6b**); *(3*R*)-[1,3-bis(ethoxycarbonyl)[3-$^2H_1$,3-$^3H_1$]propyl]triphenylphosphonium triflate* (**7b**); *diethyl (3*R*)-2-methylidene[3-$^2H_1$,3-$^3H_1$]glutarate* (**8b**); *(3*R*)-2-methylidene[3-$^2H_1$,3-$^3H_1$]glutaric acid* (**1a**).

2.2. *2-([^{14}C]Methylidene)glutaric Acid* (**1f**). This compound was derived from diethyl 2-([^{14}C])methylidene)glutarate, which was synthesised from [1,3-bis(ethoxycarbonyl)propyl]triphenylphosphonium triflate and [^{14}C]formaldehyde (0.1 mCi/ml) in the manner described for compounds **1a** and **1b**. Hydrolysis of the diethyl 2-([^{14}C]methylidene)glutarate afforded **1f** with an activity of 12.8 μCi/mmol.

3. Control Experiment: Oxidation of Dimethylmaleic Anhydride with Permanganate in Tritiated Water. – To dimethylmaleic anhydride (4.0 mg, 31 μmol) in 0.4 ml of 0.25M NaOH was added tritiated water (5 μl, 30 mCi/ml) and 1.5 ml of 0.2M $KMnO_4$. The mixture was left overnight at r.t. and then treated with 3% H_2O_2 (2.0 ml) to reduce the excess $KMnO_4$. Finally, the mixture was boiled to remove unreacted H_2O_2. The cooled mixture was filtered and the filtrate was passed through a 5 ml *Dowex 1X8* (200–400 mesh) column, Cl^- form. The resin was washed with H_2O (150 ml) and acetate was recovered by elution with 7.5 ml of 0.5M HCl. The acidic soln. was neutralised with NaOH and freeze-dried twice to afford 300 mg (mainly NaCl) of a white solid. Acetate was determined enzymatically with acetyl-CoA synthase [20]. This sample, which contained 38.7 μmol (62%) acetate, was counted against a blank of AcONa and NaCl. No deviation from the background was found for either sample. Blank: 26 dpm; sample: 28 dpm (for a complete exchange 3.6×10^5 dpm would have been expected).

4. Purification of *apo*-2-Methylideneglutarate Mutase and 3-Methylitaconate Isomerase. – Overexpression of the gene coding for 2-methylideneglutarate mutase (*mgm*) in *Escherichia coli* DH5α, transformed with the plasmid pBB2, was performed essentially as described in [21]. Since *E. coli* lacks adenosylcobalamin, the *apo*-enzyme could be purified aerobically and without particular precautions to exclude light. All manipulations were carried out at 4°. Cells (20 g wet mass) were harvested by centrifugation (6000 × g, 15 min), resuspended in buffer *A* (50 mM K_3PO_4, 1 mM EDTA, pH 7.4) containing 1 mM methylphenylsulfonyl fluoride, and disrupted by sonication (*Branson sonifier B15*, USA). Cell debris and membranes were removed by two consecutive ultracentrifugation steps (200,000 × g, 30 min). The enzyme was further purified by chromatography on a *Pharmacia* FPLC system equipped with *Pharmacia* columns (D-Freiburg). The supernatant of the second

ultracentrifugation step was loaded onto DEAE Sepharose (column type *HiLoad 26/10*) equilibrated with buffer A (3 ml/min). After washing with 100 ml buffer *A*, *apo*-2-methylideneglutarate mutase was eluted with a linear gradient of 0–1M NaCl in buffer *A*. The fractions eluting at *ca.* 300 mM NaCl contained *apo*-mutase activity. $(NH_4)_2SO_4$ was added to a final concentration of 1M to the pooled fractions. The clear soln. was loaded onto phenyl-*Sepharose* HP column (*HiLoad 26/10*) equilibrated with buffer *B* (buffer *A* supplemented with 1M $(NH_4)_2SO_4$) operated at a flow rate of 3 ml/min. A gradient of 100–50% buffer *B* in buffer *A* (100 ml), 50–0% buffer *B* in buffer *A* (200 ml) and 200 ml buffer *A* eluted the *apo*-mutase after *ca*. 400 ml. Fractions which exhibited activity were concentrated in an *Amicon* chamber with *PM30* membrane (*Amicon*, D-Witten) and loaded on a *Superdex 200* prep-grade column (type *35/600*) equilibrated with buffer *A* supplemented with 100 mM NaCl (0.5 ml/min). *apo*-Mutase fractions eluted at a volume of *ca*. 250 ml. The purity of the concentrated enzyme was 95–99% as determined by *SDS-PAGE*. Purified *apo*-mutase was stable for several months when stored at protein concentrations of 1–40 mg/ml at –80°. The protein content was determined by the *Bradford* method [22].

The gene *mii* coding for (*R*)-3-methylitaconate isomerase was cloned by PCR from pBB1 into pBluescript SK+ cosmid yielding the plasmid pGB1. Restriction analysis showed that the 5′-region of the *mii* gene was oriented to the T7-promoter of the cloning vector. For the overexpression of the *mii* gene, *E. coli* BL21(λDE3) [23] was transformed with the pGB1 plasmid. The *E. coli* clone was grown and induced by the procedure described in [21]. The purification of the overproduced enzyme was essentially the same as described above for the 2-methylideneglutarate mutase, except that the size-exclusion column (*Superdex 200 prep-grade*) was omitted. The enzyme with a purity of 95–99% could be stored at –80% for months at protein concentrations of 1–40 mg/ml without loss of isomerase activity.

The activity of *apo*-2-methylideneglutarate mutase was measured spectrophotometrically at 25° in a cuvette, $d = 1$ cm, total volume of 1.0 ml, containing 100 mM K_3PO_4, pH 7.4, 1 mM dithiothreitol, *ca*. 1 U 3-methylitaconate isomerase, and 5 mM 2-methylideneglutarate. After addition of the *apo*-enzyme, the reaction was started with 10 µM adenosylcobalamin (final concentration) and was followed at $\lambda = 256$ nm ($\varepsilon = 0.66$ mM^{-1} cm^{-1}). The activity of 3-methylitaconate isomerase was determined with a similar assay in which 2-methylideneglutarate and adenosylcobalamin were replaced by 5 mM (*R*,*S*)-3-methylitaconate. The reaction was started by addition of the isomerase.

5. Enzymology. – 5.1. *Conversion of (*R*)-2-Methylidene[3-$^2H_1$,3-$^3H_1$]glutarate to Chiral Acetate.* The incubation contained in a total volume of 1.4 ml: 50 mM K_3PO_4, pH 7.4; 10 mM (*R*)-2-methylidene[3-$^2H_1$,3-$^3H_1$]glutarate (*ca*. 116000 dpm/µmol); 40 mM 2-([^{14}C]methylidene)glutarate (*ca*. 12000 dpm/µmol); 1.5 U 2-methylideneglutarate mutase; 5 U 3-methylitaconate isomerase. A 7-µl sample was withdrawn from a mixture containing only the two 2-methylideneglutarates (460 µl) and its radioactivity was determined: ^3H: 24388 dpm; ^{14}C: 9511 dpm; $^3H/^{14}C = 2.56$. The reaction was started by addition of 28 µl of 1 mM adenosylcobalamin. The formation of 2,3-dimethylmaleate was followed by the increase of the UV absorbance at $\lambda = 270$ nm ($\varepsilon = 0.085$ mM^{-1} cm^{-1}). After 134 s, an increase in absorbance of 0.106 (determined in a cuvette with 5-mm path length) had occurred corresponding to a 5% (2.5 mM/50 mM) conversion of 2-methylideneglutarate, the reaction was quenched with 600 µl of 8M (25%) aq. HCl. The resulting soln. was extracted wtih CH_2Cl_2 (4 × 10 ml) and dried ($MgSO_4$). The solvent was removed to afford 2,3-dimethylmaleic anhydride.

The dimethylmaleic anhydride (*ca*. 0.4 mg) was oxidised with alkaline $KMnO_4$ to acetate as described above (*Sect. 3*), but on a 10-fold smaller scale. Radiochemical analysis of the acetate soln. (100 µl, 5.73 µmol, *ca*. 90% yield) gave: ^3H: 7123 dpm; ^{14}C: 2825 dpm; $^3H/^{14}C = 2.55$. According to analysis by 500-MHz ^1H-NMR and UV/VIS spectroscopy, no residual 2,3-dimethylmaleate was present.

5.2. *Analysis of Chiral Acetate from (*R*)-2-Methylidene[3-$^2H_1$,3-$^3H_1$]glutarate.* The incubation contained in a total volume of 0.5 ml: 33 mM $KHCO_3/K_2CO_3$, pH 9; 2.0 mM chiral acetate from above; 4 mM glyoxylate; 4 mM ATP; 4 mM $MgCl_2$; 1 mM Na_2EDTA; 2 U malate synthase; 2 U acetyl-CoA synthase (the malate synthase and acetyl-CoA synthase both contained less than 0.1 mU fumarase). The reaction was started by addition of 0.3 mM CoASH and monitored for the disappearance of glyoxylate. The assay was based on the reduction of glyoxylate to hydroxyacetic acid (glycolate) mediated by NADH with a non-specific lactate dehydrogenase from rabbit muscle. The reaction soln. contained 100 mM K_3PO_4 buffer, pH 7.4, 20 mM NADH, 15 µl of the sample to be analysed and 6 µl of lactate dehydrogenase (8760 U/ml). The reaction was followed by monitoring the decrease of absorption at $\lambda = 340$ nm ($\varepsilon_{340} = 6.2$ mM^{-1} cm^{-1}): 0/0, 20/2.04, 30/2.05 (time [min]/glyoxylate consumed [µmol] from an initial 3.93 µmol). The reaction mixture was diluted to 20 ml and passed through a *Dowex 8 × 1* (formate form, 20 ml), which was washed with H_2O (50 ml). Malic acid was eluted from the column with 1M HCO_2H, collected in 7 fractions (10 ml each) and counted. Radioactivity was present in

fractions 3 and 4. The combined radioactive fractions were evaporated to dryness under vacuum. The residue was dissolved in *ca*. 1 ml of 50 mM phosphate buffer, pH 7.4. A sample (200 µl) of this soln. was counted. Fumarase (10 U, 14 µl) was added to the rest of the mixture and the resulting soln. was incubated at 40°. Samples (200 µl) were taken at different times and briefly boiled in order to inactivate the enzyme. Each sample was diluted with 30 ml of H_2O and brought to dryness (this was done twice). Eventually H_2O (200 µl) was added and the whole sample counted to give the following results: 0/2.14/0, 60/1.03/48.1, 90/1.12/52.3, 120/1.07/50.0 (time [min]/ratio of ^3H to ^{14}C/% change compared to malate).

5.3. *Conversion of (*S*)-2-Methylidene[3-$^2H_1$,3-$^3H_1$]glutarate to Chiral Acetate.* This was performed in the manner described for (*R*)-2-methylidene[3-$^2H_1$,3-$^3H_1$]glutarate. A 7-µl sample was withdrawn from a mixture containing only the two 2-methylideneglutarates (*i.e.*, before adding the other components) and its radioactivity was determined: ^3H: 25778 dpm; ^{14}C: 9539 dpm; $^3H/^{14}C = 2.70$. Acetate was determined by the acetyl-CoA synthase method: 5.43 µmol (*ca*. 78% yield). Radiochemical analysis of the acetate soln. (100 µl) gave: ^3H: 7748 dpm; ^{14}C: 2884 dpm; $^3H/^{14}C = 2.71$.

5.4. *Analysis of Chiral Acetate from (*S*)-2-Methylidene[3-$^2H_1$,3-$^3H_1$]glutarate.* The final sample was counted to give the following results: 0/2.23/0, 60/1.08/48.4, 90/1.10/49.3, 120/1.08/48.4 (time [min]/ratio of ^3H to ^{14}C/% change compared to malate).

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