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Stereochemistry of *si*-Citrate Synthase and ATP-Citrate-Lyase Reactions

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1. A new synthesis of *R*- and *S*-acetic acid (*R*- and *S*-[$^2H_1$, $^3H_1$]acetate) is described.
2. *R*-Acetic acid after conversion into citrate on *si*-citrate synthase and cleavage of the citrate with citrate lyase regenerates an acetate which is still predominantly *R*.
3. *S*-Acetic acid when put through the same sequence yields acetic acid which is predominantly *S*.
4. Essentially the same results as in (2) and (3) are obtained when ATP-citrate lyase is substituted for citrate lyase.
5. The conclusion is drawn that on *si*-citrate synthase, the replacement of hydrogen by oxaloacetic acid proceeds with inversion of configuration at the methyl group.
6. It also follows that ATP-citrate lyase, like citrate lyase, cleaves citrate with inversion of configuration.
7. It is further deduced that there is a normal intramolecular deuterium-isotope effect, with respect to the chiral-methyl group, in the condensation of chiral acetate with oxaloacetate on *si*-citrate synthase.

The enzymes *si*-citrate synthase (for nomenclature see preceding paper [7]), ATP-citrate lyase and citrate lyase catalyse the formation of citrate from acetyl-CoA and oxaloacetate [Eqn (1)], the reverse of this synthesis at the expenditure of ATP [Eqn (2)],

$$\text{acetyl-CoA} + \text{oxaloacetate} + H_2O \rightleftharpoons \text{citrate} + \text{CoA-SH} \quad (1)$$

$$\text{citrate} + \text{CoA-SH} + \text{ATP} \rightleftharpoons \text{acetyl-CoA} + \text{oxaloacetate} + \text{ADP} + P_i \quad (2)$$

$$\text{citrate} \rightleftharpoons \text{acetate} + \text{oxaloacetate} \quad (3)$$

and the more freely reversible formation of acetate and oxaloacetate from citrate [Eqn (3)], respectively. In each of these reactions the acetate unit is attached to the *si* side [1] of oxaloacetate and detached from the *pro-S* group of citrate [2–4].

The synthesis of highly labelled chiral acetates of known configuration and configurational assay of asymmetric methyl groups [5,6] opened the possibility of investigating the mechanism of action of these enzymes in more detail. Thus if formation and cleavage of the C-C bond are both stereospecific,

Enzymes. Acetate kinase (EC 2.7.2.1.); ATP-citrate lyase (EC 4.1.3.8); citrate lyase (EC 4.1.3.6); *si*-citrate synthase (EC 4.1.3.7); fumarase (EC 4.2.1.2); lactate dehydrogenase (EC 1.1.1.27); malate dehydrogenase (EC 1.1.1.37); malate synthase (EC 4.1.3.2); phospho-transacetylase (EC 2.3.1.8).

Table 1. *Expected configuration of product acetate formed from chiral acetate by combination of two enzymic reactions*

Substrate	Stereochemical course		Product
	on citrate synthase	on citrate lyase	
R-Acetate	inversion	inversion	*R*-acetate
R-Acetate	inversion	retention	*S*-acetate
R-Acetate	retention	inversion	*S*-acetate
R-Acetate	retention	retention	*R*-acetate

R-acetate used as a substrate for the *si*-synthase reaction [Eqn (1)] must yield either *R*- or *S*-acetate on cleavage of the product citrate by citrate lyase [Eqn (3)] (Table 1).

From the results of the configurational assay of the product acetate it should be possible to reduce the stereochemical ambiguities from four to two. Since, however, citrate lyase is known to produce inversion of configuration [7], the formation of *R*-acetate in the above sequence would establish inversion and that of *S*-acetate would establish retention of configuration on the *si*-synthase. The chirality of the product acetate from, for example, *R*-acetate on combination of the *si*-synthase [Eqn (1)] with the ATP-citrate lyase reaction [Eqn (2)], will then likewise reveal the corresponding stereochemistry of this enzyme.

MATERIALS AND METHODS

ENZYMES AND SUBSTRATES

Enzymes and biochemicals were from Boehringer Mannheim GmbH (Mannheim, Germany) with the following exceptions. Dowex-1 (X8, formate form, 200—400 mesh) and Dowex-50 (X8, 200—400 mesh) were from Serva (Heidelberg). [1-^{14}C]Acetate (specific activity 52.9 Ci/μmol) and uniformly labelled *S*-[^{14}C]-malate (specific activity approx. 50 Ci/μmol) were from the Radiochemical Centre (Amersham *via* Buchler, Braunschweig). Malate synthase (specific activity 25 $\mu mol \times min^{-1} \times mg^{-1}$) was purified from bakers' yeast [8].

ATP-Citrate Lyase

ATP-citrate lyase was purified [9] from chicken liver and obtained free of lactate dehydrogenase by gel filtration. All solvents and buffer solutions contained 5 mM mercaptoethanol and 1 mM $MgCl_2$ unless indicated otherwise.

Extraction. Frozen chicken liver (50 g) was homogenized in a blender (Starmix, Bosch) with 500 ml of 0.4 N KCl in 20% ethanol for 2 min at 0—4 °C [10] and the homogenate was centrifuged at $13000 \times g$ (Sorvall RC II) for 15 min. The supernatant contained 12—15 mg protein/ml.

Isoelectric Precipitation. The supernatant was decanted through two layers of cheese cloth for separation of a fat layer and the clear solution was adjusted to pH 5.3 by adding approx. 3 ml 1 M citrate solution pH 4.0. The solution was stirred for 5 min and then centrifuged for 5 min at $13000 \times g$. The precipitate was extracted with 200 ml 0.03 M Tris buffer pH 7.5 in a mechanically stirred Potter-Elvehjem homogenizer and the extract was centrifuged for 20 min at $20000 \times g$. The supernatant (200 ml), pH 7, containing 6—7 mg protein/ml was adjusted to pH 6.5 with 1 M citrate solution pH 4.0.

Gel Adsorption. Aluminum-hydroxide gel (about 18.5 ml, 22 mg dry weight per ml) was added and the solution was stirred for 10 min, then centrifuged for 5 min at $10000 \times g$). The gel was washed twice with 40 ml 0.03 M phosphate buffer pH 7 containing 5 mM mercaptoethanol and eluted five times with 40 ml 0.05 M citrate solution pH 7.5 to yield 200 ml of eluate containing 1.1—1.5 mg protein/ml.

Salt Precipitation. Solid ammonium-sulphate (25.6 g per 100 ml solution) was stirred into the solution within 10 min and the 0.45-saturated solution was centrifuged for 15 min at $20000 \times g$ after a 30-min equilibration. The precipitate was dissolved in 1.5 ml 0.1 M Tris buffer pH 7.5 and gave 2.1 ml solution containing 30—36 mg protein.

Gel Filtration. A Sephadex-G-200 column (450 ml) was prepared [11] and washed with 1 l 0.03 M Tris buffer pH 7.5 containing 10 mM mercaptoethanol and 1 mM $MgCl_2$. The same buffer was used to elute (5 ml fractions, approx. 15 ml/h) the protein applied to the column. The lyase, after a forerun of about 150 ml, appeared in the first protein-containing fractions; lactate dehydrogenase was eluted later.

Salt Precipitation. The combined lyase-containing effluents (50 ml) were treated with ammonium sulphate in exactly the same manner as described above. The precipitate was dissolved in 1.5 ml 0.1 M triethanolamine buffer pH 7.6 containing 10 mM mercaptoethanol and 1 mM $MgCl_2$ and dialysed for 1 h against 1 l 0.01 M solution of the same buffer but without mercaptoethanol. Centrifugation for 10 min at $20000 \times g$ resulted in 1.5 ml of clear solution containing 6—7 mg protein/ml. The preparation was free of lactate dehydrogenase and could be kept for two weeks under nitrogen at 2 °C [11] without loss of activity. Fast inactivation (up to 40% within 3 h) was observed with these preparations on exposure to air. A summary of the purification procedure is given in Table 2.

Table 2. *Purification of ATP-citrate lyase*

Step	Total protein	Total activity	Specific activity	Yield
	mg	units	units/mg	%
Crude extract	5900	107	0.018	100
Isoelectric precipitation	1260	102	0.08	96
Gel adsorption	250	51	0.20	47
Salt precipitation	73	33	0.45	31
Gel filtration	18	12	0.06	11
Salt precipitation	9	8	0.87	7

NEW PREPARATION OF CHIRAL ACETATES

The flow diagram is shown in Fig. 1. Measurements of radioactivity in this preparation were made in a Packard model-3375 scintillation counter in solutions previously described, for chemical products [6]. Gas-liquid-chromatographic columns A and B were also as described; in addition, column C (5 ft. $\times$ 1/8 in., 3% ethylene glycol succinate on 100—120 mesh Chromosorb W, flow rate 30 ml/min, temperature 80 ° → 150 °C at 8 °C/min) was used as indicated. Where not mentioned here, the stereospecificity of the procedures employed has already been discussed [5].

(E)-[2'-$^3H_1$]Ethenylbenzene

(*E*)-2'-Bromoethenylbenzene (*trans*-β-bromostyrene) was recrystallized from pentane at −78 °C in the dark until analysis by gas-liquid chromatograph (Column B) showed < 1% of the *Z*-isomer. A portion (4.0 g, 21.9 mmol) was dissolved in pentane (100 ml), stirred and treated at room temperature in the dark with *n*-butyl-lithium in hexane (62 ml, 110 mmol). After 1 h, tritiated water (2.0 ml, 2 Ci) was added over 15 min; after a further 5 min, more

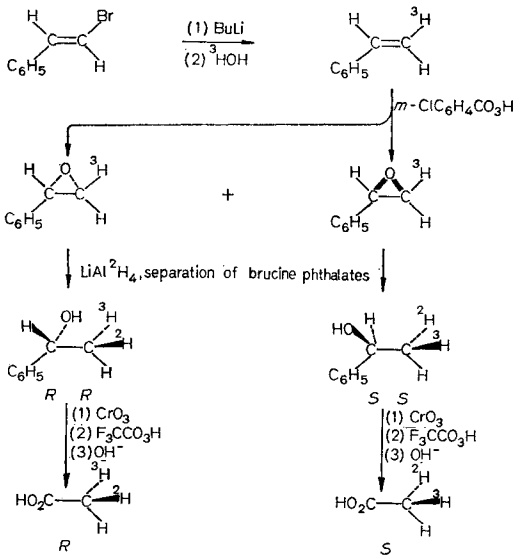

Fig. 1. *New preparation of chiral acetates*

water (2.0 ml) was added. The reaction mixture was washed with water and the organic layer was dried (K_2CO_3). Non-radioactive ethenylbenzene (2.0 g) was added and the paraffins were distilled through a 20-cm helix-packed column. The residue was distilled to yield 3.84 g, b.p. 54—92 °C/100 mm, assaying 80% ethenylbenzene by analysis with gas-liquid chromatography (Column A), and showing 1.07 μCi/μmol on counting.

A parallel experiment using deuterium oxide instead of tritiated water gave (*E*)-[2'-$^2H_1$]ethenylbenzene, free from the (*Z*) form as shown by nuclear-magnetic-resonance measurements [5], but containing 2—3% styrene, presumably from lithium hydroxide in the butyl-lithium. The stereochemistry of the tritiated ethenylbenzene is assigned on the basis of this experiment. The conditions of reaction with butyl-lithium are critical: appreciable amounts of the (*Z*) form were detectable if the reaction mixture was left for 2 h before quenching with deuterium oxide.

1',2'-Epoxy-[2'-^3H]ethylbenzene

Unlabelled styrene (1.0 g) along with the above product (3.84 g) in chloroform (160 ml) was treated during 25 min at 0 °C with *m*-chloroperoxybenzoic acid (13.7 g, 57.8 mmol by iodometric assay) and stirred for 22 h at 4 °C. The chloroform solution was washed with sodium-carbonate solution (2×150 ml of 5%), water, and brine, and the chloroform was removed. The residue was distilled at 1 mm pressure to yield a distillate of styrene oxide (2.22 g).

(1R,2R)- and (1S,2S)-1-Phenyl-[2-$^2H_1$,2-$^3H_1$]ethanol

The above preparation of styrene oxide (2.22 g) in ether (10 ml) was added to lithium aluminum deuteride (0.315 g, 7.5 mmol, 99% ^{2}H) which had been refluxing for 20 min with peroxide-free dry ether (75 ml) under argon. Reflux under argon was continued for 90 min, and water (0.31 ml) followed by sodium hydroxide (0.31 ml of 15%) and more water (0.93 ml) were added. After a further 15 min under reflux the mixture was filtered and the solid washed with ether, the filtrate dried ($CaSO_4$) and the ether removed, leaving the racemic 1-phenylethanol (2.02 g). Gas-liquid-chromatography analysis (Column A) indicate the presence of 14% 2-phenylethanol.

The optical resolution of this product was carried out exactly as described previously [5] and yielded the (+)-alcohol (1*R*,2*R*) (0.29 g) and the (−)-alcohol (1*S*,2*S*) (0.31 g).

The optical purity of these alcohols (with respect to C-1) was tested by gas-liquid-chromatography analysis of the (−)-menthylcarbonates. *RS*-1-Phenylethanol (1 mmol) in dry pyridine (1.0 ml) with (−) menthoxycarbonyl chloride (1.2 mmol, 0.6 M in toluene) [12] were left for 1 h at room temperature and shaken with water. The organic layer was separated, dried ($MgSO_4$) and examined by gas-liquid-chromatography analysis (Column C). In the conditions specified above, (−)-menthyl(−)-1-phenylethyl carbonate had a retention time of 28 min and (−)-menthyl(+)-1-phenylethyl carbonate had a retention time of 30 min. Smaller quantities of the optical isomers, described above, were converted into the (−) menthylcarbonates in the same way. Gas-liquid-chromatography examination showed that each sample contained $< 1\%$ of the diastereoisomeric form. The optical purity of the above alcohols is hence $> 99\%$.

R- *and* S-[$^2H_1$,$^3H_1$]*Acetylbenzenes*

Each optically active alcohol (2 mmol) was separately dissolved in dry tetrahydrofuran (6 ml), cooled to −30 °C treated all at once with chromic-acid solution [13] (1.2 ml, 4.8 m-atom "active oxygen"), and stirred vigorously without cooling for 65 sec. The supernatant was removed and the residue washed with tetrahydrofuran (2×2 ml). These solutions were at once added to water (10 ml) and extracted with ether (3×8 ml). The ether extracts were washed with water and brine, dried ($MgSO_4$) and filtered. The ether was removed at ambient pressure (bath below 44 °C) and the tetrahydrofuran at lower pressure (20—30 mm, bath below 33 °C) to leave the acetophenones as a liquid residue.

Potassium R- *and* S-[$^2H_1$,$^3H_1$]*Acetates*

Oxidation of the two acetophenones by peroxytrifluoroacetic acid, saponification of the phenyl acetates, and isolation of the acetic acids by steam distillation, were carried out exactly as described

previously [5]. The *R*-acetic acid originating from (1*R*,2*R*)-1-phenyl-[2-$^2H_1$,2-$^3H_1$]ethanol required 15.2 ml of 0.1 N KOH for neutralization (76% overall yield) and the *S*-acetic acid from (1*S*,2*S*)-1-phenyl-[2-$^2H_1$,2-$^3H_1$]ethanol required 13.7 ml (69% overall yield). The potassium acetates were stored as sterile 0.1 M solutions as previously described [5]. Direct counting of the aqueous solutions indicated specific radioactivities of 1.090 and 1.096 Ci/mol for the *R*- and *S*-acetates, respectively. Conversion into, the 4-bromophenacyl esters [5] and subsequent counting of these gave, however, 0.821 and 0.845 Ci/mol for the *R* and *S* esters, respectively (average of 3 assays each), agreeing (with allowance for the one dilution) with the specific radioactivity of the parent ethenylbenzene. The differences may be due to the use of different scintillator solutions for potassium acetate on the one hand, and for ethenylbenzene and 4-bromophenacyl acetates on the other.

Stability of Aqueous Potassium Acetate

A sample of potassium *R*-acetate (50 µmol in 500 µl, specific activity 0.075 Ci/mol), which had been kept for 8 months in a sealed ampoule at room temperature, was carefully distilled through a short-path apparatus at 20 mm pressure, the receiver being cooled and protected from atmospheric water. The water was collected in two fractions (0.1 and 0.4 ml) which were counted. The count of the first distillate indicated that 0.063% of the original radioactivity could now be in the form of water; the second distillate however gave a figure of 0.0055%. It was concluded that exchange of tritium with the medium had been insignificant.

3S-[4-$^2H_1$,4-$^3H_1$]Citrates Derived from Chiral Acetates on si*-Citrate Synthase*

This synthesis was performed by the method of Bergmeyer and Möllering [14] in a range of 2 to 10 µmol. The incubation mixture, volume 6.0 ml, at 25 °C contained 1.3 mmol triethanolamine buffer pH 8.5, 2—10 µmol acetate, 83 µmol *S*-malate, 12 µmol $MgCl_2$, 12 µmol Mg-EDTA, 18 µmol NAD, 12 µmol ATP, 1.2 µmol coenzyme A, 30 U malate dehydrogenase, 64 U acetate kinase, 60 U phosphotransacetylase and 30 U *si*-citrate synthase. A sample was used to follow the reaction at 366 nm (d = 0.2 cm) against a control cuvette containing all components but acetate. The enzymes were inactivated (3 min, 100 °C) after the reaction went to completeness (50—110 min) and trace amounts of ^{14}C-labelled *S*-malate (approx. 10^5 counts/min) were added to the solution. Residual malate and formed citrate were isolated on Dowex-1 (1×13 cm) by elution with 120 ml 1 N formic acid and with 35 ml 4 N formic acid, respectively. The citrate-containing eluates were brought to dryness *in vacuo* and the residue was dissolved in water; citrate was determined enzymically.

The yield of 3*S*-citrates derived from *R*-acetate (5.0 µmol, specific activity 7.5×10^5 counts×min×µmol^{-1}), *S*-acetate (8.85 µmol, specific activity 77600 counts×min^{-1}×µmol^{-1}) and [$^3H_1$]acetate (5.3 µmol, specific activity 1.18×10^5 counts×min^{-1}×µmol^{-1}) in this procedure was 4.6 µmol (specific activity 7.05×10^5 counts×min^{-1}×µmol^{-1}), 7.0 µmol (specific activity 61000 counts×min^{-1}×µmol^{-1}) and 4.6 µmol (specific activity 1.06×10^5 counts×min^{-1}×µmol^{-1}), respectively.

2S-[3-$^2H_1$,3-$^3H_1$]Malates Derived from 3S-[4-$^2H_1$,4-$^3H_1$]Citrates with Citrate Lyase and Malate Synthase

The incubation mixture in a total volume of 5.0 ml at 25 °C contained 100 mM Tris buffer pH 8, 1 mM $MgCl_2$, 1 mM Mg-EDTA, 3—4 µmol NADH, 1—3 µmol citrate, 1 U citrate lyase, 18 U malate dehydrogenase and 10 U lactate dehydrogenase. The latter enzyme was added to trap any pyruvate formed from oxaloacetate by decarboxylation and thus to secure the analytic procedure for cleavage of citrate. The absorbance at 366 nm (d = 0.2 cm) of a sample was used to follow the reaction from the consumption of NADH.

The three specimens of 3*S*-citrate derived from *R*-acetate, *S*-acetate and [$^3H_1$]acetate (1.83, 3.01 and 2.09 µmol, respectively, specific activities as described above) were used for the cleavage reaction (NADH consumption 1.47[1], 3.0 and 2.12 µmol, respectively) and the enzymes were inactivated by adding 1.5 ml 1 M sulphuric acid after complete reaction had occurred. [^{14}C]Acetate (specific activity 86000 counts×min^{-1}×µmol^{-1}) was added to establish a $^3H/^{14}C$ ratio between 1 and 2 and the acetates were isolated by steam distillation [5] to yield 4.6, 3.1 and 3.8 µmol ($^3H/^{14}C$ ratio 1.77, 1.21 and 1.16) acetate derived *via* citrate from *R*-acetate, *S*-acetate and [$^3H_1$]acetate, respectively. The isolated acetates were converted to the malates and analysed with fumarase as usual [5].

2S-[3-$^2H_1$,3-$^3H_1$]Malates Derived from 3S-[4-$^2H_1$,4-$^3H_1$]Citrates with ATP-Citrate Lyase and Malate Synthase

The incubation mixture in a total volume of 2.0 ml at 25 °C contained 50 mM triethanolamine buffer pH 7.6, 0.5—1.5 mM citrate, 1.3 mM coenzyme A, 10 mM ATP, 1.9 mM NADH, 10 mM $MgCl_2$, 0.5 mM Mg-EDTA, 3 mM glyoxylate, 35 U, malate dehydrogenase, 0.6—1.1 U ATP-citrate lyase and 7 U malate synthase. The reaction was followed from the decrease in NADH concentration (absorb-

[1] This initially pure specimen of highly [^3H]labelled citrate derived from *R*-acetate most likely had suffered partial self-radiolysis [7] during an 8-weeks storage at −15 °C.

ance at 366 nm) and also from the decrease in glyoxylate concentration [5]. Malate was isolated by chromatography on Dowex-1.

Representative results are as follows: citrate used (2.0 and 2.0 μmol), NADH consumed (2.12 and 1.96 μmol), glyoxylate consumed (1.91 and 1.74 μmol), malate isolated (3.38 and 3.33 μmol). Note that the labelled malate formed from acetyl-CoA and glyoxylate is diluted by unlabelled malate derived from oxaloacetate.

The three specimens of 3*S*-citrate (1.83, 2.0 and 1.79 μmol, derived from *R*-acetate, *S*-acetate and [$^3H_1$]acetate, respectively, specific activities as described above) when put through the procedure yielded 2.56 μmol malate (specific activity 3.07×10^5 counts $\times$ min^{-1} $\times$ μmol^{-1}) derived from *R*-acetate *via* citrate, 3.18 μmol malate (specific activity 30400 counts $\times$ min^{-1} $\times$ μmol^{-1}) derived from *S*-acetate *via* citrate, and 2.52 μmol (specific activity 50000 counts $\times$ min^{-1} $\times$ μmol^{-1}) derived from [$^3H_1$]acetate *via* citrate. The isolated malates were diluted with trace amounts of ^{14}C-labelled *S*-malate to establish a $^3H/^{14}C$ ratio between 1.5 and 2 and further with unlabelled *S*-malate to a total of 75 μmol used in a total volume of 1.50 ml for the incubation with fumarase [5].

Assay of Chiral Acetates by Use of Chemically-Prepared Acetyl-CoA

The specimens of acetyl-CoA were those used for the studies on *re*-citrate synthase [24] and prepared from *R*-acetate and *S*-acetate *via* the mixed anhydride formed with ethyl chloroformate. They were diluted with unlabelled acetyl-CoA and purified at 4 °C by chromatography on Dowex-1 (1 $\times$ 15 cm per 10 μmol) [15]. "Acetyl phosphopantetheine" (the presence of other acetyl-thioester impurities was not excluded) was eluted with the formic acid gradient (0—4 N), and acetyl-CoA with the formic acid-formate gradient (4 N formic acid and 4 N formic acid + 0.2 M ammonium formate). The fractions containing acetyl-CoA were lyophilized after Dowex-50 treatment and the residue was dissolved in water. The enzymically [21] determined recovery was 80%.

The incubation mixtures which were kept at 25 °C for 2 h initially contained 20 μmol pyrophosphate buffer pH 8.0, 1 μmol $MgCl_2$, 5 μmol glyoxylate, the purified acetyl-CoA (1.2 μmol each) specific activities 62000 and 98000 counts $\times$ min^{-1} $\times$ μmol^{-1}, respectively, of acetyl-CoA derived from *R*-acetate and *S*-acetate) and 2 U malate synthase. ^{14}C-labelled *S*-malate (50000 counts/min) was then added to each incubation mixture and the doubly labelled malates were isolated by chromatography on Dowex-1. They were further diluted with unlabelled *S*-malate to a total of each 100 μmol and then subjected to the usual exchange with fumarase. See Table 3 for results.

Table 3. *Loss of tritium from labelled malates in the presence of fumarase*

The three specimens of malate were incubated with fumarase and samples were taken at different times. After purification, each sample was dissolved in 1.5 ml water, and 1.0 ml was used to measure the $^3H/^{14}C$ ratio. 0_1, 0_2 and 0_3 are triplicate determinations before the addition of fumarase

Malate derivation	Time	$^3H/^{14}C$ ratios			
		Actual	Average		*cf.* Initial
	min				%
From *R*-acetate *via* chemically synthesized acetyl-CoA	0_1	1.115			
	0_2	1.108	1.121		100
	0_3	1.141			
	15	0.861		76.80	
	45	0.853		76.09	
	75	0.861		76.80	
	105	0.859	0.858	76.62	76.6 ± 0.7
	120	0.860		76.71	
	150	0.857		76.44	
From *S*-acetate *via* chemically synthesized acetyl CoA	0_1	2.080			
	0_2	2.068	2.082		100
	0_3	2.097			
	15	0.540		25.93	
	45	0.536		25.74	
	90	0.545	0.538	26.17	25.9 ± 0.5
	105	0.544		26.12	
	120	0.535		25.69	
	150	0.533		25.60	

The same specimens of acetyl-CoA without purification prior to the configurational analysis led to "malates" which on incubation with fumarase retained 82% (malate derived from *R*-acetate) and 43% (malate derived from *S*-acetate) of their tritium label. The tritium-labelled "acetyl phosphopantetheine", formed by chemical acetylation of the impure coenzyme A charges used, is not attacked by malate synthase. On isolation of malate by chromatography on Dowex-1 "acetyl phosphopantetheine" and malate are eluted together [16] (1 N formic acid) and therefore appear in the incubation mixtures for the fumarase exchange. Here on purification of the malates *via* barium-salt treatment [5] both compounds remain in solution and finally appear in the samples prepared for counting of radioactivity. The $^3H/^{14}C$ ratios of the malates after the exchange with fumarase consequently appear too high, and this was observed.

EQUIPMENT

Counting of radioactivity was done on a Packard-3380 scintillation counters as described previously [5]. The photometer and spectrophotometer were those used previously [5].

DETERMINATION OF ENZYMES AND SUBSTRATES

ATP-citrate lyase activity was determined [10] at 25 °C in a total volume of 1.0 ml containing 100 mM

Tris buffer pH 7.3, 20 mM citrate pH 7.5, 5 mM ATP, 10 mM $MgCl_2$, 10 mM mercaptoethanol, 0.2 to 0.3 mM NADH, 14 U malate dehydrogenase and 0.01—0.05 U ATP-citrate lyase. Absorbance was measured at 366 nm, 1-cm path length in a photometer Eppendorf. Other enzymes and substrates were determined as described in the literature: citrate lyase [17], *si*-citrate synthase [18], fumarase [19], malate synthase [20], acetyl-CoA [21], citrate [17], malate [5]. Protein was determined according to Warburg and Christian [22].

RESULTS AND DISCUSSION

Combination of si-*Citrate Synthase with Citrate Lyase*

For information on the outlined stereochemical questions, chiral acetate must be transformed through the sequence

acetate → acetyl-CoA → citrate → acetate (or acetyl-CoA) → acetyl-CoA → malate → fumarate, (4)

where citrate provides the substrate for citrate lyase and ATP-citrate lyase. This synthesis was achieved with *R*-acetate, *S*-acetate and [$^3H_1$]acetate by the method of Bergmeyer and Möllering [14]. The isolated specimens of 3*S*-citrates in the presence of NADH and malate dehydrogenase were cleaved by citrate lyase to the acetates and oxaloacetate, trapped as malate. After completion of the cleavage reaction, ^{14}C-labelled acetate was added to the incubation mixtures and the doubly labelled acetates were isolated. Following the procedures described previously [5] the three specimens of acetate were analysed for chirality by conversion to the malates and incubation with fumarase to yield the results shown in Table 4.

The sequence starting with *R*-acetate gave malate retaining 65.0 ± 1.3% of its tritium on incubation with fumarase. *R*-Acetate had therefore been generated from *R*-acetate and the formation of citrate on the *si*-synthase consequently proceeds with inversion. The sequence acetate → citrate → acetate is shown in Fig. 2. Non-dissymmetric [$^3H_1$]acetate, put through this procedure, gave the statistically expected result (50.1 ± 1.0%) for retention of tritium in malate. The *S*-acetate used for a parallel experiment was of less optical purity. The malate derived from it in the sequence acetate → citrate → acetate → malate showed 40.7 ± 1.6% retention of tritium and *S*-acetate had hence been generated from *S*-acetate.

Combination of si-*Citrate Synthase with ATP-Citrate Lyase*

ATP-citrate lyase produces acetyl-CoA, not acetate, from citrate [Eqn (2)]. It was desirable therefore to circumvent the isolation of the acetate unit and to convert it *in situ* to malate. This genera-

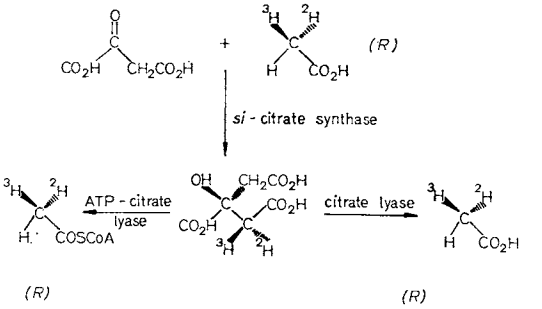

Fig. 2. *Stereochemistry of* si-*citrate synthase and of ATP-citrate lyase*

Table 4. *Loss of tritium from labelled malates in the presence of fumarase*

The three specimens of malate were derived from *R*-, *S*- or [^3H]acetate in the sequence acetate → citrate → acetate → malate with *si*-citrate synthase, citrate lyase and malate synthase. They were incubated with fumarase and samples were taken at different times. After purification, each sample was dissolved in 1.5 ml water, and 1.0 ml was used to measure the $^3H/^{14}C$ ratio. 0_1, 0_2 and 0_3 are triplicate determinations before the addition of fumarase

Malate derivation	Time	$^3H/^{14}C$ ratios			
		Actual	Average		*cf.* Initial
	min				%
R-Acetate	0_1	1.507			
	0_2	1.540	1.524		100
	0_3	1.525			
	4	1.234			81.0
	10	1.057			69.4
	20	1.000		65.6	
	35	0.993	0.990	65.2	65.0 ± 1.3
	60	0.978		64.2	
S-Acetate	0_1	1.005			
	0_2	1.050	1.035		100
	0_3	1.051			
	8	0.550			53.1
	11	0.486			47.0
	20	0.428		41.4	
	35	0.421	0.421	40.7	40.7 ± 1.6
	60	0.414		40.0	
[$^3H_1$]Acetate	0_1	1.054			
	0_2	1.043	1.049		100
	0_3	1.049			
	4	0.734			70.0
	10	0.546			52.0
	20	0.520		49.6	
	35	0.523	0.525	49.9	50.1 ± 1.0
	60	0.532		50.7	

tion of malate from citrate in one incubation mixture was achieved by combining the cleavage of citrate [Eqn (5)]

citrate + ATP + CoA-SH
⇋ acetyl-CoA + oxaloacetate + ADP + P_i (5)

oxaloacetate + NADH + H^+
⇋ malate + NAD (6)

acetyl-CoA + glyoxylate + H_2O
⇋ malate + CoA-SH (7)

citrate + glyoxylate + NADH + H^+ + H_2O
→ 2 malate + NAD + ADP + CoA-SH + P_i (8)

with NADH and malate dehydrogenase [Eqn (6)] and also with glyoxylate and malate synthase [Eqn (7)]. The overall reaction [Eqn (8)] was followed from (a) the decrease in NADH concentration, and (b) the consumption of glyoxylate, and was found to proceed quantitatively. It was important for this conversion to obtain ATP-citrate lyase free of lactate dehydrogenase, and a corresponding brief purification procedure is given in Experimental Procedures. In the presence of lactate dehydrogenase the overall reaction [Eqn (8)] would be significantly inhibited by reduction of glyoxylate with NADH. Note that the radioactive malate generated from chiral acetate in the sequence of Eqn (4) on cleavage of citrate is diluted two-fold by unlabelled malate derived from citrate *via* oxaloacetate.

The three specimens of radioactive citrates synthesized from the acetates on the *si*-synthase by use of the combined optical test [Eqn (8)] were converted to the malates. These were diluted with ^{14}C-labelled malate after isolation and analysed as usual by incubation with fumarase. The results are summarized in Table 5.

Citrate derived from *R*-acetate in the sequence of Eqn (4) yielded a malate which retained 68.4 ± 0.6% of its tritium on incubation with fumarase. Thus *R*-acetate had been generated from *R*-acetate and the cleavage of citrate by ATP-citrate lyase, like that by citrate lyase, proceeds with inversion. The sequence is also shown in Fig. 2. The malate derived from non-dissymmetrical tritium-labelled acetate gave the statistically expected result, 49.9 ± 0.9% retention of tritium and that derived from *S*-acetate of initially less optical purity retained 39.7 ± 1.3%; hence *S*-acetate had been generated from *S*-acetate.

Table 5. *Loss of tritium from labelled malates in the presence of fumarase*

The three specimens were derived from *R*-, *S*- or [$^3H_1$]-acetate in the sequence acetate → citrate → acetyl-CoA → malate with *si*-citrate synthase, ATP-citrate lyase and malate synthase. They were incubated with fumarase and samples were taken at different times. After purification, each sample was dissolved in 1.5 ml water, and 1.0 ml was used to measure the $^3H/^{14}C$ ratio. 0_1, 0_2 and 0_3 are triplicate determinations before the addition of fumarase

Malate derivation	Time	$^3H/^{14}C$ ratios			
		Actual	Average		*cf.* Initial
	min				%
R-Acetate	0_1	1.581			
	0_2	1.607	1.599		100
	0_3	1.608			
	4	1.280			80.1
	10	1.132			70.8
	20	1.094		68.4	
	35	1.092	1.093	68.3	68.4 ± 0.6
	60	1.094		68.4	
S-Acetate	0_1	1.958			
	0_2	1.988	1.976		100
	0_3	1.981			
	4	1.167			59.1
	10	0.865			43.8
	20	0.788		39.9	
	35	0.794	0.784	40.2	39.7 ± 1.3
	60	0.771		39.0	
[$^3H_1$]Acetate	0_1	1.988			
	0_2	1.966	1.961		100
	0_3	1.930			
	4	1.361			69.4
	10	1.022			52.1
	20	0.994		50.7	
	35	0.967	0.979	49.3	49.9 ± 0.9
	40	0.976		49.8	

Remarks on the Analytic Procedure

As mentioned in the preceding sections the chiral acetates used as starting material in this study were of different optical purity. The original samples ("old acetates") on analysis showed 69 and 31% retention of tritium in the malates derived from *R*- and *S*-acetate, respectively [5]. This compares with 76.5 ± 0.3 and 25.3 ± 1.5%, the data obtained with *R*- and *S*-acetate, respectively [23,24], prepared by the modified chemical synthesis. The results may indicate less optical purity of the "old specimens" but could also arise from (a) fast decomposition or racemisation of the chiral acetates on storage, (b) insufficient precision of the analytic procedure, and (c) a dependence of the isotopic effect k_H/k_D for the malate-synthase reaction on the concentration of acetyl-CoA.

The absence of significant exchange, over 8 months, of hydrogen between chiral acetates and the medium has been demonstrated above. Reanalysis of the "old *R*- and *S*-acetates" which were kept in storage for nearly a year led to malates which on incubation with fumarase retained 68.1 ± 0.1% (*versus* 68.6 ± 0.8% [5]) and 33.7 ± 0.2% (*versus*

30.8 ± 0.5% [5]) of their tritium label. The results are sufficient to exclude points (a) and (b). Point (c) deserves further comment: Assuming the operation of a normal isotopic effect the ratio of (2*S*,3*S*)-[3-$^3H_1$,3-$^2H_1$]malate and (2*S*,3*R*)-[3-$^3H_1$]malate formed from *R*-acetate and glyoxylate on malate synthase is equal to k_H/k_D. This was determined with the "old acetates" from the results of the fumarase-exchange as 69/31 = 2.2. If the concentration of acetyl-CoA on synthesis of the malates exerts an influence on this ratio to yield *e.g.* k_H/k_D = 75/25, we would then have the relationship found on analysis of the newly synthesized acetates, *i.e.* 75 and 25% retention of tritium in the malates derived from *R*- and *S*-acetate, respectively. Though an enzymic procedure was used for the conversion of acetate to malate *via* acetyl-CoA, small changes of the pH or enzyme activities during the incubation may influence the "stationary" concentration of acetyl-CoA and this in turn could affect k_H/k_D. Moreover, in working with chemically synthesized acetyl-CoA for the malate synthase reaction, *i.e.* with initially considerably higher concentrations of acetyl-CoA than formed in the enzymic procedure, our Swiss colleagues observed 90% retention of tritium in the malate derived from *R*-acetate [6]. This result corresponds to $k_H/k_D = 9$ and the differences in quantitative terms between their and our results could be due to the use of different concentrations of acetyl-CoA. The question was clarified by use of chemically synthesized chiral acetyl-CoA (2.4 mM) for the assay. The malate derived from *R*-acetyl-CoA on incubation with fumarase retained 76.6 ± 0.7% of its tritium and that from *S*-acetyl-CoA retained 25.9 ± 0.5% (Table 3). Comparison of these data with those obtained on analysis of the malates which were synthesized from the same samples of acetate *via* enzymically generated acetyl-CoA, 76.5 ± 0.3 and 25.3 ± 1.5% retention [23,24] demonstrates that the concentration of acetyl-CoA exerts no influence on k_H/k_D. The consistency of the analytical data however indicates that the analytical procedure is reliable and of remarkably high accuracy.

Inverse Isotopic Effect

A normal intermolecular isotopic effect, $k_H > k_D$, was found in earlier studies for the malate-synthase reaction. If the intramolecular isotopic effect of the chiral methyl group is also normal, the product formed on malate synthase from *R*-acetate with inversion will consist of (2*S*,3*S*)-[3-$^2H_1$,3-$^3H_1$]malate preponderating over (2*S*,3*R*)-[3-$^3H_1$]malate in a ratio equal to k_H/k_D. If the intramolecular isotopic effect is inverse, $k_D > k_H$, the product formed from *R*-acetate with retention of configuration would consist of (2*S*,3*R*)-[3-$^3H_1$]malate preponderating over (2*S*,3*R*)-[3-$^2H_1$,$^3H_1$]malate in a ratio equal to k_D/k_H. The analytical procedure however, incubation of the malates with fumarase, cannot distinguish between (2*S*,3*S*)-[3-$^2H_1$,3-$^3H_1$]malate and (2*S*,3*S*)-[3-$^3H_1$]malate, the preponderating species formed on combination of either normal isotopic effect with inversion or inverse isotopic effect with retention of configuration. As discussed earlier, inversion of configuration with the malate-synthase reaction is only true if the isotopic discrimination is normal.

This is not so with *si*-citrate synthase where the overall experimental result (*i.e.* the chirality of the acetate obtained by cleavage) is qualitatively the same whether the isotope effect is normal, inverse, or even non-existent.

Assuming inversion of configuration at the methyl group on synthesis of citrate, the two radioactive species of citrate produced from *R*-acetate are (3*S*,4*S*)-[4-$^2H_1$,4-$^3H_1$] citrate and (3*S*,4*R*)-[4-$^3H_1$]citrate. The ratio of these two species is equal to the intramolecular k_H/k_D for the *si*-synthase. On cleavage, with inversion, by the lyase, only the species containing both deuterium and tritium yields chiral (in this case *R*) acetate; the other radioactive species yields non-chiral [$^3H_1$]acetate. Thus the overall result is that *R*-acetate yields *R*-acetate, accompanied by a lesser or greater quantity of [$^3H_1$]acetate according to whether k_H/k_D is greater or less than 1: that is, whether the intramolecular deuterium-isotope effect for the synthase is normal or inverse. If the reaction on the synthase proceeded with retention of configuration at the methyl group, the same analysis shows that *R*-acetate would yield, after cleavage, *S*-acetate accompanied by a lesser or greater amount of [$^3H_1$]acetate.

When the product acetate is then converted into malate, the non-chiral [$^3H_1$]acetate gives equal amounts of 3*R*- and 3*S*-[$^3H_1$]malate and on treatment with fumarase 50% of the tritium in these two species is therefore lost, as is found experimentally. As is known [5], any *R*-acetate in the sample gives malate retaining most of its tritium, and any *S*-acetate gives malate losing most of its tritium, on treatment with fumarase. Thus an acetate in which *R*-acetate is the only chiral species will give malate retaining >50% of its tritium on fumarase treatment, and an acetate in which *S*-acetate is the only chiral species will give malate retaining <50% of its tritium on fumarase treatment. The isotope effect, if any, influences only the magnitude of the deviation from 50%.

The degree of non-chiral material (in this case [$^3H_1$]acetate) present in *R*- and *S*-acetate can be calculated from the exchange data as follows. Let malate A, synthesized from chiral acetate of given optical purity, have a $^3H/^{14}C$ ratio of α before and a $^3H/^{14}C$ ratio of β after the incubation with fumarase. If the starting material had been completely racemic, then $\beta = \alpha/2$. Malate B was derived from chiral

acetate of less optical purity and contains a fraction x of racemic molecules. This fraction x on incubation with fumarase yields a $^3H/^{14}C$ ratio of $\alpha/2$, and only fraction $(1 - x)$ yields a $^3H/^{14}C$ ratio of β. The ratio of all molecules is given by $[x\alpha/2 + (1 - x)\beta]/(1 - x) + x = x\alpha/2 + (1 - x)\beta$. The percentage retention of tritium in the presence of fumarase is given by $100\,\beta/\alpha$, and the deviation from 50%, *i.e.* from the value found with completely "racemic" material, follows as $\Delta[A] = (\beta/\alpha \cdot 100) - 50$. The corresponding deviation for species B follows as $\Delta[B] = \{100\,[x\alpha/2 + (1 - x)\beta]/\alpha\} - 50$. Hence $\Delta[B]/\Delta[A] = 1 - x$ and $x = 1 - \Delta[B]/\Delta[A]$, where x represents the (additional) fraction of racemic molecules present in B.

An intramolecular isotopic effect of $k_H/k_D = 2$ for the *si*-synthase and inversion of configuration would lead in the sequence of Fig. 2 from optically pure *R*-acetate to *R*-acetate containing 33.3% nondissymmetric [$^3H_1$]acetate. An inverse isotopic effect, $k_D/k_H = 2$ combined with inversion of configuration however would produce *R*-acetate containing 66.6% "racemic" acetate. The configurational assay for *R*-acetate yielded 75.6% retention of tritium in the malate before, and 68.4% retention after it was regenerated in the sequence of Eqn (4). The corresponding data for *S*-acetate were 33.7 and 39.7%, respectively. The degree of "racemisation" follows as $x = 1 - 18.4/26.5 = 0.31$ for *R*-acetate, and $x = 1 - 10.3/16.3 = 0.37$ for *S*-acetate (average of both determinations $x = 0.34$). Thus 34% "racemisation" means that on synthesis of citrate 34% of the chiral acetates lose deuterium and 66% lose hydrogen. Hence $k_H/k_D = 66/34 = 1.94$, and this excludes the operation of an inverse isotopic effect on *si*-citrate synthase.

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Note Added in Proof. By configurational assay, detailed previously [5], and by an approach partially different from ours, the stereochemistry of *si*-citrate lyase reactions described here and elsewhere (preliminary account [23]) has been confirmed recently [25].

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