Stereochemical and Mechanistic Studies on the Decarboxylation of Uroporphyrinogen III in Haem Biosynthesis

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The substrate stereochemistry of porphyrinogen carboxy-lyase which catalyses the decarboxylation of the four acetate side chains of uroporphyrinogen III was determined. (2*R*)-[2-$^3H_1$,2-$^2H_1$]Succinic acid was prepared from (3*RS*)-[$^3H_1$]-2-oxoglutarate using the stereospecific exchange reaction catalysed by $NADP^+$-dependent isocitrate dehydrogenase, followed by decarboxylation. The (2*R*)-[2-$^3H_1$,2-$^2H_1$]succinic acid was incorporated into haem using a cell-free preparation obtained from anaemic chicken erythrocytes. The biosynthetic haem was degraded initially to haematinic acid and ethylmethyl maleimide. A method involving ozonolysis was developed for the further degradation of these imide rings, with minimal concomitant proton labilisation, to give acetic acid. The chirality of the asymmetric methyls in acetic acid samples was determined using malate synthetase and fumarase, which catalyse reactions of known stereochemical course. The chiral analysis established that in the porphyrinogen carboxy-lyase reaction both protons at each methylene of uroporphyrinogen III remain intact in coproporphyrinogen III and that the reaction proceeds with retention of configuration at the methylene carbons of all four acetate side chains. In the light of these results a possible mechanism for the porphyrinogen carboxy-lyase reaction is proposed

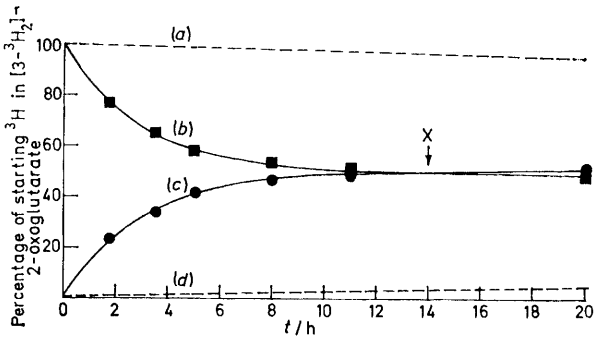
INITIAL stages in porphyrin biosynthesis involve the formation of 5-aminolaevulinic acid, two molecules of which condense to produce porphobilinogen. Through the concerted action of two enzymes, porphobilinogen is then converted into uroporphyrinogen III (4), the last common biogenetic intermediate of all naturally occurring tetrapyrrolic macrocycles. In the biosynthesis of haem,[1,2] the four acetate side chains of uroporphyrinogen III are decarboxylated by a single enzyme, porphyrinogen carboxy-lyase, giving rise to the four methyl groups of coproporphyrinogen III (5). This enzyme has been purified to homogeneity from chicken erythrocytes [3] and decarboxylates all porphyrinogen isomers I—IV but reports vary as to whether the naturally occurring I and III isomers are decarboxylated with equal facility or at different rates.[4,5]

It has been demonstrated that the partially decarboxylated species with seven, six, and five carboxy-groups [6,7] are intermediates in the reaction, which consists of successive decarboxylations starting with the carboxy group in ring D, followed by those in A, B, and finally C.[8,9]

In this paper we describe the stereochemistry of the porphyrinogen carboxy-lyase reaction through the analysis of chiral methyl groups produced by the decarboxylation of acetate side chains stereospecifically labelled at the methylene positions with deuterium as well as tritium. Part of this work has been published in a preliminary communication.[10]

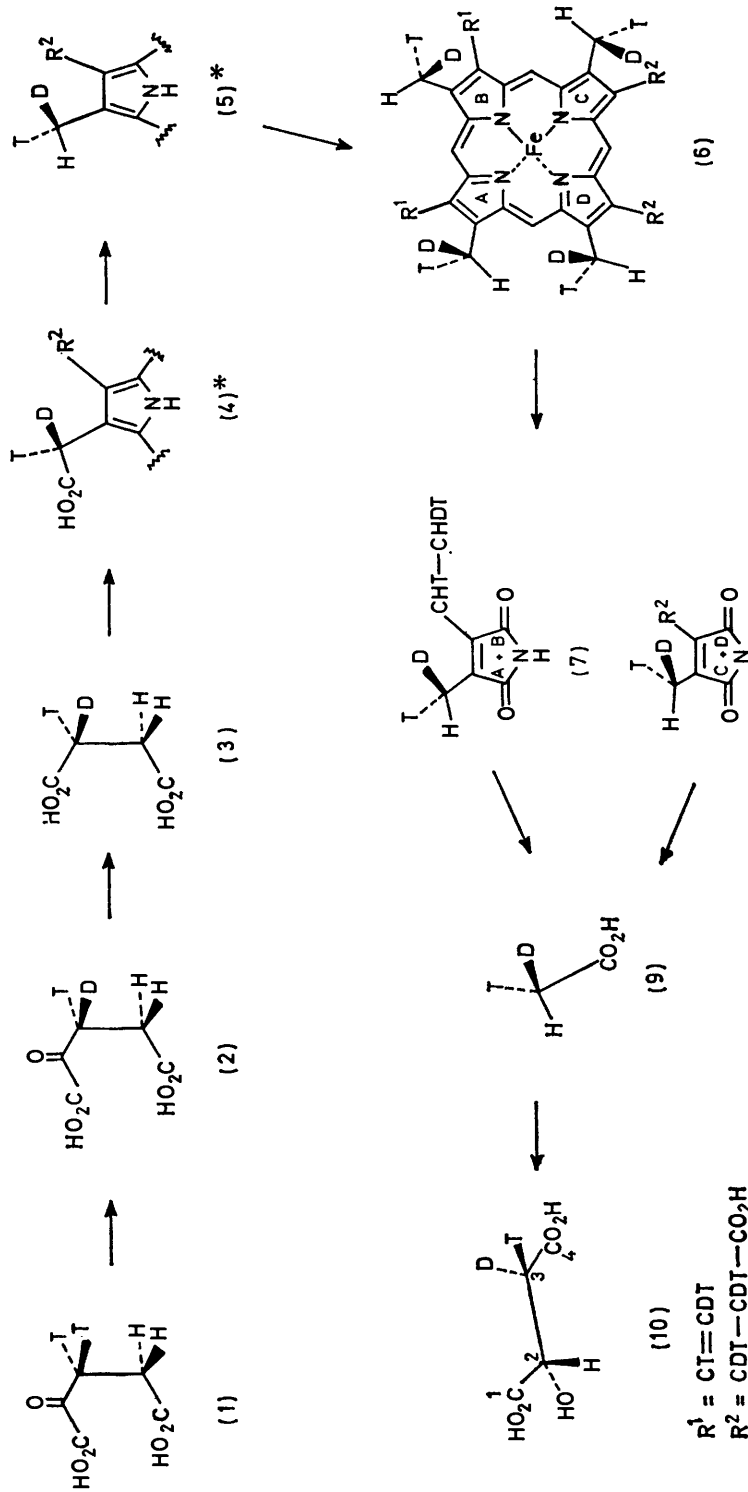
RESULTS

Preparation of (2R)-[2-$^3H_1$,2-$^2H_1$]*Succinate.*—The overall method for the determination of the stereochemistry of the porphyrinogen carboxy-lyase reaction is outlined in Scheme 1 and will now be considered sequentially. Using T_2O with a final specific activity of approximately 1 Ci ml^{-1}, a sample of [3-$^3H_2$]-2-oxoglutarate (1) having 1.57×10^7 disintegrations min^{-1} μmol^{-1} due to ^3H was prepared. For the stereospecific introduction of deuterium, isocitrate dehydrogenase which catalyses a partial reaction equilibrating [11] the C-3 *pro-S*-hydrogen of 2-oxoglutarate with the medium was used in D_2O to generate (3*R*)-[3-$^3H_1$,3-$^2H_1$]-2-oxoglutarate (2). Representative results of such an experiment are shown in the Figure. It should be noted that once 50% labilisation



Isocitrate dehydrogenase-catalysed removal of tritium from [3-$^3H_2$]-2-oxoglutarate in D_2O. The design of the incubation medium and detailed methodology for the determination of radioactivity released in the medium as $[^3H]^+$ and that associated with the recovered 2-oxoglutarate is described in the Experimental section. Control incubation (without NADPH) (*a*) ^3H in 2-oxoglutarate; (*d*) ^3H as medium water. Experimental incubation (complete system); (*b*) ^3H in 2-oxoglutarate; (*c*) ^3H as medium water. At point ' X ' the bulk of the incubation was removed for the isolation of [3-$^3H_1$,$^2H_1$]-2-oxoglutarate, while a portion was further supplemented with 0.6mM-NADPH and isocitrate dehydrogenase (5 units) to determine the chemical rate of tritium removal.

of ^3H from [3-$^3H_2$]-2-oxoglutarate had occurred (at ' X ', see Figure), further addition of the enzyme did not increase the exchange rate beyond that resulting from the non-enzymic process, thus proving that the replacement of tritium by deuterium in the initial fast reaction was stereospecific. Decarboxylation of the resulting (3*R*)-[3-$^3H_1$,3-$^2H_1$]-2-oxoglutarate with hydrogen peroxide gave (2*R*)-[2-$^3H_1$,2-$^2H_1$]-succinate (3). The deuterium content of the succinate was determined by mass-spectrometric analysis of its



SCHEME 1 An overall reaction sequence for the introduction of ^3H, ^2H, and ^1H into the methyl groups of haem and the determination of the chirality of acetic acid.* (4) and (5) represent partial structures of uroporphyrinogen III and coproporphyrinogen III, respectively

TABLE 1

Deuterium analysis of the sample of (2*R*)-[2-$^3H_1$,$^2H_1$]succinate used in the biosynthetic work. The (*R*)-succinate was prepared enzymically as detailed in the Figure and the Experimental section and subjected to mass-spectrometric analysis using the method originally developed by Cornforth *et al.*[12a]

	% of				
Source of succinic anhydride	^1H	$^2H_1$	$^2H_2$	$^2H_3$	$^2H_4$
Unlabelled succinic acid	100	0	0		
(2*R*)-[2-$^3H_1$,$^2H_1$]succinate	5	63	31	1	0

anhydride as detailed by Cornforth *et al.*[12a] The results in Table 1 show that the biosynthetic sample of succinate contained up to 31% [$^2H_2$]succinate in addition to the expected [$^2H_1$]-species. The observed production of the [$^2H_2$]-species may be interpreted to be the consequence of a primary isotope effect operating against cleavage of C–T in the chemical enolisation of [3-$^3H_1$]-2-oxoglutarate. This isotope effect can be evaluated as follows. From the Figure the chemical rate of C–T bond cleavage in D_2O was 0.25% h^{-1} which after 14 h would produce a maximum of 3.5% [$^2H_2$]-species. Therefore the experimentally determined value of 30.8% for the species must represent those molecules arising by both C–T and C–H cleavage. The C–H cleavage can thus be estimated as 30.8 − 3.5 = 27.3% in 14 h. This represents a tritium isotope effect, K_H/K_T, of 27.3/3.5 = 7.7 for the chemical enolisation of 2-oxoglutarate. It should be stressed that the presence of [2-$^2H_2$]-succinate in the sample of (2*R*)-[2-$^3H_1$,2-$^2H_1$]succinate is irrelevant because analysis of the stereochemistry of methyl groups in (6) is based on a tritium assay.

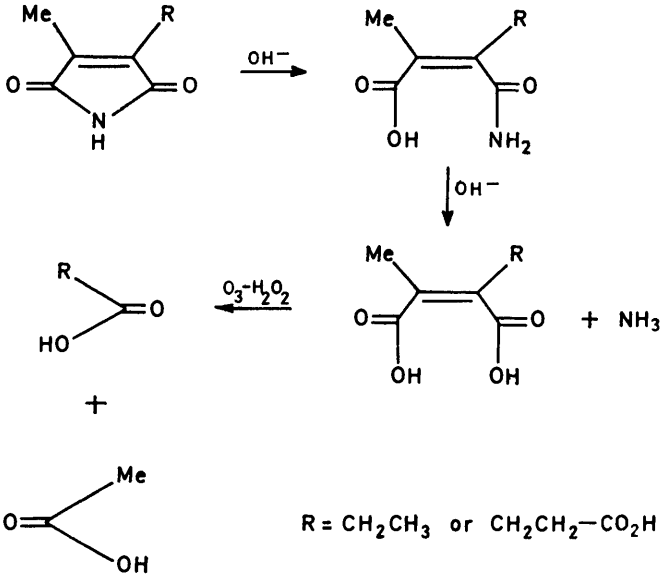
Incorporation of (2R)-[2-$^3H_1$,2-$^2H_1$]*Succinate into Haem.*—(2*R*)-[2-$^3H_1$,2-$^2H_1$]Succinate was incubated with a haemolysate prepared from anaemic chicken red blood cells containing a mixture of erythrocytes and reticulocytes. The presence of an active succinate thiokinase in the preparation permits the use of succinate plus CoASH as substrates (with glycine) for poryphyrin formation.

In this experiment (2*R*)-[2-$^3H_1$,2-$^2H_1$]succinate was incorporated into haem (6), performing *in situ* the decarboxylation of uroporphyrinogen III (4) into coproporphyrinogen III (5). The incubation being carried out in H_2O, hydrogen from the medium is incorporated into each of the four methyl groups. These groups, therefore, can potentially contain all three isotopes of hydrogen.

Degradation of the Biosynthetic Haem and Determination of the Chirality of the Acetate.—Haem was degraded *via* established procedures to protoporphyrin IX then mesoporphyrin IX, followed by chromic acid oxidation which converted the A + B rings of haem into ethylmethyl maleimide (7) and the C + D rings into haematinic acid (8).

The next stage in our degradation required the isolation of the methyl groups of both haematinic acid and ethylmethyl maleimide as the methyl group of acetic acid, under conditions which did not promote racemisation or loss of isotopic hydrogen atoms from the potentially chiral centre.

The procedure which finally satisfied this prerequisite is as follows. The imide rings of (7) and (8) were hydrolysed with NaOH and the course of the reaction was followed by quantitatively determining the amount of NH_3 released using glutamate dehydrogenase. The hydrolysed derivatives were then subjected to ozonolysis in the presence of H_2O_2 at pH 3. The pH of the incubation was critical; to minimise proton labilisation an acidic pH was essential. In such experiments acetic acid was produced in a radiochemical yield which was about 40% of theoretical, with <1% of the original tritium associated with water. It should be born in mind that in the degradation of ethylmethyl maleimide (Scheme 2) the method of isolation of the products does not separate acetic acid from propionic acid. It was envisaged that subsequent enzyme specificities in the

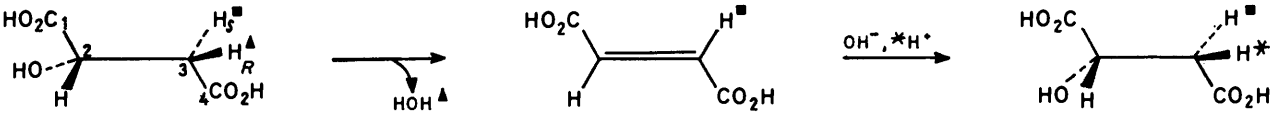


SCHEME 2 A degradative Scheme for the isolation of acetic acid from maleimides

conversion of acetate to malate would circumvent the problem of contamination of acetate by propionate, since malate synthetase is specific for its substrate, acetyl CoA (see ref. 12*b*).

The analysis of the chirality of the asymmetric methyl groups of acetic acid was based on the elegant approach originally developed by the schools of Cornforth [13] and Arigoni,[14] which lays down the rule that in the malate synthetase reaction the tritium from (*S*)- and (*R*)-acetates occupies the (3*R*) and (3*S*) positions respectively in malate. The stereochemistry of the labelled hydrogen at C-3 of malate may then be determined by an exchange reaction catalysed by fumarase [15,16a] (Scheme 3).

Accordingly, [$^3H_1$,$^2H_1$]acetates (10 μmol) deriving from haematinic acid or ethylmethyl maleimide were admixed with [^{14}C]-acetate and separately converted to (2*S*)-malate (7 μmol) using essentially the system as detailed by Rose (see ref. 16*b*) converting acetate *in situ* to acetyl phosphate, acetyl CoA and finally (2*S*)-malate. It was found that



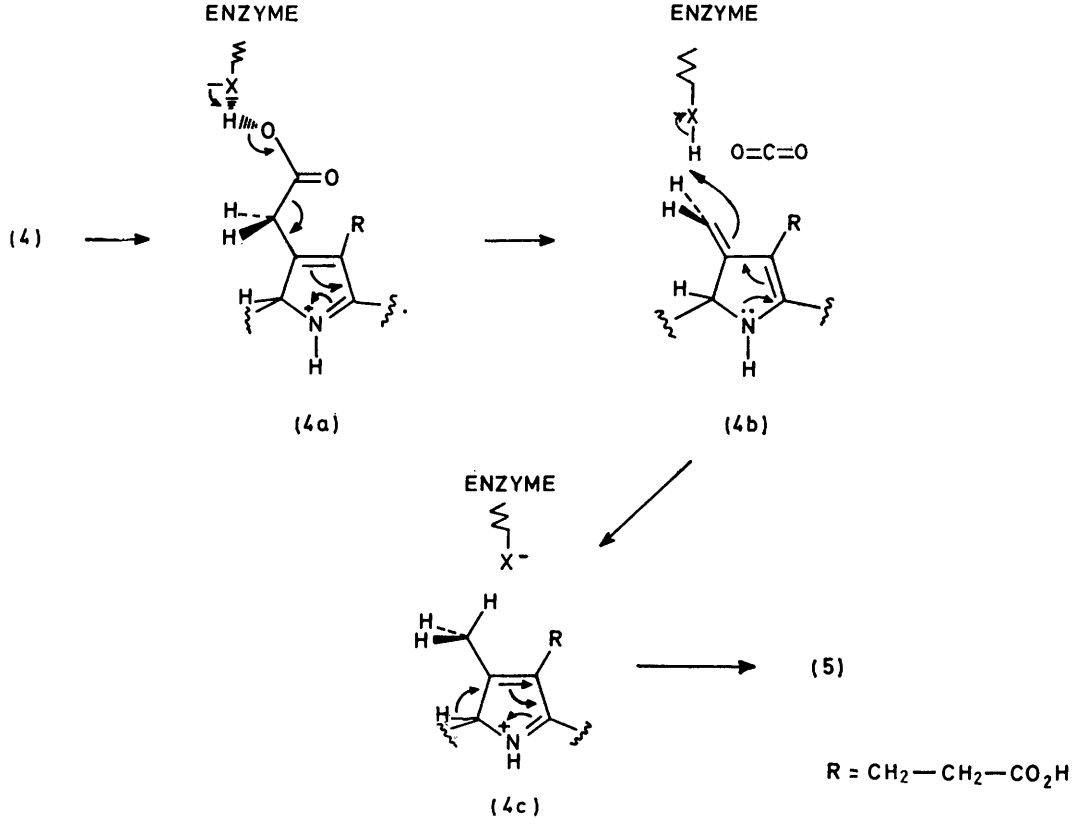
SCHEME 3 Fumarase-catalysed exchange of the C-3 *pro-R*-hydrogen of malate with the protons of medium (*H⁺)

TABLE 2

Stereochemical analysis of the methyl groups from rings A + B and C + D of haem, biosynthesised from [2-$^3H_2$]succinate or (2*R*)-[2-$^3H_1$,2-$^2H_1$]succinate. Haem was biosynthesised from the two species of [^{3}H]succinate and degraded as described in the text. The results below were confirmed in three sets of completely independent experiments. The predicted value for the retention of ^{3}H upon equilibration of malate derived from (*S*)-acetate is *ca.* 30%

Source of acetate	$^3H/^{14}C$ value in		Retention of ^{3}H after equilibration
[2-$^3H_2$]Succinate→haem→ethylmethyl maleimide→acetate (Rings A + B)	malate	0.65	
	equilibrated malate	0.33	50.8 ± 2%
(2*R*)-[2-$^3H_1$,2-$^2H_1$]Succinate→haem→ethylmethyl maleimide→acetate (Rings A + B)	malate	0.42	
	equilibrated malate	0.125	29.7 ± 1.3%
[2-$^3H_2$]Succinate→haem→haematinic acid→acetate (Rings C + D)	malate	2.10	
	equilibrated malate	1.04	49.4 ± 0.3%
(2*R*)-[2-H_1,2-$^2H_1$]Succinate→haem→haematinic acid→acetate (Rings C + D)	malate	1.26	
	equilibrated malate	0.38	30.2 ± 0.5%

glyoxylate severely inhibited the formation of acetyl CoA, therefore glyoxylate was added in aliquots throughout the incubation to prevent its accumulation. To locate the distribution of tritium at the C-3 of malate one-third of the material from each source was admixed with non-radioactive carrier (2*S*)-malate and crystallised for the determination of radioactivity. The remaining two-thirds from each specimen was incubated with fumarase to exchange the C-3 *pro-R*-hydrogen of malate with the protons of the medium.[15,16a] Isolation and recrystallisation of these malate samples allowed comparison of the T/C values before and after fumarase equilibration. In parallel control experiments (2*RS*)-[$^3H_1$]succinate was incorporated into haem and the derived [^{3}H]acetate mixed with [^{14}C]acetate and converted into malate. These experiments showed close to the predicted 50% retention of tritium upon fumarase equilibration (Table 2). With samples of malate deriving ultimately from stereospecifically (2*R*)-[2-$^3H_1$,2-$^2H_1$]succinic acid (3) *via* haem (6), 70% tritium at the C-3 of malate was lost during the fumarase-catalysed equilibration reaction, thus showing that the tritium in malate was predominantly in the *pro-R*-position (Scheme 1). This was the case whether the acetate originated from ethylmethyl maleimide or haematinic acid. Such malate samples (10) therefore must be derived from (2*S*)-[$^3H_1$,$^2H_1$, $^1H_1$] acetate (9).[13,14] With the assumption that no change in chirality occurred during the degradation of haem, then the porphyrinogen carboxy-lyase reaction proceeds with a retention of configuration at all four rings of uroporphyrinogen III. Had the ' rearranged ' D-ring followed a different stereochemical course of decarboxylation, the malate deriving from haematinic acid would have been racemic and would have lost 50% of its tritium upon fumarase equilibration.



SCHEME 4 A hypothetical mechanism for the porphyrinogen carboxy-lyase reaction

Mechanism.—A possible mechanism of decarboxylation is shown in Scheme 4. In contrast to most decarboxylation reactions, the porphyrinogen carboxy-lyase has no known absolute cofactor requirement. In Scheme 2 the structure of the porphyrinogen nucleus, after a preliminary protonation at the α-position of the pyrrole ring, has been utilised to facilitate decarboxylation, the overall reaction occurring through the sequences (4) ⟶ (4a) ⟶ (4b) ⟶ (4c) ⟶ (5) (Scheme 4). With a retention of configuration it is tempting to speculate that the group on the enzyme involved in either removal of the hydrogen from the O–H bond of the carboxy, or in the binding of an already dissociated carboxy, may also participate in the protonation of the intermediate (4b).*

It has been suggested that the overall conversion of uroporphyrinogen III into coproporphyrinogen III is kinetically a biphasic process in which the first carboxy group is removed at a faster rate than the remaining three.

uroporphyrinogen III $\xrightarrow[\text{fast}]{\text{decarboxylation at ring D}}$ heptacarboxylic acid intermediate $\xrightarrow[\text{slow}]{\text{decarboxylation at the remaining three rings}}$ coproporphyrinogen III

The further observation that the two stages are differently susceptible to conditions such as pre-heating, high NaCl concentration, and glutathione has led to the proposal that the enzyme contains two distinct catalytic sites.[3] Our results indicate that whether the removal of all the four carboxy groups occurs on a single or multiple site, the stereochemical mechanisms for the decarboxylations at all the four centres are the same.

EXPERIMENTAL

Materials.—Isocitrate dehydrogenase ($NADP^+$) from pig heart, citrate synthetase, L-glutamate dehydrogenase, catalase, phosphotransacetylase, 2-oxoglutaric acid disodium salt, triethanolamine, NADPH, imidazole, sodium glyoxylate, dithiothreitol, and Dowex 1 × 2-100 Cl^-, were obtained from Sigma (London) Chemical Co. Ltd. Acetate kinase and fumarase were obtained from Boehringer Corp. (London) Ltd. Deuterium oxide (99.8%) was obtained from British Oxygen Co. Ltd. Sodium [$^3H_1$]acetate, sodium [^{14}C]acetate, and ^3H_2O were obtained from the Radiochemical Centre, Amersham, Bucks. 1-M-Hyamine hydroxide in methanol, 10% palladium on charcoal, and crystallised haemin were obtained from Koch–Light Laboratories Ltd. Perspex chips were obtained from Griffin and George Ltd. (London). Coenzyme A lithium salt was obtained from P-L Biochemicals Inc. Milwaukee, U.S.A. Liquid scintillation fluid NE 250 was obtained from Nuclear Enterprises, Edinburgh, Scotland. All other chemicals were obtained from the British Drug Houses Ltd., Poole.

* The mechanistic proposal using a single base to carry out two chemical steps stems from the reasonable, but unproven, assumption that catalytic sites of enzymes have evolved with due consideration for economy. Other classes of reactions which have been interpreted to use one catalytic group where two or more could have been employed include 1,3-allylic and aza-allylic rearrangements and aldose–ketose isomerisations. These and other such examples are discussed in detail by K. R. Hanson and I. A. Rose, *Accounts Chem. Res.*, 1975, **8**, 1.

Methods.—[3-$^3H_2$]-2-Oxoglutarate was prepared by autoclaving 2-oxoglutaric acid disodium salt in T_2O.[17] [2-$^3H_2$]-Succinate was prepared by decarboxylation of [3-$^3H_2$]-2-oxoglutarate, as detailed below.

Preparation of (3R)-[3-$^3H_1$,3-$^2H_1$]-2-*oxoglutarate* (2) *and* (2R)-[2-$^3H_1$,2-$^2H_1$]*succinate* (3). Samples (10 ml) containing 100mM-triethanolamine–DCl buffer in D_2O; isocitrate dehydrogenase (1 ml, 50 units; dialysed against the deuteriated buffer to remove H_2O); disodium [3-$^3H_2$]-2-oxoglutarate (68.5 μmol); NADPH (6 μmol) and $MgCl_2 \cdot 6H_2O$ (6 μmol) were incubated at 29 °C. The course of tritium removal was followed as detailed by Zaman and Akhtar [18a] which involved applying 0.1 ml samples of the reaction mixture to a Dowex 1 × 2-100 Cl^- column (30 × 5 mm); the labilised tritium was eluted with 30 ml of water and 2-oxoglutarate with 50mM-HCl (30 ml). Samples (0.1 ml) from the two fractions were then used to determine the specific radioactivity.

When 50% of the tritium had been labilised, 5% H_2O_2 (6.6 ml) was added to the reaction mixture, which was set aside at room temperature for 30 min, and then applied to a Dowex 1 × 8, 20—50, Cl^- column (130 × 13 mm). After washing with water to remove H_2O_2 and the buffer, the compound was eluted from the column with 50mM-HCl and [2-$^3H_1$,2-$^2H_1$]succinate obtained by evaporation of the fraction *in vacuo* at 25 °C.

Preparation of succinic anhydride for mass-spectral analysis. Freshly distilled acetyl chloride (0.6 ml) was added to succinic acid (2 mg) and the solution heated under reflux for 1 h, with exclusion of moisture. After cooling, acetyl chloride was removed *in vacuo* at 25 °C to leave succinic anhydride, which was dissolved in dry acetone and aliquots analysed by mass spectrometry.

Haem biosynthesis from succinic acid.[18] To red cells obtained from blood [18] (25 ml) was added: a buffer (5.5 ml), pH 7.4, containing 0.15M-Na_2HPO_4 and 0.15M-KH_2PO_4 in 0.483M-KCl; penicillin (1.5 mg); streptomycin (1.5 mg); sodium malonate (530 μmol); glycine (330 μmol); $MgCl_2 \cdot 6H_2O$ (50 μmol); $FeSO_4 \cdot 7H_2O$ (15 μmol); and (*R*)-[2-$^3H_1$,2-$^2H_1$]succinate (40 μmol). The flask was incubated at 37 °C for 15 h with shaking. In a typical experiment, when incubation was performed with (2*R*)-[2-$^3H_1$,2-$^2H_1$]-succinate (40 μmol; 7.5×10^6 disintegrations min^{-1} μmol^{-1} of ^3H), haem (80.6 μmol) with an average specific radioactivity of 2.85×10^4 disintegrations min^{-1} μmol^{-1} was isolated. It should be born in mind that the bulk of haem arises from that already present in the red blood cells. Haem was isolated following the method of Labbe *et al.*[19] and counted for tritium radioactivity as detailed elsewhere.[18a]

Degradation of haem to ethylmethyl maleimide and haematinic acid. Iron was removed from haem by the method of Falk [20] to give protoporphyrin IX which was then hydrogenated to mesoporphyrin IX.[21] The procedure of Shemin [22] was adopted for chromic acid oxidation of mesoporphyrin IX to give haematinic acid and ethylmethyl maleimide, which were purified by repeated sublimation. The latter imides were quantified [23] by using molar extinction coefficients, in water at 290 nm, of 420 for ethylmethyl maleimide and 465 for haematinic acid. As an example 80.6 μmol of stereospecifically [^3H,^2H]haem (2.85×10^4 disintegrations min^{-1} μmol^{-1}) on degradation yielded, after purification, 106 μmol of haematinic acid (8.04×10^3 disintegrations min^{-1} μmol^{-1}) and 60 μmol of ethylmethyl maleimide (7.97×10^3 disintegrations min^{-1} μmol^{-1}).

Degradation of haematinic acid and ethylmethyl maleimide to isolate acetic acid. Haematinic acid (*ca.* 6 μmol) or ethylmethyl maleimide (*ca.* 6 μmol) were separately treated with 10mM-NaOH (1.0 ml) for 1 h to hydrolyse the imide rings. Liberation of ammonia was monitored using glutamate dehydrogenase.[24] The reaction mixtures were then adjusted to pH 2.7—3.0 with phosphoric acid and supplemented with 30% hydrogen peroxide (50 μl). Ozonised oxygen was passed through the solutions at 22 °C to give 100 : 1 molar ratios of ozone : substrates. After neutralisation with 10mM-NaOH, catalase was added to decompose H_2O_2, and when effervescence ceased the pH was adjusted to 2.5 with 10mM-phosphoric acid. The reaction mixture was then freeze-dried trapping the aqueous fraction which was adjusted to pH 7—8 with 10mM-NaOH and the solution again freeze-dried to give the solid sodium acetate. By the methods outlined above haematinic acid (70 μmol, 8.04×10^3 disintegrations min^{-1} μmol^{-1}) was degraded to acetate (*ca.* 25 μmol, 2.8×10^3 disintegrations min^{-1} μmol^{-1}). Similarly ethylmethyl maleimide (60 μmol, 7.97×10^3 disintegrations min^{-1} μmol^{-1}) gave acetate (*ca.* 10 μmol, 2.6×10^3 disintegrations min^{-1} μmol^{-1}).

Biosynthesis of malate from [$^3H_1$,$^2H_1$,$^1H_1$]*acetate.*—Incubations based on the system of Rose [16b] contained in 2 ml final volume: 50 mM-tris(sulphonate)–KOH buffer, pH 7.4; 50mM-potassium chloride; 16mM-magnesium chloride; 10mM-neutralised ATP; 10mM-dithiothreitol; acetate kinase (11 units); phosphotransacetylase (24 units); 5mM-sodium acetate; and CoASH (2 μmol).

After an optimal conversion of acetate to acetyl CoA had occurred (see below) the formation of malate was initiated by the addition of sodium glyoxylate (2 μmol) and malate synthetase (2—3 units). Threee further portions each containing sodium glyoxylate (3.4 μmol) were added at 30, 45, and 60 min intervals. After 75 min from the initiation of malate synthesis the incubation was terminated by applying the solution to a column of Dowex 1 × 2—100 Cl^- (1.3 × 18 cm). The column was washed with water (40 ml), unreacted acetate and glyoxylate were eluted with 5mM-HCl (80 ml), and the malate (7 μmol) was eluted with 10mM-HCl (60 ml) and recovered by freeze-drying.

Assay for the formation of acetyl CoA. In a final volume of 0.33 ml, incubation for the enzymic formation of acetyl CoA contained: 50mM-tris(sulphonate)–KOH buffer, pH 7.4; 50mM-KCl; 15mM-$MgCl_2 \cdot 6H_2O$; 10mM-neutralised ATP; acetate kinase (2 units); of phosphotransacetylase (4 units); 1.0mM-CoASH; 15mM-sodium acetate; and 0—40mM-glyoxylate as required. Reactions were started by the addition of acetate and incubated at 22 °C. At various time intervals, samples (10 μl) from the preceding mixture were removed and added to a 1-ml spectrophotometer cell containing: H_2O (0.7 ml); 1M-Tris–HCl buffer (0.1 ml), pH 7.8; 5mM-5,5′-dithiobis-(2-nitrobenzoic acid) (0.1 ml); and 5mM-disodium oxaloacetate. The total change in 412 nm gave the concentration of free CoASH (the mM extinction coefficient of 5-thio-2-nitrobenzoic acid at pH 7.8 being 13.6). Citrate synthetase (1 μl) was then added to the cuvette and the new change in optical density at 412 nm was taken to represent the CoASH which was esterified as acetyl CoA.

Purification of malate synthetase. Malate synthetase from *Saccharomyces cerevisiae* was purified and assayed, essentially following the method of Dixon and Kornberg.[26] Owing to the instability of the enzyme, the method was modified to allow rapid purification of small amounts of the enzyme as required. The resulting preparation had no detectable fumarase activity and catalysed the formation of 1—2 μmol of malate per min per mg of protein.

The procedure employed, at 0—4 °C, was as follows: Three batches of fresh baker's yeast (each 150 g), each in 5mM-Tris–HCl buffer, pH 8.0 (300 ml), containing 1mM-$MgCl_2 \cdot 6H_2O$ were ultrasonicated with a 19-mm diameter probe, in an ice–salt mixture, twice for 23 min, with cooling in between ultrasonications. The cell debris was removed by centrifugation at 23 000g for 30 min, to leave a turbid brown supernatant. Crushed solid $[NH_4]_2[SO_4]$ was added from 0—51% saturation over *ca.* 1 h and the solution was gently stirred for 30 min. The precipitate was collected by centrifugation at 23 000g for 30 min and forced into dialysis tubing of diameter 24/32 for dialysis against of 5mM-Tris–HCl buffer, pH 8.0 (2 × 5 000 ml) containing 1mM-$MgCl_2 \cdot 6H_2O$ for 16 h. The dialysate was clarified by ultracentrifugation at 110 000G for 2.5 h, collecting the lower one-third of the contents of the tube, which contained the majority of the malate synthetase activity. Aliquots of the ultracentrifugate containing 300—500 mg of protein were passed directly onto a DEAE cellulose column (DE 22) (0.8 × 10 cm), which had been thoroughly equilibrated with 5mM-Tris–HCl buffer, pH 7.4, containing 1mM-$MgCl_2 \cdot 6H_2O$. The enzyme was washed through with equilibrating buffer and collected in 0.5-ml fractions. Malate synthetase was amongst the first of the non-retained proteins to pass through the column. The first fractions with sufficient activity (and with a specific activity of at least 1.0 μmol of acetyl CoA consumed per min per mg protein) were used directly in the incubations converting acetate to malate.

Equilibration of malate with fumarase. To neutralised (2S)-malate (5 μmol) in water (0.5 ml) was added 1M-imidazole–HCl buffer, pH 7.0 (50 μl), and fumarase (5 μl, 7.2 units). The solution was incubated at 25 °C for 110 min and then passed directly onto a column of Dowex 1 × 2—100 Cl^- (0.5 × 6.0 cm) which had been pre-treated with 1M-HCl (6.0 ml) and washed with water until the pH stabilised. The column was washed with water (10 ml) to remove buffer and labilised tritium. Malate was eluted with 10mM-HCl, and after the addition of non-radioactive carrier (2*S*)-malate (30 mg) the solution was freeze-dried. Malate was recrystallised from ether–light petroleum (b.p. 60—80 °C) and counted in a mixture of methanol (0.5 ml) and 0.6% butylPBD (10 ml) [5-(biphenyl-4-yl)-2-(4-t-butylphenyl)-1,3,4-oxadiazole] in toluene.

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REFERENCES

[1] A. R. Battersby and E. McDonald, in 'Falk's Porphyrins and Metalloporphyrins,' 2nd edn., ed. K. M. Smith, Elsevier, Amsterdam, 1975.

[2] (*a*) M. Akhtar, M. M. Abboud, G. Barnard, P. Jordan, and Z. Zaman *Phil. Trans.*, 1976, **B273**, 117; (*b*) M. Akhtar and P. Jordan, 'Comprehensive Organic Chemistry,' Pergamon Press, 1979, vol. 5, p. 1121.

[3] R. C. Garcia, L. C. San Martin De Viale, J. M. Tomio, and M. Grinstein, *Biochim. Biophys. Acta*, 1973, **309**, 203 and references cited therein.

[4] P. Cornford, *Biochem. J.*, 1964, **91**, 64.

[5] D. Mauzerall and S. Granick, *J. Biol. Chem.*, 1958, **232**, 1141.

[6] C. A. M. Del Batlle and M. Grinstein, *Biochim. Biophys. Acta*, 1964, **82**, 1.
[7] L. C. San Martin De Viale and M. Grinstein, *Biochim. Biophys. Acta*, 1968, **158**, 79.
[8] A. H. Jackson, H. A. Sancovich, A. M. Ferramola, N. Evans, D. E. Games, S. A. Maltin, G. M. Elder, and S. G. Smith, *Phil. Trans.*, 1976, **B273**, 191.
[9] (*a*) A. R. Battersby, E. Hunt, M. Ihara, E. McDonald, J. B. Paine III, F. Satoh, and J. Saunders, *J.C.S. Chem. Comm.*, 1974, 994; (*b*) A. R. Battersby, E. Hunt, E. McDonald, J. B. Paine III, and J. Saunders, *J.C.S. Perkin I*, 1976, 1008.
[10] G. F. Barnard and M. Akhtar, *J.C.S. Chem. Comm.*, 1975, 494.
[11] G. E. Lienhard and I. A. Rose, *Biochemistry*, 1964, **3**, 185.
[12] (*a*) J. W. Cornforth, R. H. Cornforth, C. Donninger, and G. Popjak, *Proc. Roy. Soc.*, 1966, **B163**, 492; (*b*) G. H. Dixon and H. L. Kornberg in ' Methods in Enzymology,' vol. V, eds. S. P. Kolowick and N. O. Kaplan, Academic Press, New York, 1962, p. 633.
[13] J. W. Cornforth, J. W. Redmond, H. Eggerer, W. Buckle, and C. Gutschow, *Nature*, 1969, **221**, 1212.
[14] J. Luthy, J. Rétey, and D. Arigoni, *Nature*, 1969, **221**, 1213.
[15] F. A. L. Anet, *J. Amer. Chem. Soc.*, 1960, **82**, 994.
[16] (*a*) O. Gawron and T. P. Fondy, *J. Amer. Chem. Soc.*, 1959, **81**, 6333; (*b*) I. A. Rose, *J. Biol. Chem.*, 1970, **245**, 6052.
[17] Z. B. Rose, *J. Biol. Chem.*, 1960, **253**, 928.
[18] (*a*) Z. Zaman and M. Akhtar, *Europ. J. Biochem.*, 1976, **61**, 215; (*b*) E. I. B. Dresel and J. E. Falk, *Biochem. J.*, 1954, **56**, 156; (*c*) E. I. B. Dresel and J. E. Falk, *Biochem. J.*, 1956, **63**, 72.
[19] R. F. Labbe and G. Nishida, *Biochim. Biophys. Acta*, 1957, **26**, 437.
[20] J. E. Falk, ' Porphyrins and Metalloporphyrins,' Elsevier, Amsterdam, 1964, p. 130.
[21] Ref. 20, p. 178.
[22] D. Shemin, in ' Methods in Enzymology,' vol. IV, eds. S. P. Colowick and N. O. Kaplan, Academic Press, New York, 1957, p. 643.
[23] H. M. Muir and A. Neuberger, *Biochem. J.*, 1949, **45**, 163.
[24] D. Cooney, R. Davis and G. Van Atta, *Analyt. Biochem.*, 1971, **40**, 312.