Stereochemistry of Porphyrinogen Carboxy-lyase Reaction in Haem Biosynthesis

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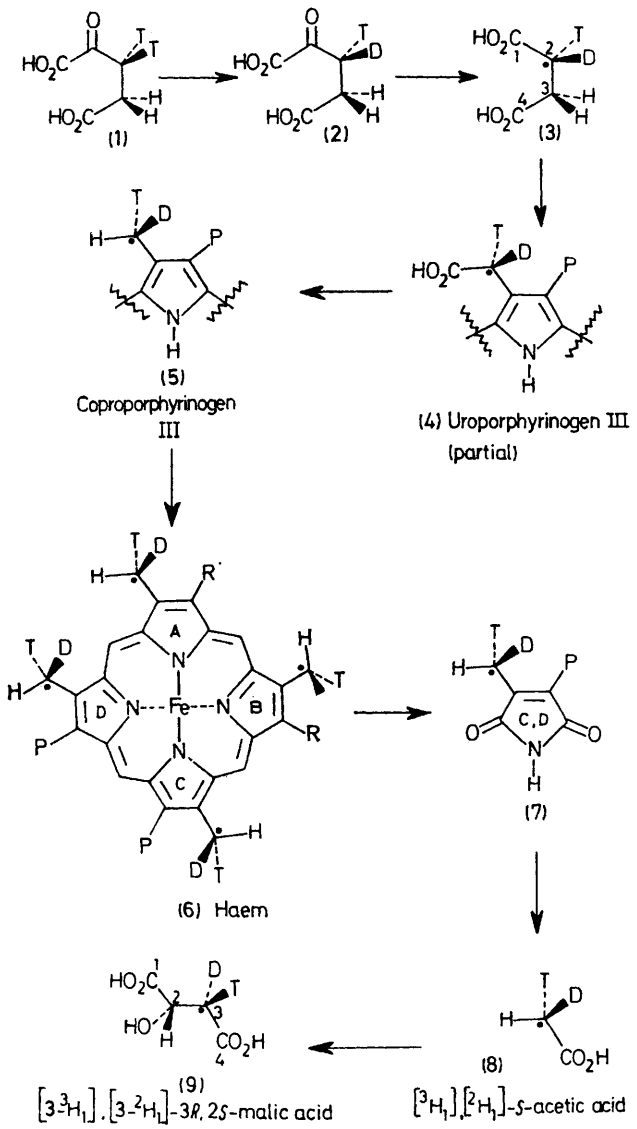
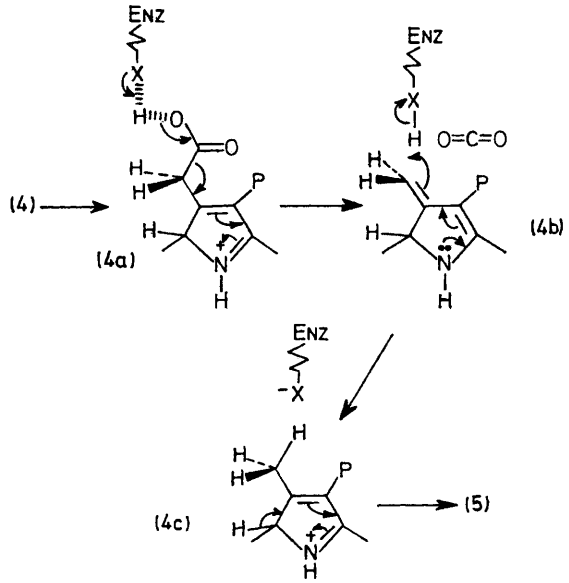
Summary Stereospecifically deuteriated and tritiated succinate was incorporated into the acetate residues of uroporphyrinogen III which on decarboxylation generated asymmetric methyl groups in coproporphyrinogen III and then in haem; degradation of the latter yielded chiral acetate deriving from the C and D rings of haem and configurational analysis of this derived acetate shows that the carboxy-lyase reaction proceeds with a retention of configuration.

IN porphyrin biosynthesis[1] the enzyme porphyrinogen carboxylase[2] catalyses a stepwise transformation of the four acetate residues of uroporphyrinogen III into the methyl groups of coproporphyrinogen III [Scheme 1, (**4**) → (**5**), T=D=H]. We now report the stereochemistry of this decarboxylation reaction at the C and D rings during haem biosynthesis using a haemolysed preparation of chicken erythrocytes.[3] Our approach for the investigation involved the generation during the haem biosynthesis of acetate side-chains in uroporphyrinogen III which were stereospecifically labelled with deuterium as well as tritium. The decarboxylation reaction in H_2O then produced coproporphyrinogen III in which each methyl group contained all the three isotopes of hydrogen residing at the single carbon atom. The methyl groups from rings C and D of haem were isolated as acetic acid and the determination of the chirality of this ^3H, ^2H, ^1H-acetic acid has shown that the porphyrinogen carboxy-lyase reaction proceeds with retention of configuration.



SCHEME 1. R = CT=CDT, P = CDTCDTCO$_2$H

For our biosynthetic experiments we required chiral succinate in which one of the two methylene groups was stereospecifically labelled with both tritium and deuterium (**3**). This synthesis was achieved by incubating [3-$^3H_2$]-2-oxoglutarate (**1**) in D_2O with NADPH and isocitrate dehydrogenase (NADPH dependent) when a time-dependent enzymic exchange of the tritium with the deuterium of the medium occurred, which ceased after 50% of the original ^3H radioactivity of the acid had been labilised. Since it is known[4] that the isocitrate dehydrogenase stereospecifically exchanges the *pro-S* hydrogen of 2-oxoglutarate, the sample produced may be formulated as [3-$^3H_1$],[3-$^2H_1$]-3*R*-2-oxoglutaric acid (**2**). On treatment with H_2O_2 this gave [$^3H_1$],[$^2H_1$]-*R*-succinic acid (**3**), which when incubated with the haemolysed erythrocyte preparation gave stereospecifically tritiated, deuteriated haem (**6**). The biosynthesised haem was degraded[1] to ethyl methyl maleimide and haematinic acid (**7**) by the sequence, haem → protoporphyrin IX → mesoporphyrin IX → ethyl methyl maleimide + haematinic acid.



SCHEME 2

We also needed to develop a method by which haematinic acid (**7**) would be oxidised to acetic acid in the absence of enolisation, so that the chirality of the methyl group was maintained. After examining several approaches it was found that when haematinic acid was ozonised at pH 3·0 in the presence of H_2O_2, acetic acid was produced in a radiochemical yield which was 40% of theoretical.† To determine the chirality of the acetic acid the approach developed by Cornforth *et al.*[5] and by Luthy *et al.*[6] was adopted. 10 μmoles of [$^3H_1$],[$^2H_1$]-acetic acid (**8**) was admixed with [^{14}C]-acetate and incubated with acetate kinase, phosphotransacetylase and malate synthetase essentially as detailed by Rose[7] to produce about 7 μmoles of malate. To locate the distribution of tritium at the C(3) position of the biosynthesised malate, one third of the

† In such experiments less than 1% of the original radioactivity was associated with water.

TABLE[a]

Source of acetate				% Retention of ^3H
Exp 1a				
Haem from [$^3H_2$]-succinic acid	T/C[b]	Malate	$=1\cdot053\pm0\cdot02$	
	T/C	Equilibrated Malate	$=0\cdot555\pm0\cdot01$	$=52\cdot7\pm0\cdot5\%$
Exp 1b				
Haem from [$^3H_1$],[$^2H_1$]-*R*-succinic acid	T/C	Malate	$=0\cdot981\pm0\cdot01$	
	T/C	Equilibrated Malate	$=0\cdot302\pm0\cdot00$	$=30\cdot8\pm0\cdot5\%$
Exp 2a				
[$^3H_1$]-acetate from Amersham	T/C	Malate	$=1\cdot637\pm0\cdot02$	
	T/C	Equilibrated Malate	$=0\cdot825\pm0\cdot01$	$=50\cdot4\pm1\cdot4\%$
Exp 2b				
Haem from [$^3H_1$],[$^2H_1$]-*R*-succinic acid	T/C	Malate	$=1\cdot108\pm0\cdot01$	
	T/C	Equilibrated Malate	$=0\cdot350\pm0\cdot01$	$=31\cdot6\pm1\cdot0\%$

[a] For all experiments the conversion of a stereochemical sample of acetate→malate → fumarate was accompanied by the simultaneous conversion of one of the non-stereochemical samples under identical conditions.

[b] T/C ratios refer to recrystallised malate, before and after fumarase equilibration, and containing at least 3,000 dpm of both ^3H and ^{14}C.

material was admixed with non-radioactive carrier and crystallised to give malate which had a $^3H:^{14}C$ ratio of 0·981 (Table, Exp 1b). The remaining two thirds was incubated with fumarase to exchange the C(3) *pro-R* hydrogen of the biosynthesised malate with protons of the medium. This fumarase-equilibrated malate after recrystallisation had a $^3H:^{14}C$ ratio of 0·302. This demonstrated that most of the tritium was located in the *pro-R* position and the malate biosynthesised as detailed above was therefore [3-$^3H_1$],[3-$^2H_1$]-3*R*-2*S*-malic acid (**9**).‡ It has been shown[5,6] that when malate synthetase condenses glyoxylate with acetyl CoA, the reaction proceeds with an inversion of configuration. When Cornforth *et al.*[5] converted [$^3H_1$],[$^2H_1$]-*S*-acetic acid (**8**) to [3-$^3H_1$],[3-$^2H_1$]-3*R*-2*S*-malic acid (**9**) subsequent equilibration with fumarase yielded malate which had retained only 30·8% of its ^3H radioactivity. Our results which show a similar predominant labilisation of ^3H therefore establish that the sample of acetic acid containing the methyl groups originally present in rings C and D of the biosynthesised haem has *S*-chirality. Thus the decarboxylation reaction converting uroporphyrinogen III to coproporphyrinogen III (**4**) → (**5**) must have occurred with a retention of configuration.

In the light of this stereochemical information 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 carboxyl or in the binding of the already dissociate carboxyl may also participate in the protonation of the intermediate (**4b**) by the sequence of reactions shown in Scheme 2, in which steps (**4**) → (**4a**) and (**4c**) → (**5**) occur *via* enzyme-catalysed protonation and deprotonation respectively.

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‡ This data and conclusion was confirmed in a completely independent experiment in which another batch of [$^3H_1$],[$^2H_1$]-*R*-succinic acid was carried through the above sequence of events (Table, Exp. 2b). In a parallel control experiment succinate randomly tritiated at position 2 [see (**3**)], was incorporated into haem, and the derived [$^3H_1$]-acetate mixed with [^{14}C]-acetate and converted into malate to give a $^3H:^{14}C$ ratio of 1·053 (Table, Exp. 1a). This sample on incubation with fumarase gave equilibrated malate having a $^3H:^{14}C$ ratio of 0·555 giving close to the expected 50% retention of ^3H radioactivity. This figure for the retention of tritium assured us that during the complex series of chemical and biological reactions involving the biosynthesis of haem from succinate, isolation of acetic acid and the determination of its chirality, no unforeseen artefact had contributed to the result.

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