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Factors Determining the Sequence of Oxidative Decarboxylation of the 2- and 4-Propionate Substituents of Coproporphyrinogen III by Coproporphyrinogen Oxidase in Rat Liver

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Coproporphyrinogen oxidase (EC 1.3.3.3) catalyses the oxidative decarboxylation of the 2- and 4-propionate substituents of coproporphyrinogen III to form protoporphyrinogen IX. A 4-propionate-substituted porphyrinogen, harderoporphyrinogen, which is also a substrate for coproporphyrinogen oxidase, is formed during the reaction. Synthetic [^{14}C]coproporphyrinogens III, specifically labelled in the carboxyl carbon atoms of either the 2- or 4-propionate substituents, were used to measure the rate of decarboxylation of each substituent by rat liver coproporphyrinogen oxidase. The experimental results, together with the recognition that in all known substrates of coproporphyrinogen oxidase only those propionate groups flanked by a specific arrangement of substituents are decarboxylated, indicate that the 4-propionate group of coproporphyrinogen III cannot be attacked until the 2-propionate group has been decarboxylated. Production of $^{14}CO_2$ from the substrate labelled in the 2-propionate group therefore measures the formation of harderoporphyrinogen, whereas $^{14}CO_2$ from the 4-propionate-labelled substrate measures protoporphyrinogen IX formation. The rate of harderoporphyrinogen formation is about twice that of protoporphyrinogen, and this ratio is unchanged by varying the concentration of coproporphyrinogen III or by competitive inhibition of the enzyme. When coproporphyrinogen III is present in an excess, two fractions of harderoporphyrinogen can be distinguished. One accumulates during the reaction, and the other, which is destined to become protoporphyrinogen IX, does not equilibrate with added harderoporphyrinogen. It is suggested that both decarboxylations take place at the same active centre, which becomes temporarily inaccessible to coproporphyrinogen III and added harderoporphyrinogen, and that the molecule rotates after the first decarboxylation to allow the second to take place.

Coproporphyrinogen oxidase (EC 1.3.3.3) catalyses the oxidative decarboxylation of coproporphyrinogen III to protoporphyrinogen IX, the penultimate precursor of haem. A tripropionate monovinylporphyrinogen is formed during the course of this reaction (Sano & Granick, 1961) in which the 2- and 4-propionate substituents of coproporphyrinogen III are converted into vinyl groups. Corresponding tripropionic acid porphyrins have been isolated from the harderian gland of the rat (Kennedy *et al.*, 1970), which is an organ with a high rate of protoporphyrin synthesis (Strand *et al.*, 1970), and from avian erythrocyte haemolysates during the conversion of coproporphyrinogen III into protoporphyrinogen IX (Games *et al.*, 1976) and identified as 4-propionic acid 2-vinyldeuteroporphyrin IX ('harderoporphyrin') (Kennedy *et al.*, 1970; Games *et al.*, 1976). The finding that this porphyrin was a single compound and not a mixture of the two possible position isomers, harderoporphyrin and isoharderoporphyrin (2-propionic acid 4-vinyldeuteroporphyrin IX), led to the suggestion that the 2- and 4-propionate side chains of coproporphyrinogen III are decarboxylated in sequence, with that at the 2-position being metabolized first (Kennedy *et al.*, 1970). Additional evidence that this is the preferred route of synthesis in plants and animals comes from experiments that show that both an enzyme extract from *Euglena gracilis* and avian erythrocyte haemolysates convert harderoporphyrinogen more readily than isoharderoporphyrinogen into protoporphyrin IX (Cavaleiro *et al.*, 1973; Games *et al.*, 1976).

The mechanism that determines the sequence of decarboxylation of the side chains is obscure. By

using [$^{14}C_1$]coproporphyrinogen III in which the carboxyl carbon atom of either the 2- or the 4-propionate side chain is labelled, it is possible to measure the rate of decarboxylation of each side chain separately. Here we report the results of experiments using this approach that are compatible with a mechanism that enables the sequence of reactions to be completed at a single active centre.

Experimental

Materials

[^{14}C]Coproporphyrins. These were prepared from 2-formyl-4,6,7-tris-(2-methoxycarbonylethyl)-1,3,5,8-tetramethylporphin, 4-formyl-2,6,7-tris-(2-methoxycarbonylethyl)-1,3,5,8-tetramethylporphin, both synthesized as described by Couch *et al.* (1976) and 2,4-diformyl-6,7-di-(2-methoxycarbonylethyl)-1,3,5,7-tetramethylporphin, prepared from protoporphyrin IX dimethyl ester by a modification (Elder & Evans, 1978) of the method of Sparatore & Mauzerall (1960). The following general method, involving condensation with malonic acid to give an acrylic acid-substituted porphyrin (Sparatore & Mauzerall, 1960) that is then reduced to coproporphyrin III, was used. A trace of piperidine was added to a solution of formylporphyrin methyl ester (0.14mmol) and [1-^{14}C]malonic acid (about 5mol/mol of formyl group) in pyridine (2.0ml) and the mixture was refluxed for 6.5h. After cooling, dilution with chloroform and addition of an excess of diazomethane in diethyl ether, the solvents were removed by evaporation under reduced pressure, with methanol as an azeotrope, to give a dark residue. This residue was dissolved in chloroform and adsorbed on neutral aluminium oxide (activity III) (3.0g) by evaporation under reduced pressure. This material was added to the top of a neutral aluminium oxide (activity III) column (approx. 15cm×2cm), packed in light petroleum (b.p. 60–80°C), and washed, in the dark, with light petroleum (b.p. 60–80°C) (50ml) followed by light petroleum (b.p. 60–80°C)/benzene (1:1, v/v) (about 10ml), light petroleum (b.p. 60–80°C)/benzene (1:3, v/v) (50ml) and benzene (50ml). A brown band was then eluted with chloroform (50ml) which was evaporated to give an acrylic acid-substituted porphyrin methyl ester as a dark residue. The residue was dissolved in tetrahydrofuran (5ml). Triethylamine (6 drops) was added and the mixture hydrogenated over 10% (w/w) Pd/$BaSO_4$ (about 5mg) for 24h at room temperature (15–22°C). It was then diluted with chloroform, filtered and treated with 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (about 3mg) to reoxidize any porphyrinogen formed during the reduction. The solvent was removed by evaporation under reduced pressure and the resultant residue was chromatographed on a neutral aluminium oxide (activity III) column (approx. 15cm×2cm), packed in chloroform, with chloroform as eluent to give a single porphyrin band that was fractionated by t.l.c. on silica gel in benzene/ethyl acetate (2:1, v/v). The major pink band was eluted with chloroform and evaporated under reduced pressure to give a porphyrin methyl ester with an adsorption spectrum in chloroform identical with that of coproporphyrin III tetramethyl ester. Yields of [^{14}C]coproporphyrin III tetramethyl ester from the formylporphyrin methyl esters ranged from 8 to 27%.

[^{14}C]Coproporphyrin III tetramethyl esters, ^{14}C-labelled in the carbonyl carbon atom of either the 2- or 4-(2-methoxycarbonylethyl) side chains, were further purified by t.l.c. in chloroform/kerosene/methanol (100:50:3, by vol.) to remove a small amount of an unidentified ^{14}C-labelled tricarboxylic acid porphyrin trimethyl ester. Each ester was hydrolysed, and the free porphyrin, after further purification by solvent partition, was dried *in vacuo* and dissolved in a small volume of 0.01M-KOH, as described previously (Elder & Evans, 1978). The specific radioactivity of each compound, measured as described by Elder & Evans (1978), was 4.85mCi/mmol compared with a theoretical value of 4.75mCi/mmol calculated from the specific radioactivity of the [1-^{14}C]malonic acid (9.5mCi/mmol) used for the condensation.

The prefixes [2-^{14}C], [4-^{14}C] and [2,4-^{14}C] will be used to show the position of the isotope in coproporphyrins and coproporphyrinogens ^{14}C-labelled in the carboxyl carbon atoms of either the 2-propionate group, the 4-propionate group or both groups respectively.

Other porphyrins. Harderoporphyrin and isoharderoporphyrin trimethyl esters were gifts from Professor G. W. Kenner and Dr. K. M. Smith, Department of Organic Chemistry, University of Liverpool, Liverpool, U.K. Coproporphyrin III was synthesized as described by Jackson *et al.* (1965).

Porphyrinogens. Porphyrins in 0.01M-KOH were reduced with 4% (w/v) sodium/mercury amalgam, and the resulting porphyrinogen solutions mixed with equal volumes of 0.25M-Tris/HCl buffer, pH7.2, containing 0.2M-sodium thioglycollate and used within 5min of preparation (Elder *et al.*, 1976).

Other materials. [1-^{14}C]Malonic acid (specific radioactivity 9.5mCi/mmol) was obtained from The Radiochemical Centre, Amersham, Bucks., U.K. Activated aluminium oxide for column chromatography and precoated silica-gel 60 plates (20cm×20cm×0.025cm) for t.l.c. were obtained from Merck, Darmstadt, W. Germany. Soluene-350, PPO (2,5-diphenyloxazole) and POPOP [1,4-bis-(5-phenyloxazol-2-yl)benzene] were from Packard Instruments, Caversham, Berks., U.K. Solvents were AnalaR grade, except for toluene (scintillation grade) (BDH Chemicals, Poole, Dorset, U.K.) and kerosene

(white) (Hopkin and Williams, Romford, Essex, U.K.).

Methods

Measurement of rate of $^{14}CO_2$ release from [^{14}C]-coproporphyrinogen III. Rates of $^{14}CO_2$ production from [^{14}C]coproporphyrinogen III were measured as described by Elder & Evans (1978), with homogenates of rat liver (0.1 g or 0.05 g wet wt./ml) in 50 mM-Tris/HCl buffer, pH 7.3, as enzyme preparation. The reaction mixture consisted of 0.03 ml of 0.25 M-Tris/HCl buffer, pH 7.2, containing bovine serum albumin (33.3 mg/ml), 0.05 ml of enzyme preparation, 0.01 ml of 0.01 M-KOH and 0.01 ml of porphyrinogen solution diluted with buffer containing sodium thioglycollate. For experiments in which two porphyrinogens were present in the incubation mixture, the corresponding porphyrins were mixed in the appropriate proportions before reduction and then reduced, diluted with buffer containing sodium thioglycollate, and added to the reaction mixture as a single solution at the start of the reaction.

Isolation and determination of specific radioactivity of porphyrins from incubation mixtures. Reactions were stopped by the addition of ethyl acetate/acetic acid (3:1, v/v), and porphyrins extracted and converted into their methyl esters (Elder *et al.*, 1976). The methyl esters were separated by t.l.c. in chloroform/kerosene/methanol (100:50:3, by vol.), eluted from the plates in chloroform/methanol (4:1, v/v), and the solvent was evaporated in a stream of N_2 at 50°C. The methyl ester was then dissolved in a known volume of chloroform and its concentration estimated spectrophotometrically by using ε_{mM} given by Falk (1964) or, for harderoporphyrin and isoharderoporphyrin trimethyl esters, 180 litre·mmol^{-1}·cm^{-1} (Suckling, 1970). Samples of the solution were transferred to glass vials, dried and dissolved in Soluene-350 for determination of radioactivity (Elder & Evans, 1978).

Separation of isomeric tripropionic acid porphyrin methyl esters. Harderoporphyrin and isoharderoporphyrin trimethyl esters were separated by high-pressure liquid chromatography on a 316 stainless-steel column [0.2 cm (internal diam.) × 100 cm] of Corasil-II (Waters Associates, Stockport, Cheshire, U.K.) in cyclohexane/ethyl acetate (4:1, v/v), with a Waters model 6000 pump with septum injector, and detected at 402 nm with a Cecil CE 272 spectrophotometer fitted with a 0.01 ml flow cell (Evans *et al.*, 1976). The eluate from the column was collected in fractions (2–11 ml), which were transferred to glass vials and evaporated at 50°C in a stream of N_2. Soluene-350 (0.5 ml) was added to each vial, and, after mixing and being left for 30 min, toluene (15 ml) containing PPO (4 g/litre) and POPOP (100 mg/litre) was added. Radioactivity was then measured by

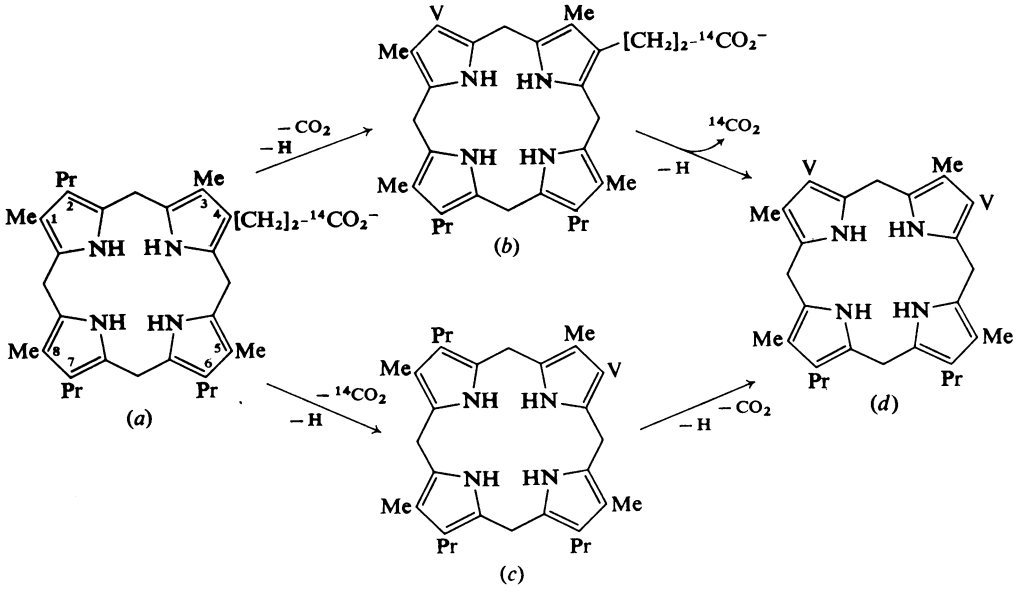

Fig. 1. *Possible routes for the formation of protoporphyrinogen IX*

The fate of the ^{14}C atom in [4-^{14}C]coproporphyrinogen III (*a*) is shown. (*b*) Harderoporphyrinogen; (*c*) isoharderoporphyrinogen; (*d*) protoporphyrinogen IX; Pr, propionate; V, vinyl; Me, methyl.

using the ^{14}C channel of a Packard Tri-Carb liquid-scintillation spectrometer.

Results and Discussion

Evidence that the 2-propionate group of coproporphyrinogen III is decarboxylated before the 4-propionate group

Formation of harderoporphyrinogen during the conversion of coproporphyrinogen III into protoporphyrinogen IX. If the 2-propionate group of coproporphyrinogen III is decarboxylated by coproporphyrinogen oxidase before the 4-propionate group, the tripropionate porphyrinogen formed from [4-^{14}C]coproporphyrinogen should have the same specific radioactivity as the substrate from which it comes, whereas that from [2-^{14}C]coproporphyrinogen should not be radioactive (Fig. 1). Measurements of the specific radioactivities of the tripropionic acid porphyrin trimethyl ester fractions isolated after incubation of each of the [^{14}C]coproporphyrinogens with rat liver homogenate are shown in Table 1. In these experiments porphyrinogens were oxidized to porphyrins, converted into their methyl esters, and porphyrin trimethyl esters separated from coproporphyrin tetramethyl ester and protoporphyrin IX dimethyl ester by t.l.c. in chloroform/kerosene/methanol (100:50:3, by vol.), a solvent system that does not separate the trimethyl esters of harderoporphyrin and isoharderoporphyrin. The specific radioactivity of the fraction obtained from [4-^{14}C]-coproporphyrinogen III does not differ significantly ($P = 0.1$–0.05) from that of the substrate, whereas that from [2-^{14}C]coproporphyrinogen has a much lower specific radioactivity. Both fractions were purified further by high-pressure liquid chromatography. In each case a single peak in the position of harderoporphyrin trimethyl ester was seen (Fig. 2). In addition, this procedure clearly separated harderoporphyrin trimethyl ester, which was not radioactive, from a radioactive compound that was also present in the fraction obtained from [2-^{14}C]-coproporphyrinogen (Fig. 2). This compound had the same retention time on high-pressure liquid chromatography as an unidentified impurity in the sample of [2-^{14}C]coproporphyrin tetramethyl ester used to prepare the substrate.

Fig. 2 also shows that there is no increase in radioactivity in the column fraction that would be expected to contain isoharderoporphyrin ester. At the specific radioactivity of the substrate used (4.85mCi/mmol), the presence of 2% isoharderoporphyrin trimethyl ester would have produced an additional 80c.p.m., assuming a counting efficiency of 90%. Thus these experiments, which use a technique for the detection of isoharderoporphyrin that is at least as sensitive as any used previously (Evans *et al.*, 1976; Games *et al.*, 1976), confirm that harderoporphyrin is the only tripropionate porphyrin that can be isolated from tissues synthesizing protoporphyrin IX.

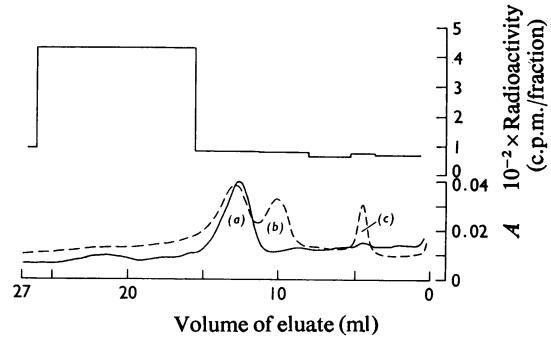

Fig. 2. *Separation of porphyrin methyl esters by high-pressure liquid chromatography*

Analyses of a mixture of harderoporphyrin (*a*), isoharderoporphyrin (*b*) and protoporphyrin (*c*) methyl esters (----) and of the tripropionic acid porphyrin trimethyl ester fraction (——) obtained by incubating [2-^{14}C]coproporphyrinogen III with rat liver homogenate are shown. The harderoporphyrin methyl ester fraction contained 430pmol of porphyrin, and the fraction eluted between 15.1 and 31.2ml contained approx. 45pmol. High-pressure liquid chromatography was carried out and radioactivity of the eluate determined as described in the Experimental section.

Table 1. *Specific radioactivity of porphyrins isolated from incubation medium*

[^{14}C]Coproporphyrinogens III (20μM) were incubated with rat liver homogenate (equivalent to 50mg wet wt. of liver) under the conditions used for measurement of $^{14}CO_2$ production (see the Experimental section) in a total volume of 1.0ml. After 60min the reaction was stopped and porphyrins were extracted, fractionated, estimated and counted for radioactivity as described in the Experimental section. Results are means±S.E.M. of four measurements.

Substrate	Porphyrin methyl ester fraction	Specific radioactivity (mCi/mmol)
[2-^{14}C]Coproporphyrinogen III	Coproporphyrin	4.61±0.08
	Tripropionic acid porphyrin	0.84±0.23
[4-^{14}C]Coproporphyrinogen III	Coproporphyrin	4.88±0.05
	Tripropionic acid porphyrin	4.49±0.21

It is known that harderoporphyrinogen can be converted into protoporphyrinogen IX (Cavaleiro *et al.*, 1973; Games *et al.*, 1976), and decarboxylation of the 2-propionate group of coproporphyrinogen III by coproporphyrinogen oxidase is regarded as the first stage in the major or, possibly, only route of protoporphyrinogen formation (Kennedy *et al.*, 1970; Cavaleiro *et al.*, 1973; Games *et al.*, 1976). At present there is no evidence that coproporphyrinogen oxidase can decarboxylate the 4-propionate group of coproporphyrinogen III. However, failure to identify isoharderoporphyrinogen in systems synthesizing protoporphyrin does not exclude this possibility. Thus the formation of protoporphyrinogen via isoharderoporphyrinogen would probably not be detectable if steady-state amounts of this compound were 1% or less than those of harderoporphyrinogen. Protoporphyrinogen was formed more rapidly from harderoporphyrinogen than from isoharderoporphyrinogen by the rat liver homogenates used in our experiments, the rates being 28.2 and 2.6nmol of protoporphyrin formed/h per g wet wt. respectively. Similarly, Cavaleiro *et al.* (1973) and Games *et al.* (1976) found a higher percentage incorporation of [^{3}H]harderoporphyrinogen than [^{3}H]isoharderoporphyrinogen into protoporphyrin IX. Unless a 2-propionate-substituted intermediate other than isoharderoporphyrinogen is involved, it seems unlikely that decarboxylation of the 4-propionate group of coproporphyrinogen III would initiate a quantitatively important route of protoporphyrinogen formation.

Substrate specificity of coproporphyrinogen oxidase. Table 2 shows that all the known substrates of this enzyme contain substituents in the sequence methyl-methyl-propionate-methyl, except for harderoporphyrinogen, in which the methyl group furthest from the propionate substituent is replaced by a vinyl group, and 4-propionate deuteroporphyrinogen IX, in which the same position is unsubstituted. Isoharderoporphyrinogen has sequences in common with both coproporphyrinogen III and harderoporphyrinogen. Related porphyrinogens, which are not substrates, do not contain these sequences (Table 2). Thus it appears that a propionate group will be decarboxylated by coproporphyrinogen oxidase only if it is flanked by two methyl groups, one of which must have either a methyl or vinyl group, an unsubstituted position or, possibly, an ethyl group as its neighbour. Although it has been suggested that the 6- and 7-propionate groups are involved in binding coproporphyrinogen III to the enzyme (Jackson & Games, 1975; Gidari & Levere, 1977), mesoporphyrinogen VI does not contain acidic substituents in these positions and their occurrence in tetracarboxylate substrates may merely be a consequence of the presence elsewhere in the molecule of the sequence described above. The structural requirements for

Table 2. *Substrate specificity of coproporphyrinogen oxidase*

Numbers refer to the β-positions on the hexahydroporphin macrocycle. Substituents are abbreviated as follows: ethyl (Et), methyl (Me), propionate (Pr), acetate (Ac) and vinyl (V). The sequences underlined indicate important common sequences mentioned in the text.

Compound	Substrate	Position and nature of substituent 8	1	2	3	4	5	6	7	Reference
Not metabolized										
Coproporphyrinogen I		Pr	Me	Pr	Me	Pr	Me	Pr	Me	Sano & Granick (1961)
Coproporphyrinogen II		Me	Me	Pr	Pr	Me	Me	Pr	Pr	Granick & Mauzerall (1958)
	Coproporphyrinogen III	Me	Me	Pr	Me	Pr	Me	Pr	Pr	
	Coproporphyrinogen IV	Me	Pr	Me	Me	Pr	Me	Pr	Pr	Porra & Falk (1964)
	Pentacarboxylate porphyrinogen III	Me	Me	Pr	Me	Pr	Ac	Pr	Pr	Elder & Evans (1978)
Isocoproporphyrinogen		Me	Me	Et	Me	Pr	Ac	Pr	Pr	
	Harderoporphyrinogen	Me	Me	V	Me	Pr	Me	Pr	Pr	
	Isoharderoporphyrinogen	Me	Me	Pr	Me	V	Me	Pr	Pr	
	4-Propionate deuteroporphyrinogen IX	Me	Me	H	Me	Pr	Me	Pr	Pr	Porra & Falk (1964)
	Mesoporphyrinogen VI	Me	Me	Pr	Me	Pr	Me	Et	Pr	Jackson *et al.* (1976*a*)
Mesoporphyrinogen IX		Me	Me	Et	Me	Et	Me	Pr	Pr	Sano & Granick (1961)

decarboxylation by coproporphyrinogen oxidase are not met by the 4-propionate group of coproporphyrinogen III. We therefore propose that this group cannot be attacked by the enzyme until it is exposed by prior decarboxylation of the 2-propionate substituent. If this argument is correct, a 4-propionate-substituted tripropionate porphyrinogen must be an obligatory intermediate in the enzymic conversion of coproporphyrinogen III into protoporphyrinogen IX, a reaction sequence that is consistent with the failure to find isoharderoporphyrin in Nature.

Nature of the sequential decarboxylation process

Time course of decarboxylation of [2-^{14}*C*]- *and* [4-^{14}*C*]-*coproporphyrinogen III.* Fig. 3(*a*) shows that the 2-propionate group of coproporphyrinogen III is decarboxylated almost twice as fast as the 4-propionate group, production of CO_2 from each group being linear with time for at least 30 min at a substrate concentration of 18–21 μM. Reproducible measurements of CO_2 production could not be made less than 2 min after the start of the reaction. Therefore the possibility that the ratio of the CO_2 production rate for the 2- and 4-propionate groups changes during this time cannot be excluded.

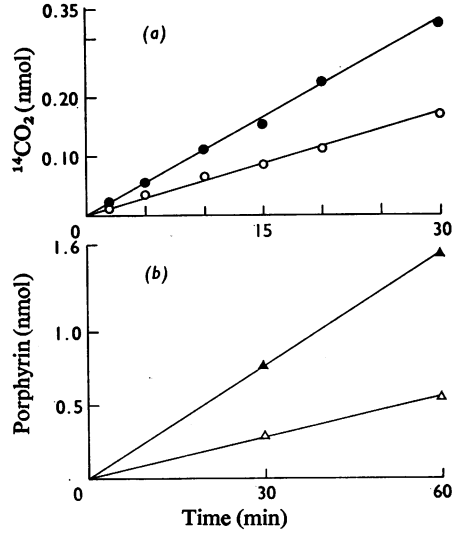

Fig. 3. *Formation of CO_2, harderoporphyrin and protoporphyrin IX from coproporphyrinogen III*

$^{14}CO_2$ production from [2-^{14}C]coproporphyrinogen III (●) and [4-^{14}C]coproporphyrinogen III (○) was measured as described in the Experimental section at substrate concentrations of 21 and 18 μM respectively (*a*). Identical reaction mixtures, except that the total volume was 1.0 ml, were used to measure the formation of protoporphyrin IX (▲) and harderoporphyrin (△) from 25 μM-coproporphyrinogen III (*b*). Porphyrins were isolated as their methyl ester derivatives as described in the Experimental section. Means of duplicate measurements are shown.

This experiment provides independent support for the chemical evidence that the 2-propionate group of coproporphyrinogen III is decarboxylated first. Thus the existence of a rate difference (Fig. 3*a*) is incompatible with decarboxylation of either group at random, whereas the more rapid decarboxylation of the 2-propionate group is consistent with the isolation of only harderoporphyrin from the reaction mixture. If, as argued above, decarboxylation of the 2-propionate group must precede decarboxylation of the 4-propionate group, it follows that the rate of formation of $^{14}CO_2$ from the 4-^{14}C-labelled substrate measures the rate of protoporphyrinogen formation, whereas $^{14}CO_2$ production from [2-^{14}C]coproporphyrinogen III reflects the formation of a 4-propionate-substituted tripropionate porphyrinogen, some of which is then converted into protoporphyrinogen. The difference between the rates will then represent the progressive formation of a tripropionate porphyrinogen as an additional reaction product. If this compound is harderoporphyrinogen, its accumulation can be explained on the assumption that it is in equilibrium with coproporphyrinogen III which, being present in relative excess, effectively prevents its conversion into protoporphyrinogen IX by competitive inhibition.

This interpretation of the experiment with ^{14}C-labelled substrates is supported by the finding that the formation of both harderoporphyrin and protoporphyrin is linear with time under similar conditions (Fig. 3*b*). However, the rate of harderoporphyrin formation (Fig. 3*b*), relative to the rate of protoporphyrin formation, is lower than that calculated for the tripropionate product from the $^{14}CO_2$ production rates (Fig. 3*a*). This difference is unexplained, but may be due to the technical difficulty of obtaining high recoveries of harderoporphyrin. There is some evidence for the formation of protein-bound porphyrinogen intermediates during this reaction, which may not be extracted by the usual procedures (Porra & Falk, 1961).

The rate of formation of protoporphyrinogen IX remains constant as the concentration of harderoporphyrinogen increases (Fig. 3). This observation suggests that the conversion of coproporphyrinogen III into protoporphyrinogen IX does not take place at separate active centres with the formation of a single pool of harderoporphyrinogen as the intermediate. For, if this was so, either a progressive increase in the rate of formation of the end product or attainment of a steady-state concentration of the intermediate would have been observed. The results in Fig. 3 are also incompatible with decarboxylation of both groups at a single active centre if coproporphyrinogen III, and all the harderoporphyrinogen formed from it,

is able to mix freely. In this case, while coproporphyrinogen III remained in excess, little protoporphyrinogen would have been formed because of competition between two substrates for which coproporphyrinogen oxidase has similar affinities (Elder & Evans, 1977).

Effect of harderoporphyrinogen on the rate of $^{14}CO_2$ formation from [4-^{14}C]coproporphyrinogen III and [2,4-^{14}C]coproporphyrinogen III. Fig. 4 shows the effect of approximately equimolar concentrations of harderoporphyrinogen on the rate of $^{14}CO_2$ release from [2,4-^{14}C]coproporphyrinogen III and [4-^{14}C]-coproporphyrinogen III. The difference between the rates for the two [^{14}C]coproporphyrinogens gives the rate of decarboxylation of the 2-propionate group (Table 3). Harderoporphyrinogen has an equal effect on the rate of release of $^{14}CO_2$ from both substrates and therefore from both propionate substituents, decreasing the rate by 25% in each case. If harderoporphyrinogen had equilibrated with the ^{14}C-labelled 4-propionate-substituted tripropionate porphyrinogen destined to become protoporphyrinogen IX, it would have greatly decreased its specific radioactivity, and so the rate of $^{14}CO_2$ production from the 4-propionate group would have been much lower. A similar result was obtained when harderoporphyrinogen was replaced by isoharderoporphyrinogen, the decrease in $^{14}CO_2$ production from each substrate being approx. 12%.

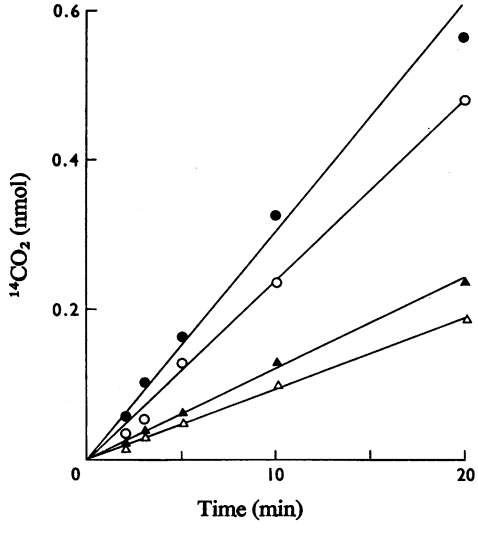

Fig. 4. *Effect of harderoporphyrinogen on the release of $^{14}CO_2$ from [^{14}C]coproporphyrinogens III*

The formation of $^{14}CO_2$ from [2,4-^{14}C]coproporphyrinogen III (20μM) (●, ○) and from [4-^{14}C]-coproporphyrinogen III (20μM) (▲, △) was measured in the absence (●, ▲) and presence (○, △) of harderoporphyrinogen (24μM) as described in the Experimental section.

These results are compatible with the deductions made from the time-course measurements and indicate that either the fraction of harderoporphyrinogen that undergoes decarboxylation to protoporphyrinogen IX does not equilibrate with added harderoporphyrinogen or harderoporphyrinogen is not the true intermediate in the reaction sequence but a side product formed from it. Studies of the mechanism of formation of the vinyl groups of protoporphyrinogen IX support the first of these alternatives. Thus stereospecific loss of a hydrogen atom from the β-methylene group of the propionate side chain and decarboxylation is believed to occur simultaneously (Zaman *et al.*, 1972; Battersby *et al.*, 1972; Jackson *et al.*, 1974). Since the 2-propionate substituent is decarboxylated before the 4-propionate, this type of mechanism must lead to formation of harderoporphyrinogen as the intermediate.

Dependence of the rate of decarboxylation of both the 2- and 4-propionate groups on the affinity of the enzyme for coproporphyrinogen III. Fig. 4 shows that the ratio of the rates of formation of $^{14}CO_2$ from the 2- and 4-propionate groups is not changed by the addition of an alternative substrate for coproporphyrinogen oxidase to the incubation medium. This ratio also remained constant when the concentration of the two [$^{14}C_1$]coproporphyrinogens was varied from 0.5 to 22.0μM, giving K_m values for the two compounds that are in reasonable agreement both with each other and with that measured previously for

Table 3. *Kinetic constants for [^{14}C]coproporphyrinogens III*

Initial rates were measured by determining the rate of $^{14}CO_2$ production over periods of 5–30min from the start of the reaction. Incubation conditions are described in the Experimental section. Rat liver homogenate (0.05g wet wt./ml) was used as enzyme. K_i values for coproporphyrin III were determined from plots of $1/v$ against coproporphyrin III concentration (three concentrations in the range 8–24μM) at two concentrations of [^{14}C]coproporphyrinogen III (5 and 20μM). Results for [2,4-^{14}C]coproporphyrinogen III are from Elder & Evans (1978).

Substrate	K_m (μM)	$V_{max.}$ (nmol of CO_2/min per g wet wt.)	K_i (μM)
[2-^{14}C]Coproporphyrinogen III	1.4	4.7	8.5
[4-^{14}C]Coproporphyrinogen III	2.0	2.5	9.5
[2,4-^{14}C]Coproporphyrinogen III	1.2	6.5	7.6

[2,4-^{14}C]coproporphyrinogen III (Table 3). Coproporphyrin III, a competitive inhibitor of coproporphyrinogen oxidase (Elder & Evans, 1978), also affected both rates equally (Table 3). These results show that the overall rate of protoporphyrinogen formation is determined by the binding of coproporphyrinogen III to the enzyme and suggest that decarboxylation of the 2-propionate group initiates a reaction sequence which, when coproporphyrinogen is present in excess, leads to the formation of harderoporphyrinogen and protoporphyrinogen in predetermined proportions.

Conclusion: decarboxylation of both propionate groups at the same active centre

The results discussed above indicate that the sequential decarboxylation of coproporphyrinogen III has three main characteristics. First, protoporphyrinogen IX is formed exclusively via harderoporphyrinogen. Secondly, under conditions of substrate excess such that initial rates are measured, more harderoporphyrinogen is formed than protoporphyrinogen, and the fraction of harderoporphyrinogen destined to form protoporphyrinogen is not in equilibrium with added harderoporphyrinogen. Thirdly, under the same conditions, protoporphyrinogen and harderoporphyrinogen are formed in a constant proportion at rates that are determined by binding of coproporphyrinogen III to the enzyme.

Although the experiments described here do not show conclusively that coproporphyrinogen oxidase contains a single type of active centre, they are compatible with the following mechanism whereby both decarboxylations take place at the same active centre, which becomes temporarily inaccessible to added harderoporphyrinogen, and to coproporphyrinogen III, because it is occupied by harderoporphyrinogen in the process of transformation to protoporphyrinogen IX. The reaction starts when the near-planar coproporphyrinogen III molecule enters a cleft on the enzyme surface that contains the active centre, the configuration of which is such that it only accepts the 2-propionate substituent. Decarboxylation of this polar group to a hydrophobic vinyl group with dissociation from the active centre is followed by a rapid anti-clockwise rotation of the substrate so that the 4-propionate group enters the active centre. The substrate does not leave the enzyme cleft during this change in position, which is directed by interaction between polar and non-polar groups on the substrate and enzyme surface. During this stage some loss of harderoporphyrinogen from the cleft is likely to occur, particularly when an excess of coproporphyrinogen is present and able to replace any intermediate that moves too far from the active centre. Harderoporphyrinogen lost in this way will accumulate while concentrations of coproporphyrinogen remain high and effectively compete with it for uptake by the enzyme. Finally, the 4-propionate group is decarboxylated and the product, which no longer contains a propionate group susceptible to attack, leaves the enzyme surface. Some support for this mechanism is provided by the observation that more tricarboxylate intermediate is formed with coproporphyrinogen IV as substrate than with coproporphyrinogen III (Frydman & Frydman, 1975; Al-Hazimi *et al.*, 1976; Mombelli *et al.*, 1976), for here the arrangement of the side chains does not allow a simple rotation to bring the second propionate group into the active centre (Table 1).

A mechanism similar to the one described here may also determine the ordered sequential decarboxylation of the acetate groups of uroporphyrinogen III (Jackson *et al.*, 1976*b*) for, here again, intermediates accumulate when substrate is present in an excess (Kushner *et al.*, 1975). Sequential reactions taking place in this way at a single active centre are likely to be least efficient in terms of end-product formation when the concentration of initial substrate is high relative to the concentration of enzyme. *In vivo*, where substrate concentrations are normally low in relation to the amount of enzyme, little or no accumulation of intermediates, which are also substrates for the enzyme, would be expected. However, this would not necessarily be so when substrate concentrations increase in the presence of enzyme defects. For example, the activity of hepatic uroporphyrinogen decarboxylase is decreased in porphyria cutanea tarda, a condition that is characterized by massive overproduction of the initial substrate and intermediates of the reaction catalysed by this enzyme (Kushner *et al.*, 1976).

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