Investigations of rat liver uroporphyrinogen decarboxylase

Comparisons of porphyrinogens I and III as substrates and the inhibition by porphyrins

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1. The decarboxylations of uroporphyrinogens, hepta-, hexa- and penta-carboxy-porphyrinogens I and III by porphyrinogen carboxy-lyase (EC 4.1.1.37) in rat liver supernatant have been compared as functions of substrate concentrations. Although K_m and V_{max} (for total porphyrinogens formed) were estimated, porphyrinogens and CO_2 produced at $1\mu M$ were considered to be a better indication of real relative rates, owing to substrate/product inhibitions. Uroporphyrinogen III was the best substrate by the criteria of K_m/V_{max} and decarboxylation at $1\mu M$ and was converted into coproporphyrinogen more quickly than its series-I isomer. 2. The difference between uroporphyrinogens I and III as substrates was confirmed by using a mixture of [$^{14}C_8$]uroporphyrinogens, the discrimination occurring principally in the first decarboxylation. 3. Porphyrins, especially oxidation products of the substrates, inhibited the enzyme. Heptacarboxyporphyrin III was the most effective inhibitor of both uroporphyrinogen III and heptacarboxyporphyrinogen III conversion into coproporphyrinogen. 4. Rapid analysis of the livers from rats made porphyric with hexachlorobenzene demonstrated that substantial quantities of the tetrapyrroles were present *in vivo* as the porphyrinogens (21–42%). 5. Enzymic decarboxylation of uroporphyrinogen III in 2H_2O -containing buffer gave [2H_4]coproporphyrinogen. 6. Rats treated with cycloheximide for 10h showed no decrease in uroporphyrinogen decarboxylase activity/mg of protein, suggesting a relatively slow turnover of the enzyme.

Uroporphyrinogen decarboxylase (porphyrinogen carboxy-lyase, EC 4.1.1.37) converts uroporphyrinogen into coproporphyrinogen via hepta-, hexa- and penta-carboxyporphyrinogens (Mauzerall & Granick, 1958). Jackson *et al.* (1976a, 1977) showed that the decarboxylations of the acetate side chains occur randomly with uroporphyrinogen I but in a clockwise manner with uroporphyrinogen III (Schemes 1 and 2). The relative rates of decarboxylation of these isomers have been investigated by a number of workers with conflicting results using erythrocytes (Mauzerall & Granick, 1958; Cornford, 1964; Rasmussen & Kushner, 1979; de Verneuil *et al.*, 1980), mouse spleen (Romeo & Levin, 1971) and pig liver (Kushner *et al.*, 1975). Previously, using rat liver (Smith & Francis, 1979), we explored the accumulation of products during the decarboxylation of uroporphyrinogens I and III; although uroporphyrinogen III was converted into coproporphyrinogen a little faster than isomer I, heptacarboxyporphyrinogen

accumulated much more with isomer III. These results, with other evidence, confirmed that the first decarboxylation of uroporphyrinogen III is faster than the second (Garcia *et al.*, 1973) and suggested that this might be due to the inverted position of the carboxy group in ring D.

We have now examined the decarboxylations in detail using all of the naturally expected substrate porphyrinogens. Uroporphyrinogen III was a better substrate than isomer I as judged by both coproporphyrinogen and total porphyrinogen formation. Product inhibition and the relative abilities of the porphyrin analogues of the substrates to inhibit uroporphyrinogen decarboxylase have also been investigated. These effects are of importance in the elucidation of the mode of action of the enzyme and its activity during the development of hexachlorobenzene-induced porphyria (Elder, 1978). In the latter context we have shown that besides porphyrins the livers of porphyric rats contain substantial quantities of porphyrinogens.

Scheme 1. *Decarboxylation sequence in the conversion of uroporphyrinogen I (a) into coproporphyrinogen I (e)*
Ac, Acetate; Pr, propionate; Me, methyl. Step (4) is slow.

Scheme 2. *Probable decarboxylation sequence in the conversion of uroporphyrinogen III (g) into coproporphyrinogen III (k)*
Ac, Acetate; Pr, propionate; Me, methyl. Step (1) is fast.

Experimental

Animals

Female AGUS rats (150–200g) were killed by decapitation and male LACA mice (30–35g) were killed by cervical dislocation and then bled. Animals were bred in the M.R.C. Toxicology Unit. Rats fed hexachlorobenzene received it mixed in a 41B diet containing 2% arachis oil.

Rat liver supernatant

Livers were homogenized in 0.1M- Na_2HPO_4 / NaH_2PO_4 buffer (pH 6.8)/0.1mM-EDTA and centrifuged at 40000g for 30min at 4°C. The supernatant contained about 21mg of protein/ml [measured by the method of Lowry *et al.* (1951) with bovine serum albumin as a standard] and was used as the source of enzyme for the experiments *in vitro*. It could be frozen in liquid N_2 and stored at -70°C until required without loss of uroporphyrinogen decarboxylase activity. Supernatant of mouse spleen was similarly prepared (17.8 mg of protein/ml).

Porphyrins

Uroporphyrins I and III (>90% III) and hepta- and penta-carboxyporphyrins I were purchased from Porphyrin Products (Logan, UT, U.S.A.). Hepta-, hexa- and penta-carboxyporphyrins III were isolated from the livers and faeces of rats that had been treated with hexachlorobenzene (San Martin de Viale *et al.*, 1970; Jackson *et al.*, 1976a). The proportions of isomers I and III in these isolated porphyrins were determined by decarboxylation in 1M-HCl under vacuum at 190°C (Edmondson & Schwartz, 1953) and the isomeric compositions of the resulting coproporphyrins were examined by t.l.c. on silica gel developed with 2,6-lutidine/water (10:3, v/v) in an atmosphere of NH_3 (Jensen, 1963). The coproporphyrin isomers were eluted with 1M-HCl/methanol (1:1, v/v) and the concentrations were estimated by the absorption at the Soret peak (Falk, 1964). The hepta- and hexa-carboxyporphyrins III contained only trace amounts of the I-series isomers, but the pentacarboxyporphyrin III from faeces contained some isomer I (11%). A small amount of a mixture of the two isomers of hexacarboxyporphyrin I was prepared by chemical decarboxylation of uroporphyrin I at 150°C in 1M-HCl. Synthetic pentacarboxyporphyrin III (*abd*) (j, Scheme 2) was a gift from Professor A. H. Jackson, Department of Chemistry, University College, Cardiff, Wales, U.K. (see Jackson *et al.*, 1976a). Free porphyrins were obtained from the methyl esters by hydrolysis in 5M-HCl for 1–2 days.

[^{14}C]₈Uroporphyrin labelled at positions 3 and 5 of each pyrrole ring (Shemin, 1955) was formed by incubating rat liver homogenate (from porphyric animals fed 0.01% hexachlorobenzene in their diet

for 6 months) with 5-amino[4- ^{14}C]laevulinate (sp. radioactivity 50Ci/mol; The Radiochemical Centre, Amersham, Bucks., U.K.) as described previously (Smith *et al.*, 1979). The radioactivity contents of samples were determined in Insta-Gel (10ml) (Packard Instrument Co. Inc.) using a model 6880 Searle Analytic Inc. MARK III liquid-scintillation system. Chemical decarboxylation of the [^{14}C]-uroporphyrin and separation by t.l.c. of the resulting coproporphyrin I and III isomers showed that the incorporations of radioactivity did not correspond to the proportions of the isomers as determined by spectrophotometry. Isomer I was of a much higher specific radioactivity than isomer III (Table 2).

Preparation of porphyrinogen substrates

Solutions of porphyrins (50–150 μl ; 0.7–0.8 nmol/ μl in 5mM-NaOH) were reduced with 5% sodium amalgam until no fluorescence could be observed. The supernatants were then neutralized with 5M- H_3PO_4 (1–5 μl) and diluted with 0.1M- Na_2HPO_4 / NaH_2PO_4 buffer (pH 6.8)/0.1mM-EDTA/0.1M-sodium mercaptoacetate to 0.3–0.4 nmol/ μl . Porphyrinogen concentrations were calculated from the initial porphyrins using ϵ values in 0.5M-HCl (Falk, 1964; Smith & Francis, 1979) and assuming a 100% yield of porphyrinogen. Re-oxidation of uroporphyrinogens I and III and pentacarboxyporphyrinogen III in 0.5M-HCl in the light gave 81%, 82% and 84% recoveries of porphyrin respectively.

Assay of uroporphyrinogen decarboxylase

This was performed as described previously (Smith & Francis, 1979) with 0.2ml of rat liver supernatant (4.2 ± 0.1 mg of protein; mean $\pm$ S.D. for six preparations) per incubation in a total volume of 1ml of 0.1M- Na_2HPO_4 / NaH_2PO_4 buffer (pH 6.8; Mauzerall & Granick, 1958)/0.1mM-EDTA, containing 3mM-sodium mercaptoacetate. Incubations were for 15min at 37°C in the dark under N_2 . The absence of mercaptoacetate in the system slightly decreased the yield of products. Appropriate carriers, controls and reagent blanks were included in the procedure. The activity of the enzyme was similar in all of the rats used. With uroporphyrinogen and pentacarboxyporphyrinogen III as substrates recoveries of 75% and 81% respectively were estimated for total methylated porphyrins, based on the original porphyrin concentrations before reduction. Formation of coproporphyrinogen from the pentacarboxy substrate was linear over the protein concentration range studied (0.4–5.3 mg/ml).

Decarboxylation in $^2\text{H}_2\text{O}$ -containing buffer

Uroporphyrinogen III (12nmol) was incubated for 2h with 50 μl of a reconstituted 30–40%-satd.-(NH_4)₂ SO_4 fraction of liver homogenate (3-

fold increase in activity/mg of protein over the homogenate) and buffer (0.95 ml) prepared with $^2\text{H}_2\text{O}$ (Aldrich Chemical Co., Gillingham, Dorset, U.K.). The electron-impact mass spectrum (70 eV) of the isolated coproporphyrin tetramethyl ester was determined with a VG Micromass 70/70 instrument by Dr. P. B. Farmer.

Effect of cycloheximide

Rats (88–106 g fed body wt.) were starved overnight and then injected intraperitoneally with cycloheximide (Sigma Chemical Co.) (40 mg/kg body wt.) in saline (20 mg/ml of saline) or saline (0.9% NaCl) alone. Some control animals were killed immediately and the remaining rats after 10 h. The uroporphyrinogen decarboxylase activities of the liver homogenates were then estimated with uroporphyrinogen III as the substrate; the activity was expressed as pmol of coproporphyrinogen produced/min per mg of protein.

Determination of porphyrinogens and porphyrins in the livers of porphyric rats

Rats (35–45 days old) were fed hexachlorobenzene (Organic Analytical Grade; BDH Chemicals, Poole, Dorset, U.K.) in their diet at a concentration of 0.02% for 75 days. After decapitation of an animal the liver was removed and homogenized at 4°C under dim light in 0.1 M- $\text{Na}_2\text{HPO}_4/\text{NaH}_2\text{PO}_4$ buffer (pH 7.4)/50 mM-sodium mercaptoacetate that had been deoxygenated by bubbling with N_2 . A sample of the homogenate (50 μl) was then mixed with 3 ml of 0.5 M-HCl at 4°C and the absorption spectrum between 370 and 420 nm was determined. The time taken from decapitation to spectral analysis was <2.5 min. The concentration of porphyrins, estimated as uroporphyrin, was calculated from the Soret peak (Falk, 1964). A further sample of the homogenate was shaken vigorously with a small crystal of iodine to oxidize porphyrinogens. After dilution with 0.5 M-HCl as before the new maximum absorption was used to calculate total porphyrin. The concentration of porphyrinogen was given by the difference between the two estimations. The reference cell contained supernatant from a control liver treated in an identical manner. Spectrofluorimetry (Granick *et al.*, 1975) was used to confirm total porphyrin concentrations.

Results

Comparison of porphyrinogens as substrates

Four intermediate porphyrinogens are possible in the decarboxylation of uroporphyrinogen I (a) to coproporphyrinogen I (f), i.e. a heptacarboxy- (b), two hexacarboxy- (c and d) and a pentacarboxy-porphyrinogen (e) (Scheme 1) (Jackson *et al.*, 1977).

The conversion of uroporphyrinogen III (g) into coproporphyrinogen III (k) probably occurs via the intermediate heptacarboxy- (h), hexacarboxy- (i) and pentacarboxy-porphyrinogens (j), as illustrated in Scheme 2 (Jackson *et al.*, 1976a). The decarboxylations of these porphyrinogens using rat liver supernatant were examined and it was evident that the kinetics of the enzyme action are complex. From preliminary experiments using <4 nmol of octa-, hepta-, hexa- and penta-carboxyporphyrinogens the loss of substrate appeared to be linear up to 15 min. The formation of coproporphyrinogen and total porphyrinogens as functions of substrate concentrations are shown in Figs. 1 and 2 (with the exception of the hexacarboxyporphyrinogen I mixture). Accumulations of heptacarboxyporphyrinogens with uroporphyrinogens as substrates are also illustrated. Graphical representations of the reaction rates are one of the best ways of gaining impressions of the relative rates of the decarboxylation steps. The curves contain some inhibition components, but despite this, estimates of K_m and V_{max} for total porphyrinogen formation were obtained by plots of $[S]$ against $[S]/v$ (Table 1). Experimental values gave good agreement with linear plots except at high concentrations where some deviations from linearity occurred due to substrate and product inhibitions. These were disregarded for the purposes of the calculations. Rates of formation of total product porphyrinogens and CO_2 at $1 \mu\text{M}$, maximum observed coproporphyrinogen production and enzyme efficiency (K_m/V_{max}) were also estimated. The apparent K_m value calculated for pentacarboxyporphyrinogen III (*abd*) was confirmed by double-reciprocal plots and a computer program for a best-fit curve to the Michaelis–Menten equation (Smith & Brooks, 1977). Experiments with liver supernatant diluted 10-fold gave an apparent K_m value of the same order for this substrate. It is obvious that uroporphyrinogen III is a better substrate than isomer I when the rates of formation of both coproporphyrinogen and total porphyrinogen products are compared, and by the criteria of reaction rate at $1 \mu\text{M}$ concentration and efficiency (K_m/V_{max}) it was the best substrate of all those examined. Comparison of the rates of reactions of the porphyrinogens also explains the pattern of the accumulations of the various intermediates in the time studies with uroporphyrinogens I and III (Smith & Francis, 1979).

Further proof of the greater ability of the enzyme to decarboxylate uroporphyrinogen III in preference to uroporphyrinogen I was obtained from experiments with [^{14}C]uroporphyrin containing a mixture of isomers I and III (3:7) with different specific radioactivities (Table 2), the minor component uroporphyrin I being more heavily radio-labelled than its isomer. Time-course studies of the

Fig. 1. Effects of substrate concentration on the decarboxylation of uroporphyrinogen I (a), uroporphyrinogen III (b), heptacarboxyporphyrinogen I (c), heptacarboxyporphyrinogen III (d) and hexacarboxyporphyrinogen III (e) to coproporphyrinogen (●) and total porphyrinogens (O)

Conditions were as described in the Experimental section. Total porphyrinogens refers to the sum of coproporphyrinogen and intermediate porphyrinogens formed from the substrates. Points represent single assays in one experiment. Experiments were conducted two to four times to confirm results. ▲, Accumulation of heptacarboxyporphyrinogen.

enzymic decarboxylation of the ^{14}C -labelled substrate gave initial rapid increases in the specific radioactivity of the remaining uroporphyrinogen, whereas those of the heptacarboxyporphyrinogen and coproporphyrinogen formed were similar to that of the coproporphyrinogen III prepared chemically (Table 2), although eventually they increased to nearly the original specific radioactivity of the

^{14}C uroporphyrin. In addition, the uroporphyrin III isolated from porphyrin rat livers and the heptacarboxyporphyrin product obtained after a short incubation showed different ratios of the I and III isomers (as coproporphyrins after chemical decarboxylation): uroporphyrin I/uroporphyrin III, 32:68; heptacarboxyporphyrin, no isomer I was detected. These experiments demonstrated that from

Fig. 2. Effect of substrate concentration on the decarboxylation of pentacarboxyporphyrinogen I (○), synthetic pentacarboxyporphyrinogen III (●) and pentacarboxyporphyrinogen III isolated from porphyrin rat faeces (■) to coproporphyrinogen

A mixture of the isomers (I/III, 1:4) gave similar results to those for the faecal pentacarboxy substrate. Conditions were as described in the Experimental section. Points are single values in one experiment. Experiments were repeated one to three times.

a mixture of uroporphyrinogens, isomer III is preferentially converted into coproporphyrinogen and that the discrimination against isomer I occurs mainly on the first step.

Uroporphyrinogen decarboxylase in mouse spleen

Romeo & Levin (1971), employing enzymically generated substrates formed by incubating porphobilinogen with uroporphyrinogen I synthetase with or without uroporphyrinogen III cosynthetase, reported that uroporphyrinogens I and III were converted into coproporphyrinogen at the same rates by mouse spleen. However, in our assay system isomer III was converted into both coproporphyrinogen and total porphyrinogens more quickly than isomer I as observed with rat liver, i.e. mouse spleen: total porphyrinogens (I/III 23.0:37.5); coproporphyrinogens (I/III, 8.4:9.4); cf. rat liver: total porphyrinogens (I/III, 15.5:26.5); coproporphyrinogens (I/III, 5.6:7.4) (values are in pmol/min per mg of 40 000 g supernatant protein, each value being a mean for duplicate determinations).

Inhibition by porphyrins

All the porphyrins that were investigated (includ-

ing protoporphyrin IX and mesoporphyrin IX) inhibited the conversion of uroporphyrinogen III into coproporphyrinogen, but to greatly differing extents, and the relative abilities of uroporphyrins and heptacarboxyporphyrins I and III are illustrated in Fig. 3. Although there were slight differences between the uroporphyrins and heptacarboxyporphyrin I, much greater inhibition was observed with heptacarboxyporphyrin III. With heptacarboxyporphyrinogen III as substrate, the porphyrin analogue was again the best inhibitor, when compared with uroporphyrin III and the hexacarboxyporphyrin III (Fig. 4). These results suggest that the decarboxylation sequence is particularly sensitive to the effects of heptacarboxyporphyrin III. In other experiments uroporphyrin III caused some inhibition of the decarboxylation of pentacarboxyporphyrinogen III. Pre-incubation of the liver supernatant with heptacarboxyporphyrin III for 10 min at 37°C before incubation caused no greater effect than when the porphyrin was added with the substrate. Preliminary experiments have illustrated that the nature of the inhibition is complex and it requires further investigation.

Porphyrinogens in porphyrin rat liver

During studies of porphyria in the livers of rats fed hexachlorobenzene, porphyrin concentrations estimated by spectrofluorimetry were greater than expected from initial inspection of the tissue under u.v. light, implying that substantial quantities of porphyrinogens were present in the liver *in vivo*. To investigate this, porphyrin rats were killed, the livers rapidly removed and homogenized in buffer under conditions designed to protect porphyrinogens from oxidation. Initial and total porphyrin concentrations were calculated from the absorption spectra in 0.5 M-HCl before and after oxidation; the differences were minimum estimates of porphyrinogen concentrations. Substantial quantities were found in all of the rats examined, i.e. porphyrinogen, 108 ± 39 nmol/g of liver; porphyrins, 249 ± 38 nmol/g of liver (means $\pm$ S.D. for five animals). Porphyrinogens ranged from 21 to 42% of the total porphyrins after oxidation.

Incorporation of ^2H from $^2\text{H}_2\text{O}$

Decarboxylation of the four acetate side chains of uroporphyrinogen necessitates the incorporation of four hydrogen atoms that *in vitro* must originate from the medium if no preformed reductive cofactor is involved in the action of the enzyme. To demonstrate this, uroporphyrinogen III (12 nmol) was incubated with a liver supernatant preparation in buffer prepared with $^2\text{H}_2\text{O}$ (final concentration 94%). After isolation of the coproporphyrin (as the tetramethyl ester) and examination by electron-impact mass spectrometry, a molecular ion m/z

Table 1. *Estimates of apparent K_m and V_{max} of rat liver uroporphyrinogen decarboxylase*

Values were calculated by plotting $[S]$ against $[S]/v$ for the total porphyrinogens† formed for the experiments shown in Figs. 1 and 2. Experiments were repeated at least once giving similar results to those presented. Inhibition that was seen at higher concentrations with most of the substrates was disregarded and lines were drawn through the linear portions of the plots. CO_2 formation was calculated from the expected contributions for each porphyrinogen formed during carboxylation.

Porphyrinogen substrate (see Schemes 1 and 2)	Apparent K_m (μM)	V_{max} (pmol/min per mg of protein)	$10^3 \times$ K_m/V_{max}	Maximum observed coproporphyrin- ogen formation (pmol/min per mg of protein)	Rates of total product formation at 1 μM substrate concn. (pmol/min per mg of protein)	
					Porphyrinogens†	CO_2
Uroporphyrinogen I (a)	2.1	23.0	91	6.1	7.6	30.3
Heptacarboxyporphyrinogen I (b)	2.4	30.4	79	7.6	10.3	25.7
Pentacarboxyporphyrinogen I (c)*	—	—	—	8.4	6.5	6.5
Uroporphyrinogen III (g)	2.7	46.6	58	9.7	12.9	44.0
Heptacarboxyporphyrinogen III (h)	1.9	25.6	74	10.9	8.7	18.7
Hexacarboxyporphyrinogen III (i)	7.8	60.4	129	22.8	7.0	12.5
Pentacarboxyporphyrinogen III (j)	14.1	124.3	113	59.4	8.3	8.3

* Apparent K_m and V_{max} values could not be obtained from the information for pentacarboxyporphyrinogen I because maximum activity was achieved at too low a substrate concentration for enough accurate measurements; an estimate of the rate at 1 μM was determined from Fig. 2. Preliminary experiments with the mixture of hexacarboxyporphyrinogens I gave an apparent K_m of 5 μM .

† Total porphyrinogens refers to the sum of coproporphyrinogen and any intermediate porphyrinogens formed from the substrate.

Table 2. *Specific radioactivities of [$^{14}C_8$]uroporphyrin and its chemical and enzymic decarboxylation products*

[$^{14}C_8$]Uroporphyrin was formed by incubating porphyrin rat liver with 5-amino[4- ^{14}C]laevulinic acid (Smith *et al.*, 1979). Chemical decarboxylation was carried out in 1M-HCl under vacuum at 190°C (Edmondson & Schwartz, 1953) and the resulting coproporphyrin methylated and purified by t.l.c. on silica gel (chloroform/kerosene/methanol, 20:10:1, by vol.). The isomeric coproporphyrins were separated as the free porphyrins by t.l.c. on silica gel eluting with 2,6-lutidine/water (10:3, v/v) in an atmosphere of NH_3 (Jensen, 1963). Enzymic decarboxylation was performed with 5 nmol of uroporphyrinogen and 0.5 ml of liver supernatant for 30 min as described for assaying the enzyme (Smith & Francis, 1979). Specific radioactivities were calculated using the free porphyrins or their methyl esters [ϵ values of Falk (1964) and Dowdle *et al.* (1970) respectively].

	Specific radioactivity (d.p.m./nmol)
Uroporphyrin	10367
Chemical decarboxylation	
Coproporphyrin	10549
Separated into isomer I	24590
III	5908
Enzymic decarboxylation	
Uroporphyrin remaining	19144
Heptacarboxyporphyrin	5937
Coproporphyrin	5877

714.2 was observed as opposed to m/z 710.3 for a control incubation, indicating the incorporation of four 2H atoms during the decarboxylation sequence. No incorporation of 2H was observed when coproporphyrinogen was incubated under the same conditions.

Effect of cycloheximide on uroporphyrinogen decarboxylase in vivo

To gain some information on the turnover of the enzyme *in vivo* young female rats were injected with cycloheximide (40 mg/kg body wt.) to inhibit protein synthesis. Uroporphyrinogen decarboxylase was assayed in homogenates of the liver at zero time in control animals and also in control and test rats after 10 h (Table 3). Some variation was seen when based on wet weight of tissue but none when related to protein concentration. Experiments for longer periods were not possible owing to the toxicity of cycloheximide.

Discussion

In the present work we examined the rates of decarboxylation in rat liver of both naturally occurring uroporphyrinogens, and the presumed intermediates between them and the coproporphyrinogens (Jackson *et al.*, 1976a, 1977). From these and previous results (Smith & Francis, 1979), and those of Cornford (1964) and de Verneuil *et al.*

Fig. 3. Inhibition of the formation of coproporphyrinogen III from uroporphyrinogen III (4 nmol) by uroporphyrin III (●), uroporphyrin I (○), heptacarboxyporphyrin I (■) and heptacarboxyporphyrin III (□). Incubation conditions (15 min) were as described in the Experimental section. Porphyrins were added dissolved in 5 mM-NaOH (0.7–0.8 nmol/μl at approx. pH 7). The control value is the mean of four determinations that varied less than $\pm 5\%$.

(1980) with human erythrocytes, there is now little doubt that uroporphyrinogen I is more slowly decarboxylated than its series-III isomer, both to total products and to coproporphyrinogen (Fig. 1, Tables 1 and 2). Although the difference in coproporphyrinogen formation was first shown by Mauzerall & Granick (1958) with rabbit erythrocytes, other workers using mouse spleen, pig liver and human erythrocyte preparations (Romeo & Levin, 1971; Kushner *et al.*, 1975; Rasmussen & Kushner, 1979) found no differences in the rates of decarboxylation. The discrimination between uroporphyrinogen I (a, Scheme 1) and isomer III (g, Scheme 2) in conversion into coproporphyrinogen occurs principally at the first step (Table 1), as is proven by the experiments with [^{14}C]uroporphyrinogen (Table 2), but there is also a contribution from the slow decarboxylation of pentacarboxyporphyrinogen I (Fig. 2, Table 1). The radiochemical experiments additionally demonstrate that the selection of uroporphyrinogen III also occurs from a mixture with its isomer, and illustrate that biosynthetically radiolabelled uroporphyrin isomers are not necessarily of the same specific radioactivity. The apparent K_m values estimated for the various substrates are all higher than those reported by de Verneuil *et al.* (1980) with human erythrocyte haemolysate and we have no evidence that the liver enzyme has a greater affinity for isomer-III porphyrinogens than the isomer-I series. A marked increase in apparent K_m was seen in the series heptacarboxy- (h), hexacarboxy- (i) and

Table 3. Hepatic uroporphyrinogen decarboxylase activity after cycloheximide treatment

Young female rats were starved overnight and injected intraperitoneally with cycloheximide (40 mg/kg body wt.) in saline (20 mg/ml of saline). Controls received saline alone. Liver uroporphyrinogen decarboxylase activity was measured as described in the Experimental section and is expressed as coproporphyrinogen formed (pmol) from uroporphyrinogen III/min per mg of liver homogenate protein. Values are means for three animals $\pm$ S.D. There were no significant differences between groups, as assessed by Student's *t* test, of the activities/mg of protein.

Treatment	Time (h)	Uroporphyrinogen decarboxylase	
		(pmol/min per g of liver)	(pmol/min per mg of protein)
Saline	0	724 $\pm$ 22	3.62 $\pm$ 0.33
Saline	10	832 $\pm$ 52	3.81 $\pm$ 0.38
Cycloheximide	10	691 $\pm$ 90	3.70 $\pm$ 0.65

Fig. 4. Inhibition of the formation of coproporphyrinogen III from heptacarboxyporphyrinogen III (4 nmol) by uroporphyrin III (●), heptacarboxyporphyrin III (□) and hexacarboxyporphyrin III (Δ).

Incubation (15 min) conditions were as described in the Experimental section. The uninhibited value is the mean for four determinations that varied less than $\pm 8\%$.

pentacarboxy-porphyrinogens III (j) (Scheme 2) and may possibly also exist in series I. However, although there seems to be a decrease in the affinity of the enzyme for the substrate with decarboxylation, owing to the complexity of the kinetics and likely differences in the lipophilicity of the substrates, deductions from the apparent K_m values could be misleading. The relative V_{max} values between substrates in our experiments were similar to those reported for human erythrocytes (de Verneuil *et al.*, 1980), i.e. 5 (III) > (III) > 8 (III) > 7 (I) > 7 (III) > 8 (I) > 5 (I) (number of carboxy groups and isomer series), but because of product inhibition these values do not represent the real maximum relative rates of decarboxylation *in vivo*. In fact uroporphyrinogen III was the most efficient substrate when based on the criteria of K_m/V_{max} and rates of total porphyrinogen and CO_2 formation at $1\mu M$ concentration (Table 1). The rates of reaction at $1\mu M$ and the maximum achievable velocities (Figs. 1 and 2) explain the accumulations of each porphyrinogen during incubations (Aragones *et al.*, 1972; Garcia *et al.*, 1973; Smith & Francis, 1979) and the occurrence of heptacarboxyporphyrin III in hexachlorobenzene-induced porphyria (San Martin de Viale *et al.*, 1977).

The kinetic data for the removal of carboxy groups from porphyrinogens by uroporphyrinogen decarboxylase have not revealed how the reactions occur. For instance, whether there is one central binding site with several catalytic sites around it (Jackson *et al.*, 1976a) or separate sites for each substrate depending on the number of acetate groups in the molecule as suggested by the experiments of de Verneuil *et al.* (1980). We have shown here that the porphyrin analogues of the porphyrinogens inhibit the enzyme but to differing degrees, heptacarboxyporphyrin III being a particularly effective inhibitor of the conversion of uroporphyrinogen III and heptacarboxyporphyrinogen III into coproporphyrinogen (Figs. 3 and 4). This is similar to the inhibition by the analogous porphyrinogen (Garcia *et al.*, 1973; de Verneuil *et al.*, 1980), i.e. 5 (III) > 6 (III) > 8 (III) > 7 centration curves of uroporphyrinogen III (Fig. 1b) in which the sharp decrease in coproporphyrinogen formation is concomitant with the accumulation of the heptacarboxy-intermediate. Possibly the effects of the porphyrins can be used as a tool to investigate the nature of the binding of substrates by the enzyme. The fast initial decarboxylation of uroporphyrinogen III compared with the later eliminations and that of uroporphyrinogen I (Table 1) may be due to the inverted position of the acetate side chain of ring D, which is decarboxylated first (Battersby *et al.*, 1974; Jackson *et al.*, 1976a) and implies a distinction between this step and the others. Elder & Tovey (1977) found no separation of

enzymic activities with uroporphyrinogen and pentacarboxyporphyrinogen III as substrates during partial purification of the enzyme from human erythrocytes, but some factors, e.g. NaCl, can preferentially affect the second step of erythrocyte uroporphyrinogen decarboxylase (Cornford, 1964; Aragones *et al.*, 1972). Although the formation of the isomer heptacarboxyporphyrinogen III_d (structure h in Scheme 2) is understandable given prerecognition of the ring D acetate function, it is difficult to envisage why subsequent decarboxylations occur in a clockwise fashion (Jackson *et al.*, 1976a). Most of the possible hepta-, hexa- and penta-carboxyporphyrins III have been synthesized and, as the respective porphyrinogens, are all converted into protoporphyrin IX by chicken erythrocyte haemolysate (Jackson *et al.*, 1976b; Smith *et al.*, 1976), so that the isomers which are not found *in vivo* or *in vitro* are capable of serving as substrates but are not formed by the enzyme. An interesting observation is the poor conversion of pentacarboxyporphyrinogen III *bcd* (Smith *et al.*, 1976). The reasons for this may also explain the slow decarboxylation of pentacarboxyporphyrinogen I (Fig. 2). Barnard & Akhtar (1975, 1979) have established that during decarboxylation both protons of the methylene group in each acetate moiety remain, elimination occurring with retention of configuration. The incubations with 2H_2O demonstrate that one hydrogen is incorporated from the medium for each step but further studies are required to elucidate the precise mechanism of catalysis.

The studies with cycloheximide show that synthesis of the enzyme probably occurs slowly when compared with some other enzymes of haem biosynthesis (Tait, 1978). This may be of significance with respect to the initial lag phase observed in the decrease of uroporphyrinogen decarboxylase activity during hexachlorobenzene-induced porphyria (Elder *et al.*, 1976; Smith *et al.*, 1979). One explanation might be that it is the production of the enzyme that is inhibited but that this does not become immediately apparent due to the slow turnover of the enzyme. Further inhibition of the enzymic activity may occur by the accumulating porphyrins and the analogous porphyrinogens thus contributing to the rapid progress of the porphyria once it has commenced.

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