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Decarboxylation of Porphyrinogens by Rat Liver Uroporphyrinogen Decarboxylase

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The decarboxylations of uroporphyrinogens I and III and of heptacarboxylic, hexacarboxylic and pentacarboxylic porphyrinogens III by rat liver uroporphyrinogen decarboxylase were compared, and the results suggest that the removal of the first carboxy group from uroporphyrinogen III is a more rapid step than that from isomer I or the other substrates investigated.

Uroporphyrinogen decarboxylase (porphyrinogen carboxy-lyase, EC 4.1.1.37) catalyses the conversion of uroporphyrinogen into coproporphyrinogen through the intermediate formation of heptacarboxylic, hexacarboxylic and pentacarboxylic porphyrinogens (Mauzerall & Granick, 1958). The four decarboxylation steps occur randomly with uroporphyrinogen I (Jackson *et al.*, 1977), but in a 'clockwise' manner around the porphyrinogen (commencing with ring D) with isomer III (Jackson *et al.*, 1976). The latter is the precursor of haem. The properties of this enzyme have not been greatly investigated, mainly owing to the difficulties in measuring the activity. Most investigations have been concerned with the enzyme in avian or mammalian erythrocytes (Mauzerall & Granick, 1958; Cornford, 1964; Garcia *et al.*, 1973; Rasmussen & Kushner, 1979) or mouse spleen (Romeo & Levin, 1971); little information is available on the enzyme in liver (Aragones *et al.*, 1972; Kushner *et al.*, 1975; Elder *et al.*, 1976), which is inhibited in hexachlorobenzene-induced porphyria. Mauzell & Granick (1958) and Cornford (1964) reported that uroporphyrinogen III is decarboxylated to coproporphyrinogen faster than is isomer I, whereas Romeo & Levin (1971) found no differences between the isomers, and also Kushner *et al.* (1975) and Rasmussen & Kushner (1979) have concluded that both isomers are decarboxylated at the same rate. We have examined the decarboxylation of porphyrinogens by rat liver and found that, although the maximum rate of coproporphyrinogen formation from uroporphyrinogen III was only a little more than that with isomer I, the production of heptacarboxylic porphyrinogen and the total yield of decarboxylation products was significantly greater with isomer III. These results, together with those obtained with intermediate porphyrinogens III, lead us to believe that the removal of the acetate carboxy group from ring D of uroporphyrinogen III is a faster and possibly a separate decarboxylation step from the initial decarboxylation steps of uroporphyrinogen I and heptacarboxylic porphyrinogen. This may have important implications for the understanding of the mechanism by which the unsymmetrical physiological substrate is decarboxylated.

Experimental

Porphyrins

Uroporphyrin I was purchased from Porphyrin Products (Logan, UT, U.S.A.). Uroporphyrin and heptacarboxylic, hexacarboxylic and pentacarboxylic porphyrins III were isolated from the livers and faeces of rats that had been treated with hexachlorobenzene (Jackson *et al.*, 1976). They were identified, as the methyl esters, by t.l.c. and electron-impact mass spectrometry. Decarboxylation of the free porphyrins to coproporphyrin (With, 1975) followed by chromatography (Cornford & Benson, 1963) confirmed that they were mainly isomer III. The free porphyrins were obtained from the methyl esters by hydrolysis in 5M-HCl for 1–2 days. After desiccation they were dissolved in 5mM-NaOH (0.6–0.8nmol/μl). Concentrations of solutions of uroporphyrin and coproporphyrin were estimated by using reported ε values (Falk, 1964). ε_{mM} values for heptacarboxylic, hexacarboxylic and pentacarboxylic porphyrins in 0.5M-HCl were estimated by hydrolysing known amounts of the methyl esters (Dowdle *et al.*, 1970) and then calculating the absorption at the Soret peak of a 1mM solution: values of 525, 513 and 496 $mM^{-1} \cdot cm^{-1}$ respectively were obtained.

Rat liver supernatant

Livers from female Agus rats (150–200g) were homogenized in 0.1M-Na_2HPO_4/NaH_2PO_4 buffer (pH6.8)/0.1mM-EDTA. The homogenate was centri-

fuged at 40000*g* for 30min at 4°C, and the supernatant, containing approx. 20mg of protein/ml [measured by the method of Lowry *et al.* (1951), with bovine serum albumin as standard], was used as the source of enzyme.

Preparation of porphyrinogen substrates

Solutions of porphyrins were reduced with 5% sodium amalgam. After neutralization with 5M-H_3PO_4 they were diluted (0.3–0.4nmol/μl) with the above buffer containing 0.1M-sodium mercaptoacetate.

Assay of uroporphyrinogen decarboxylase

A modification of previously published methods (Elder *et al.*, 1976; Smith *et al.*, 1979) was used. The preincubation mixtures, in 1.7cm×12cm stoppered tubes, contained 4.1mg of liver supernatant protein and were adjusted to a final volume of 1ml with 0.1M-Na_2HPO_4/NaH_2PO_4 buffer (pH6.8)/0.1mM-EDTA/3mM-sodium mercaptoacetate. The porphyrinogen substrate (1–40μl) was added under N_2 and the tubes were incubated in the dark at 37°C. Incubations were stopped by adding acetone (6ml), and the tubes were left for 30min to allow oxidation of the porphyrinogens. Ground Zerolit FF (ip) resin (100mg; BDH Chemicals, Poole, Dorset, U.K.) was added and the tubes were shaken. The porphyrins were adsorbed on the precipitated proteins and the resin. After centrifugation at 1000*g* (for 5min) the supernatants (containing less than 1% of added porphyrin) were decanted and the pellets were vacuum-desiccated for 5min. The residues were mixed thoroughly with 2ml of 14% (w/v) BF_3 in methanol (Fisons, Loughborough, U.K.) and refluxed for 30min. Chloroform (6ml) was added to the tubes and the mixtures were suction-filtered through 2cm glass-fibre discs (Whatman, Maidstone, Kent, U.K.). Porphyrin methyl esters were partitioned into the chloroform by adding water (8ml). The chloroform layers were then washed with water (8ml), diluted with ethanol (10ml) and evaporated to dryness under vacuum. The extracts, dissolved in chloroform, were applied as 1cm bands to 0.2mm Merck Kieselgel 60 aluminium sheets (BDH Chemicals) and chromatographed for 8cm with chloroform/kerosene/methanol (20:10:1, by vol.). Porphyrins were viewed by their fluorescence under u.v. light at 366nm (R_F 0.39, 0.44, 0.49, 0.54 and 0.62 for octacarboxylic, heptacarboxylic, hexacarboxylic, pentacarboxylic and tetracarboxylic porphyrins respectively), and desired bands were cut out and eluted with 1.5ml of chloroform/methanol (9:1, v/v). The concentrations of porphyrins were measured by their absorption at 400–406nm (Dowdle *et al.*, 1970).

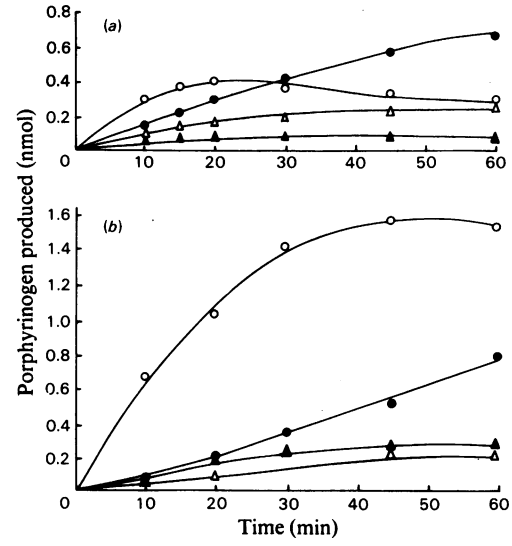

Fig. 1. *Accumulation of decarboxylation products from uroporphyrinogen I (a) and uroporphyrinogen III (b) when incubated anaerobically with rat liver supernatant*

Conditions were as described in the Experimental section, with 8nmol of substrate. ○, Heptacarboxylic porphyrinogen; ▲, hexacarboxylic porphyrinogen; △, pentacarboxylic porphyrinogen; ●, coproporphyrinogen. Product porphyrinogens were determined as the porphyrins.

Results

The decarboxylation of uroporphyrinogens I and III and the accumulation of intermediates were compared initially at a fixed substrate concentration (Fig. 1). The rates of formation of the end products, coproporphyrinogens, appeared to be similar and approximately linear over the time studied. In contrast, the first product, heptacarboxylic porphyrinogen, accumulated far more rapidly with isomer III than with isomer I. The concentrations of hexacarboxylic and pentacarboxylic porphyrinogens remained lower and more constant.

The relationships between substrate concentration and coproporphyringen production were then explored by using, not only uroporphyrinogens I and III, but also the partially decarboxylated products from the latter, i.e. heptacarboxylic, hexacarboxylic and pentacarboxylic porphyrinogens III (Figs. 2 and 3). The maximum rate of coproporphyrinogen formation was higher with uroporphyrinogen III than with uroporphyrinogen I. Inhibition at higher substrate concentrations was observed with both uroporphyrinogens, especially with isomer III. It also occurred with heptacarboxylic porphyrinogen as substrate, but not apparently with pentacarboxylic porphyrinogen. These inhibitions were not caused by oxidized

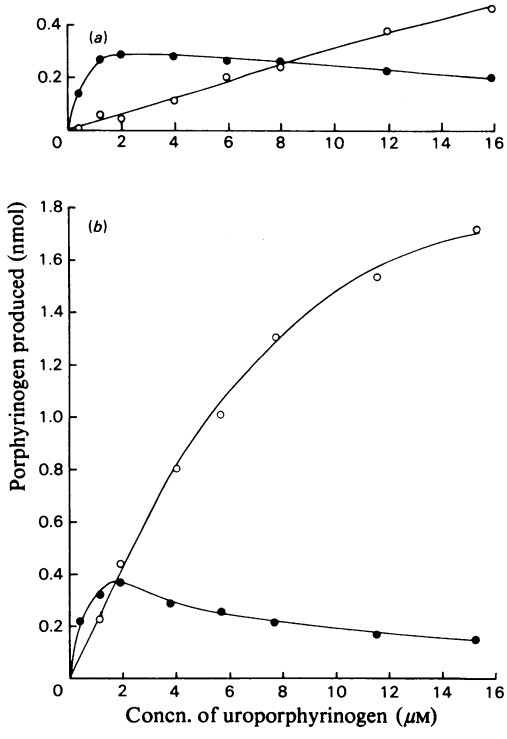

Fig. 2. *Effect of concentrations of uroporphyrinogen I (a) and uroporphyrinogen III (b) on the accumulation of heptacarboxylic porphyrinogen (○) and the production of coproporphyrinogen (●)*
Conditions were as described in the Experimental section, with an incubation time of 15 min.

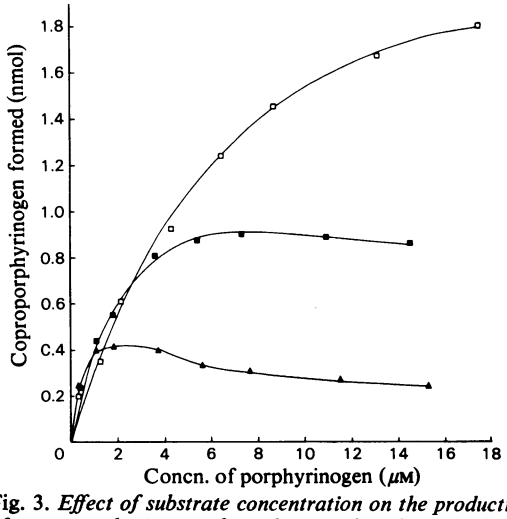

Fig. 3. *Effect of substrate concentration on the production of coproporphyrinogen from heptacarboxylic (▲), hexacarboxylic (■) and pentacarboxylic (□) porphyrinogens*
Incubations were as described in the Experimental section, for 15 min.

porphyrinogens, although many porphyrins inhibit uroporphyrinogen decarboxylase to various degrees (A. G. Smith & J. E. Francis, unpublished work), as inhibition did not increase with much longer incubation times or decrease with higher concentrations of mercaptoacetate. Increasing the concentration of uroporphyrinogen III led to a rapid accumulation of heptacarboxylic porphyrinogen, but this was not observed with isomer I (Fig. 2). Additional experiments measuring the disappearance of substrate (4μM) confirmed that uroporphyrinogen III was decarboxylated faster than was heptacarboxylic porphyrinogen III (initial rates 18.6 and 11.9pmol/min per mg of protein respectively).

The results with pentacarboxylic porphyrinogen were combined with those from a duplicate experiment and treated to give a $V_{max.}$ value (Wentworth, 1965; Smith & Brooks, 1977); estimates for the other substrates were made from the curves shown in Figs. 2 and 3, i.e. uroporphyrinogen I, 4.3, uroporphyrinogen III, 5.9, heptacarboxylic porphyrinogen, 6.5, hexacarboxylic porphyrinogen, 13.9, and pentacarboxylic porphyrinogen, 43.2 ± 1.5 (S.E.M.) pmol of coproporphyrinogen formed/min per mg of protein. An apparent K_m value of 8.1 ± 0.35 (S.E.M.)μM was calculated for the pentacarboxylic substrate and a range of about 0.5–1.5μM was estimated for the remaining porphyrinogens. The apparent K_m seemed to increase with each decrease in the number of carboxyl groups in the substrate (Figs. 2 and 3).

Our results show that the removal of the first carboxy group from uroporphyrinogen III is faster than that from isomer I or from heptacarboxylic porphyrinogen III, and, despite the difficulty in measuring accurately the apparent K_m for all the porphyrinogens, it appears that the affinity of uroporphyrinogen decarboxylase for substrates may decrease with each decarboxylation.

Discussion

In our system the maximum rate of decarboxylation of uroporphyrinogen III to coproporphyrinogen was marginally faster than that of uroporphyrinogen I, but the difference between the isomers was more marked when total products are considered. These results are in agreement with those reported for erythrocytes by Mauzerall & Granick (1958) and Cornford (1964). In contrast, Romeo & Levin (1971) with mouse spleen, Kushner *et al.* (1975) with pig liver and Rasmussen & Kushner (1979) with human erythrocytes concluded that there were no differences between the isomers. We have also examined mouse spleen and obtained similar results to those we report here for rat liver (A. G. Smith & J. E. Francis, unpublished work). We believe that the initial decarboxylation of uroporphyrinogen III does occur more rapidly than that of heptacarboxylic

porphyrinogen (Garcia *et al.*, 1973) or uroporphyrinogen I. This would imply that recognition and removal of the acetate carboxy group from the unsymmetrically arranged ring D of uroporphyrinogen III may occur on a separate active site or enzyme from the other decarboxylations. The heptacarboxylic porphyrinogen that accumulates seems to inhibit both its own and uroporphyrinogen decarboxylation (Garcia *et al.*, 1973). Finally, the results described illustrate that experiments with uroporphyrinogen decarboxylase at fixed time intervals and fixed substrate concentrations and *in vitro* should be interpreted with extreme caution.

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