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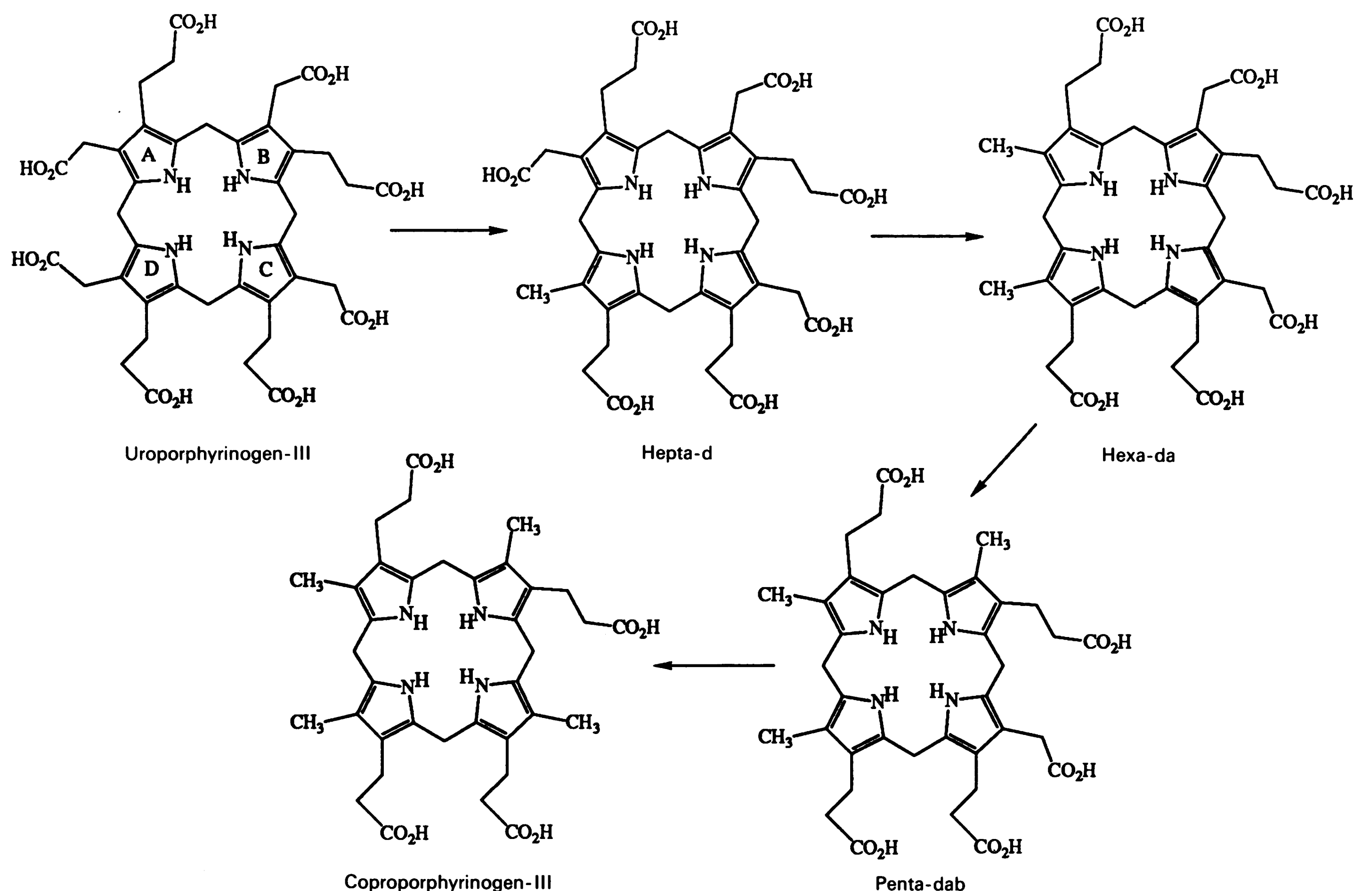
Action of uroporphyrinogen decarboxylase on uroporphyrinogen-III: a reassessment of the clockwise decarboxylation hypothesis

In the later stages of haem biosynthesis, uroporphyrinogen-III undergoes a series of four decarboxylations to give coproporphyrinogen-III. The sequential decarboxylation of uroporphyrinogen-III by uroporphyrinogen decarboxylase could take place by 24 different pathways involving 14 possible intermediates. The intermediacy of heptacarboxylate (heptas), hexacarboxylate (hexas) and pentacarboxylate (pentas) porphyrinogens has long been recognized (Batlle & Grinstein, 1964; San Martin de Viale & Grinstein, 1968). In the mid-1970's, Jackson and coworkers undertook a detailed investigation of this process (Jackson *et al.*, 1976*a*). Four heptacarboxylate porphyrinogens are possible, depending on whether initial decarboxylation occurs at the A, B, C or D ring acetate groups (designated as heptas -a, -b, -c or -d). Six hexacarboxylate porphyrinogens (hexas) might be formed and each structure is designated by a two-letter code corresponding to the acetate units that have undergone decarboxylation. Four type III pentacarboxylate isomers (pentas) are possible and these are assigned a three-letter code which corresponds to the positions of the three methyl substituents. All fourteen of the related porphyrins were synthesized by the MacDonald condensation and the b-oxobilane approach (Jackson *et al.*, 1980*a*; see also Clezy *et al.*, 1976). Surprisingly, all 14 of the porphyrinogens were metabolized by chicken red cell haemolysates, although not all of these compounds were good substrates (Jackson *et al.*, 1976*b*; Smith *et al.*, 1976; Lash, 1979). For instance, penta-bcd was shown to be a poor substrate for uroporphyrinogen decarboxylase, while the other type III pentacarboxylate porphyrinogens were rapidly converted to protoporphyrin-IX. Hence, the nature of the decarboxylation pathway between uroporphyrinogen-III and coproporphyrinogen-III could not be deduced from the substrate specificity of uroporphyrinogen decarboxylase. Hepta, hexa and penta fractions were known to accumulate in the urine and faeces of rats poisoned with hexachlorobenzene. These components were isolated and shown, on the basis of europium shift reagent proton n.m.r. studies and comparison to synthetic samples, to primarily consist of single porphyrin isomers (Jackson *et al.*, 1976*a*). These fractions corresponded to hepta-d, hexa-da and penta-dab (Scheme 1). In addition the heptacarboxylate fraction isolated from the urine of a patient suffering from porphyria cutanea tarda (PCT) was also shown to be indistinguishable from hepta-d (Jackson *et al.*, 1976*b*; Ryder, 1977). These results suggested that uroporphyrinogen-III underwent initial decarboxylation at the D ring, followed by successive decarboxylations at the A, B and C positions. It is tempting to suggest that the porphyrinogen undergoes a series of rotations on the enzyme surface; however, there is some evidence to suggest that more than one active site is involved (cf. de Verneuil *et al.*, 1980). Given that this sequence takes place in a clockwise progression around the periphery of the conventional 'Fischer' porphyrinogen structures, this process was considered to be a clockwise decarboxylation pathway (Jackson *et al.*, 1976*a*). In an independent study, Battersby and coworkers isolated a heptacarboxylate porphyrin from incubations of porphobilinogen (PBG), the precursor of uroporphyrinogen-III, with avian red cell haemolysates at high salinity (Battersby *et al.*, 1976). The high salt content was required to induce an accumulation of the heptacarboxylate fraction. N.m.r. studies and comparisons with synthetic samples again demonstrated that a single isomer, hepta-d, had been formed. It should be noted that hepta-d, hexa-da and penta-dab were all excellent substrates for uroporphyrinogen decarboxylase and the presence of the corresponding porphyrins cannot be rationalized as being due to the preferential accumulation of these isomers. Penta-dab is also believed to be a precursor to the isocoproporphyrin series (Elder, 1972; Stoll *et al.*, 1973), a group of abnormal metabolites that have been isolated from the faeces of porphyrics and hexachlorobenzene-poisoned rats. The corresponding porphyrinogens may be intermediates in an alternate route from penta-dab to harderoporphyrinogen under abnormal conditions (Elder & Evans, 1978; Jackson *et al.*, 1980*b*).

In order to analyse trace amounts of porphyrins from natural sources, partial h.p.l.c. separations of hepta, hexa and penta isomers were developed at Cardiff in the late 1970's (Jackson *et al.*, 1980*a*). Analysis of the penta fractions from normal and porphyric urine showed that at least three type III penta isomers were present (Jackson *et al.*, 1980*c*). Subsequently, Lim and coworkers developed elegant h.p.l.c. separations of penta, hexa and hepta porphyrin fractions (Lim & Rideout, 1983; Lim *et al.*, 1983*a,b*; Li *et al.*, 1987; Lim *et al.*, 1987). In each case, they demonstrated that all fourteen of the possible hepta, hexa and penta isomers were present in normal urine in similar proportions. However, they were able to confirm that the major hepta in the urine of PCT patients was hepta-d (Luo & Lim, 1990). In addition, h.p.l.c. studies at Cardiff had confirmed that the hepta, hexa and penta fractions from the urine and faeces of hexachlorobenzene-poisoned rats were single isomers, in agreement with the original n.m.r. and synthetic studies (Jackson *et al.*, 1976*a*). It seems quite likely that abnormal isomers would be excreted preferentially and the presence of trace quantities of porphyrin isomers in urine samples does not preclude the validity of Jackson's clockwise decarboxylation hypothesis.

Uroporphyrinogen-I is formed in excessive quantities in certain pathological conditions, such as congenital erythropoietic porphyria (Gunther's disease), and type I porphyrins are excreted in the urine and faeces. Uroporphyrinogen-I is metabolized by uroporphyrinogen decarboxylase to give coproporphyrinogen-I. Only one hepta and one penta can be formed in the type I series, although two hexas are possible. Analysis of the type I hexa fraction from natural sources by h.p.l.c. confirmed that both isomers were present (Jackson *et al.*, 1977). In addition, when uroporphyrinogen-I was incubated with chicken red cell haemolysates, both hexas were formed as metabolic intermediates. Hence, the decarboxylation of uroporphyrinogen-I by uroporphyrinogen decarboxylase appears to be random in nature.



Scheme 1. 'Clockwise' decarboxylation of uroporphyrinogen-III

Similar studies were carried out on the metabolism of uroporphyrinogen-III by chicken red cell haemolysates (A. H. Jackson, T. D. Lash, K. R. N. Rao & S. G. Smith, unpublished work in 1978; Lash, 1979). Although not all of the possible isomers could be separated, the results clearly demonstrated that mixtures of isomers were formed. Indeed, there was no indication of a preferred decarboxylation pathway for uroporphyrinogen-III, hepta-d, or hexa-da. Recently, a similar study was carried out for human erythrocyte uroporphyrinogen decarboxylase (Luo & Lim, 1990) and uroporphyrinogen-III was shown to form all four possible heptas in roughly equal portions. These authors suggest that their work 'provides conclusive evidence for a random, rather than ordered, decarboxylation sequence'.

If the decarboxylation of uroporphyrinogen-III is truly a random process, one must infer that hexachlorobenzene poisoning induces selectivity. Since hepta-d, hexa-da and penta-dab are good substrates for uroporphyrinogen decarboxylase, and other intermediates such as penta-bcd are relatively poor substrates, it does not seem reasonable that these structures would accumulate preferentially under pathological conditions. A similar effect would also have to operate in PCT. It may be that the hepatic enzymes, which are primarily responsible for cytochrome *P*-450 production, have a different selectivity to those derived from erythrocytes. However, hepta-d was also obtained from incubations of PBG with avian erythrocyte haemolysates containing 2 g of NaCl/100 ml (Battersby *et al.*, 1976). The high level of salinity would be unlikely to induce enzyme specificity and an alternative explanation is needed.

PBG is acted upon by hydroxymethylbilane synthase and uroporphyrinogen-III synthase to give uroporphyrinogen-III. Since these enzymes, together with uroporphyrinogen decarboxylase, are present in the cytoplasm, it may be that they act in a co-operative fashion. Once uroporphyrinogen-III is formed by the action of uroporphyrinogen-III synthase, the porphyrinogen may be 'handed on' in a specific orientation to uroporphyrinogen decarboxylase. Under these circumstances, it is proposed that a selective 'clockwise' decarboxylation process occurs to form coproporphyrinogen-III, which is then released and further metabolized by coproporphyrinogen oxidase. Leakage of free uroporphyrinogen-III from the porphyrinogen–enzyme complex, followed by recombination in a random orientation, would then lead to the formation of all fourteen possible intermediates. Since this is the situation that is encountered when uroporphyrinogen-III is used in enzymic studies, random decarboxylation products would be expected. On the other hand, studies *in vitro* using earlier biosynthetic precursors, such as δ-aminolaevulinate, PBG or the hydroxymethylbilane, would be expected to give protoporphyrin-IX by a single metabolic pathway. Hence, in view of this author, all the evidence to date is consistent with a fairly specific route for the *in vivo* sequential enzymic decarboxylation of uroporphyrinogen-III, although some leakage may occur. Clearly, further studies are needed to clarify our understanding of this neglected portion of the haem biosynthetic pathway. Enzymic studies on PBG under physiological conditions may be hampered, however, since the hepta, hexa and penta fractions are unlikely to accumulate at detectable levels.

Timothy D. LASH

Department of Chemistry, Illinois State University, Normal, IL 61761-6901, U.S.A.

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Action of uroporphyrinogen decarboxylase on uroporphyrinogen III

One of the outstanding questions concerning the enzymic decarboxylation of uroporphyrinogen III is whether the reaction is clockwise and orderly starting from the ring-D acetate group or is random. Evidence in support of a clockwise mechanism was provided by the fact that the intermediates isolated from the faeces of hexachlorobenzene-poisoned rats were 7d, 6da and 5dab, respectively (Jackson *et al.*, 1976). We have also confirmed this by h.p.l.c. analysis. On the other hand, all 14 intermediates were detected in normal human urine (Lim *et al.*, 1983) and incubation of uroporphyrinogen III with red cell haemolysates produced a mixture of isomers (Lash, 1979; Luo & Lim, 1990), indicating random decarboxylation.

Lash (1991) postulates that the uroporphyrinogen III used in the enzyme reaction may be presented randomly to the enzyme leading to random decarboxylation while with PBG as substrate the enzymically produced uroporphyrinogen III is 'handed on' to uroporphyrinogen decarboxylase in a specific orientation, resulting in specific decarboxylation of the ring-D acetate group. We accept this explanation is plausible and agree that it should be tested experimentally.

Whether the preferential accumulation of 7d in PCT and in hexachlorobenzene-poisoned rats is a result of damage to uroporphyrinogen decarboxylase, however, remains an open question.

Jinli LUO and C. K. LIM

MRC Toxicology Unit, MRC Laboratories,
Woodmansterne Road, Carshalton, Surrey, SM5 4EF, U.K.

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Inhibition of adenylate cyclase by Ca^{2+} — a counterpart to stimulation by Ca^{2+}/calmodulin

Stimulation of adenylate cyclase, mediated by Ca^{2+} acting via calmodulin, is the longest established example of potential positive 'crosstalk' between the Ca^{2+} and cyclic AMP-signalling systems [1]. Ca^{2+}, in concentrations that are achieved intracellularly, elicits a prominent stimulation of adenylate cyclase, which is variable in magnitude, depending on the tissue [2]. It is assumed that neurotransmitters that elevate $[Ca^{2+}]_i$ should also elevate cyclic AMP by this mechanism in intact neurons. Whether this actually occurs in cells has never been proven, although a considerable number of reports indicate that $[Ca^{2+}]_i$-mobilizing neurotransmitters elevate cyclic AMP in brain slice preparations [3–5]. It has also been proposed that such Ca^{2+}/calmodulin-stimulated adenylate cyclase is the form that is susceptible to neurotransmitter inhibition [2,6,7] and that this inhibition is a significant component of presynaptic autoinhibitory mechanisms [8]. Ca^{2+}/calmodulin-stimulated adenylate cyclase was first described in brain [9,10] and partially purified from this source. Development of antibodies against such preparations suggested a rather limited tissue distribution [11,12]. Further purification, leading to the recent cloning and expression of a Ca^{2+}/calmodulin-stimulated adenylate cyclase [13] has confirmed the largely neuronal distribution of the mRNA encoding this species [14].

In contrast to the stimulatory effect of Ca^{2+}/calmodulin on brain adenylate cyclase, a growing number of reports indicate that low concentrations of Ca^{2+} inhibit adenylate cyclase in plasma membranes from other sources. Adenylate cyclase from pituitary tissue [15], purified somatotrophs [16], GH3 cells [17], platelets, NCB-20 cells [18] and cardiac sarcolemma [19] is inhibited by 35–50% by Ca^{2+} in the same concentration range that stimulates brain adenylate cyclase. The magnitude of inhibition is greater than that elicited by G_i-protein-coupled receptors and is additive with their effects [15–19]. {Inhibition by Ca^{2+} in the low micromolar range is not to be confused with inhibition by Ca^{2+} in the *submillimolar* range. This latter inhibition is displayed by all adenylate cyclases regardless of their source and of their response to Ca^{2+} in the submicromolar range, including, for instance, the enzymes from brain, liver and cardiac tissue, which are stimulated, unaffected and inhibited, respectively, by submicromolar $[Ca^{2+}]$. In a system in which inhibition by Ca^{2+} is encountered both in the submicro- and submillimolar range, the two inhibitory effects are generally separated by a