Biochem. J. (1978) **169**, 205–214
Printed in Great Britain

A Radiochemical Method for the Measurement of Coproporphyrinogen Oxidase and the Utilization of Substrates other than Coproporphyrinogen III by the Enzyme from Rat Liver

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(*Received* 17 *June* 1977)

[$^{14}C_2$]Coproporphyrin III, ^{14}C-labelled in the carboxyl carbon atoms of the 2- and 4-propionate substituents, was prepared by stepwise modification of the vinyl groups of protoporphyrin IX. The corresponding porphyrinogen was used as substrate in a specific sensitive assay for coproporphyrinogen oxidase (EC 1.3.3.3) in which the rate of production of $^{14}CO_2$ is measured. With this method, the K_m of the enzyme from rat liver for coproporphyrinogen III is 1.2 μM. Coproporphyrin III is a competitive inhibitor of the enzyme (K_i 7.6 μM). Apparent K_m values for other substrates were measured by a mixed-substrate method: that for coproporphyrinogen IV is 0.9 μM and that for harderoporphyrinogen 1.6 μM. Rat liver mitochondria convert pentacarboxylate porphyrinogen III into dehydroisocoproporphyrinogen at a rate similar to that for the formation of protoporphyrinogen IX from coproporphyrinogen III. Mixed-substrate experiments indicate that this reaction is catalysed by coproporphyrinogen oxidase and that the K_m for this substrate is 29 μM. It is suggested that the ratio of the concentration of pentacarboxylate porphyrinogen III to coproporphyrinogen III in the hepatocyte determines the relative rates of formation of dehydroisocoproporphyrinogen and protoporphyrinogen IX.

During the final stages of haem biosynthesis, coproporphyrinogen III is converted into protoporphyrinogen IX by coproporphyrinogen oxidase (EC 1.3.3.3). This enzyme catalyses the oxidative decarboxylation of the propionic acid substituents at positions 2 and 4 of coproporphyrinogen III to vinyl groups. A 2-vinyl-substituted porphyrinogen, harderoporphyrinogen, is formed during the course of this reaction, suggesting that the decarboxylation of the propionate groups proceeds in sequence (Kennedy *et al.*, 1970; Cavaleiro *et al.*, 1973; Games *et al.*, 1976). The partially purified enzyme from mammalian liver (Sano & Granick, 1961; Batlle *et al.*, 1965) and yeast mitochondria (Poulson & Polglase, 1974) catalyses the conversion of both propionic acid substituents, and there is, at present, no evidence that more than one enzyme is involved in these reactions.

Apart from coproporphyrinogen III, harderoporphyrinogen is the only one of the small number of porphyrinogens known to be substrates for this enzyme (Porra & Falk, 1964; Cavaleiro *et al.*, 1973; Frydman & Frydman, 1975; Jackson *et al.*, 1976*a*) that occurs naturally. A group of previously undescribed porphyrins, named the isocoproporphyrin series, has been identified in the bile, faeces and urine of patients with porphyria cutanea tarda and in the bile and faeces of rats with porphyria caused by prolonged feeding of hexachlorobenzene (Elder, 1972, 1974; Watson *et al.*, 1975). Trace amounts of these porphyrins have also been detected in faeces from normal subjects and patients with other types of porphyria (Elder, 1975). Their structure is similar to that of the pentacarboxylate porphyrinogen immediately preceding coproporphyrinogen III in the biosynthetic pathway, except that the 2-propionate group is replaced by either a vinyl group or substituents derived from it through the action of intestinal microorganisms or by chemical modification during the extraction procedure (Stoll *et al.*, 1973; Baptista de Almeida *et al.*, 1975; Jackson *et al.*, 1976*b*). This structural relationship suggests that the pentacarboxylate precursor of coproporphyrinogen III may also be a substrate for coproporphyrinogen oxidase.

In current methods for the determination of coproporphyrinogen oxidase the rate of formation of protoporphyrinogen is measured (Sano & Granick, 1961; Batlle *et al.*, 1965; Tait, 1972; Poulson & Polglase, 1974). The protoporphyrinogen is separated from the substrate, after both have been oxidized to the corresponding porphyrins, by solvent partition, and is then determined by spectrophotometry. Such methods are laborious and lack sensitivity, since they

are least accurate when the percentage conversion of substrate into product is low. In addition, protoporphyrinogen, by virtue of its vinyl substituents, is susceptible to oxidative degradation during the oxidation and purification stages and is difficult to recover quantitatively. Coproporphyrinogen oxidase from all eukaryotic cells so far studied (Sano & Granick, 1961; Batlle *et al.*, 1965; Hsu & Miller, 1970), except for the yeast *Saccharomyces cerevisiae* (Poulson & Polglase, 1974), has an absolute requirement for molecular oxygen. *In vitro* both oxidation of substrate to coproporphyrin III and conversion into protoporphyrinogen IX proceed together, so that initial velocities at substrate concentrations approaching the K_m value are hard to measure. Values for the K_m for coproporphyrinogen III ranging from about 20 to 40 μM have been found for the enzyme from plant and animal tissues by using solvent-partition methods (Sano & Granick, 1961; Batlle *et al.*, 1965; Hsu & Miller, 1970; Poulson & Polglase, 1974), but have not been reported for other substrates.

An alternative approach to the measurement of coproporphyrinogen oxidase activity, which is described in the present paper, depends on determination of the rate of release of $^{14}CO_2$ from [$^{14}C_2$]-coproporphyrinogen III, ^{14}C-labelled in the carboxyl carbon atoms of the 2- and 4-propionate groups. [The structures of some of the compounds mentioned in this paper are shown in the next paper (Elder *et al.*, 1978).] This approach also allows comparison of the apparent K_m values for different substrates by means of mixed-substrate experiments.

Experimental

Materials

[$^{14}C_2$]Coproporphyrin III. [$^{14}C_2$]Coproporphyrin III was synthesized by stepwise modification of the vinyl substituents of protoporphyrin IX dimethyl ester. Crude 2,4-di-(1,2-dihydroxyethyl)deuteroporphyrin IX dimethyl ester (approx. 0.8mmol), prepared from protoporphyrin IX dimethyl ester (Sparatore & Mauzerall, 1960), was dissolved in pyridine (6ml) followed by methanol (300ml). Sodium periodate (6.6mmol) in distilled water (60ml) was added slowly with shaking. The reaction mixture was left in the dark at room temperature (15–22°C) for 4.5h, then diluted with chloroform (200ml) and washed with an excess of aqueous sodium thiosulphate, followed by distilled water. The solvent was removed from the organic layer by evaporation under reduced pressure with the use of methanol as an azeotrope to give a dark-coloured residue, which was chromatographed on a neutral aluminium oxide (activity IV) column (approx. 15cm×2cm), packed in chloroform, with chloroform as the eluent. The first pink band was discarded, and the major brown band was collected and evaporated under reduced pressure. The resultant residue was crystallized from chloroform/methanol to give 2,4-diformyldeuteroporphyrin IX dimethyl ester as dark crystals (0.15mmol). This compound was condensed with [1-^{14}C]malonic acid to form 2,4-di-(2-carboxyvinyl)deuteroporphyrin IX tetramethyl ester, which was reduced to give [$^{14}C_2$]coproporphyrin III tetramethyl ester (Elder *et al.*, 1978). Yields of coproporphyrin ester from the diformylporphyrin ester ranged from 24 to 27%.

Samples (0.5–1.5 μmol) of [$^{14}C_2$]coproporphyrin III ester were dissolved in 5M-HCl (2ml) and left in the dark at room temperature for 48h to complete hydrolysis. The solution was then diluted with water (10–20ml), adjusted to pH3.5 with satd. aq. sodium acetate and shaken with diethyl ether (100ml). The ether phase was washed twice with aq. 3% (w/v) sodium acetate (20ml) and twice with water (10ml). The original aqueous phase and the washings were combined and extracted with 50ml of ether, which was washed twice with water (10ml). [$^{14}C_2$]Coproporphyrin III was extracted from the combined ether extracts by shaking with 4×10ml of 0.1M-HCl. The small amount of porphyrin (Soret maximum 410nm in 1.4M-HCl) that remained in the ether layer was discarded. This procedure separated [$^{14}C_2$]coproporphyrin III from traces of mono-(2-carboxyvinyl)-mono-(2-carboxyethyl)deuteroporphyrin IX in the sample. The [$^{14}C_2$]coproporphyrin was then transferred to ether (about 20ml), which after washing with aq. sodium acetate and water, was evaporated in a stream of N_2 at 40°C, and dried *in vacuo* over solid KOH. The porphyrin was converted into its ammonium salt by brief exposure to NH_3 vapour and dissolved in 0.01M-KOH to give a solution of 2–4mM. This solution was stable for at least 1 month at 4°C in the dark. The specific radioactivity of [$^{14}C_2$]-coproporphyrin prepared in this way was 102±1% (mean±S.E.M. for 12 determinations) of that calculated from the specific radioactivity of the [1-^{14}C]malonic acid used for the synthesis.

Other porphyrins. Protoporphyrin IX dimethyl ester was prepared from haemin (Grinstein, 1947), and uroporphyrin III octamethyl ester from *Turacus* feathers (With, 1957). Penta-, hexa- and hepta-carboxylic porphyrin III methyl esters were isolated from the faeces of rats with hepatic porphyria caused by hexachlorobenzene poisoning (Elder *et al.*, 1976). Synthetic coproporphyrin I, II, III and IV tetramethyl esters were gifts from Professor A. H. Jackson, Department of Chemistry, University College, Cardiff, Wales, U.K., and synthetic isocoproporphyrin tetramethyl ester was from Professor G. W. Kenner, Department of Organic Chemistry, University of Liverpool, Liverpool, U.K.

Porphyrin esters, other than protoporphyrin IX dimethyl ester, were hydrolysed with 5M-HCl for 48h at room temperature in the dark, dried *in vacuo*

over KOH and dissolved in 0.01 M-KOH as described above. Protoporphyrin IX dimethyl ester was separated from impurities by t.l.c. on silica gel in chloroform/kerosene/methanol (200:100:7, by vol.) (Elder, 1972) and hydrolysed in 5 M-HCl for 16h at room temperature in the dark. Porphyrin was then precipitated by adjusting the pH to the isoelectric point with satd. aq. sodium acetate, recovered by centrifugation (15000*g*-min), washed three times with water, dissolved in 0.01 M-KOH and used within 4h.

Porphyrinogens. Porphyrins were reduced to porphyrinogens with 4% (w/w) sodium amalgam (0.05g/0.1ml of porphyrin solution in 0.01 M-KOH) as described previously (Elder *et al.*, 1976). A sample of porphyrinogen solution was then transferred with a 100μl Hamilton syringe to a tube containing an equal volume of 0.25 M-Tris/HCl buffer, pH7.2, containing 0.2 M-sodium thioglycollate, mixed, gassed with N_2 and stored in ice until used, usually within 5min. The yield of coproporphyrinogen, determined by oxidation to porphyrin in ethyl acetate/acetic acid (3:1, v/v) (Sano, 1966) was about 85%.

Other materials. [1-^{14}C]Malonic acid (specific radioactivity 2.5–9.5mCi/mmol) was obtained from The Radiochemical Centre, Amersham, Bucks., U.K. Soluene-350, 1.0 M-Hyamine in methanol, PPO (2,5-diphenyloxazole) and POPOP [1,4-bis-(5-phenyloxazol-2-yl)benzene] were from Packard Instruments, Caversham, Berks., U.K. Toluene (scintillation grade) and other solvents (AnalaR grade) were obtained from BDH Chemicals, Poole, Dorset, U.K., except for kerosene (white) which was from Hopkin and Williams, Romford, Essex, 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.

Methods

Determination of specific radioactivity of [$^{14}C_2$]-coproporphyrin. Samples of the solution of [$^{14}C_2$]-coproporphyrin in 0.01 M-KOH to be reduced to coproporphyrinogen for use as substrate were removed for measurement of porphyrin concentration and radioactivity. Porphyrin concentration was determined from the extinction at the Soret maximum of a sample accurately diluted in 0.1 M-HCl (1000-fold or greater), with the use of 489×10^3 litre·mmol^{-1}·cm^{-1} (Falk, 1964) as the millimolar extinction coefficient for coproporphyrin in 0.1 M-HCl. For measurement of radioactivity, 2–10μl of the [$^{14}C_2$]-coproporphyrin solution was mixed with 0.25ml of Soluene-350 in a polypropylene vial and decolorized by adding 0.15ml of H_2O_2 solution (about 30%, w/v). After 10min, 0.05ml of acetic acid was added, followed by 0.25ml of Soluene-350 and 15ml of toluene containing PPO (4g/litre) and POPOP (100mg/litre). Vials treated identically, but containing no porphyrin, were prepared for determination of the background radioactivity. Radioactivity was measured in a Packard Tri-Carb liquid-scintillation spectrometer. Counting efficiency was determined by a channels-ratio method with external quenched standards and calibrated with [^{14}C]toluene as internal standard, and varied from 88 to 92%. The coefficient of variation for specific radioactivity determinations was ±3%.

Measurement of coproporphyrinogen oxidase activity. Assays were carried out in glass vials (46mm×13mm) containing polypropylene cups, made by cutting off the bottom 12mm of polypropylene test tubes (capacity 1.5ml, W. Sarstedt Ltd., Leicester, U.K.), which supported a flat-bottomed glass tube (254mm×6.35mm) containing 0.15ml of 1.0 M-Hyamine in methanol. The standard reaction mixture contained 0.03ml of 0.25 M-Tris/HCl buffer, pH7.2 at 20°C, containing bovine serum albumin (33.3mg/ml), 0.05ml of rat liver homogenate or mitochondrial fraction in 0.05 M-Tris/HCl buffer, pH7.3 at 20°C, 0.01ml of 0.01 M-KOH and 0.01ml of porphyrinogen solution, diluted with buffer containing sodium thioglycollate as described above. The final pH varied from 7.2 to 7.3 at 37°C.

The vials containing all the reagents except the buffered porphyrinogen solution were cooled in ice. The buffered porphyrinogen solution was then added and each vial sealed with a rubber cap. The reaction was started by transferring the vials to a water bath at 37°C, in which they were shaken at 30 cycles/min in the dark. Zero-time vials were included in all assays. The reaction was stopped by freezing in methanol/solid CO_2; 0.1 M-Na_2CO_3 (0.05ml), followed by 5 M-HCl (0.05ml), was then injected through the rubber cap on to the side of each vial away from the central tube. The vials were then warmed to room temperature and shaken at 20 cycles/min for 0.75h. The Hyamine solution containing trapped $^{14}CO_2$ was transferred to polyethylene vials containing 15ml of toluene containing 4g of PPO and 100mg of POPOP/litre. Radioactivity was measured by using the ^{14}C channel of a Packard Tri-Carb liquid-scintillation spectrometer. Blank vials containing Hyamine solution and scintillant were included in each assay and channel ratios measured to detect apparent radioactivity due to chemiluminescence. The efficiency of this system for liberating, trapping and measuring $^{14}CO_2$ was tested with reaction vials set up as described but containing 0.1 M-$Na_2{}^{14}CO_3$ (2–10μl, 115–2150 d.p.m./vial) and was 88–95% (mean 90%) for five duplicate measurements over the range 100–2000c.p.m. Experimental conditions were chosen so that radioactivity measured was usually in this range.

Enzyme activities were expressed as nmol of CO_2 liberated/unit time, nmol of CO_2 being calculated by assuming that the specific radioactivity of the $^{14}CO_2$

liberated was half that of the [$^{14}C_2$]coproporphyrin III used for preparation of the substrate and that the overall efficiency of measurement of $^{14}CO_2$ was 90%.

Effect of substrate concentration on $^{14}CO_2$ liberation. For K_m measurements, the concentration of substrate was calculated by assuming that the yield of porphyrinogen from porphyrin was 100% and was varied by adding different volumes of buffered porphyrinogen solution. Appropriate volumes of 0.25M-Tris/HCl buffer, containing sodium thioglycollate, and 0.01M-KOH were added separately so that the reaction mixtures differed only in the concentrations of substrate and of NaOH (from the reaction of sodium amalgam with water). The final pH of the mixture was measured at 37°C for each substrate concentration. For mixed-substrate experiments, porphyrins were mixed before reduction so that the corresponding porphyrinogens were added to the reaction vials in a single solution.

Isolation and fractionation of porphyrins. In some experiments porphyrins were isolated from a reaction mixture identical with that described above. The reaction was stopped by cooling the vials in ice and adding ethyl acetate/acetic acid (3:1, v/v; 5.0ml/ml of reaction mixture). Porphyrins were then extracted and fractionated as described by Tait (1972) or converted into their methyl esters (Elder *et al.*, 1976) and fractionated by t.l.c. in chloroform/kerosene/methanol (100:50:3, by vol.) (Elder, 1972). Recoveries were calculated from the amount of porphyrin used to prepare the substrate by assuming that the yield of porphyrinogen was 100%.

Preparation of rat liver homogenate and mitochondrial fraction. All procedures were carried out at 4°C. Livers were removed from exsanguinated female Wistar rats (150–200g body wt.) that had been killed by decapitation. Liver tissue, cut into small pieces, was homogenized in 9vol. of 0.05M-Tris/HCl buffer, pH7.2 at 20°C, by five strokes of an Aldridge homogenizer. The homogenate was either used immediately or stored for up to 10 weeks at −20°C. For the preparation of mitochondrial fractions, livers were homogenized in 5vol. of 0.25M-sucrose, containing 10mM-Tris, adjusted to pH7.4 at 20°C with HCl, and centrifuged at $850g_{av.}$ for 10min. The supernatant was then centrifuged at $7000g_{av.}$ for 10min in the 3×30ml aluminium swing-out rotor of a Christ Omega II ultracentrifuge. The pellet was washed twice with buffered sucrose solution and then resuspended in 0.05M-Tris/HCl buffer, pH7.2, to give 15–20mg of mitochondrial protein/ml.

Other methods. The concentration of porphyrin and porphyrin methyl ester solutions was measured by spectrophotometry (Falk, 1964); 180×10^3 litre·mol^{-1}·cm^{-1} was used as ε for harderoporphyrin trimethyl ester (Suckling, 1970). For other porphyrins, ε_{mM} values given by Falk (1964) and Doss (1974) were used, isocoproporphyrin and related porphyrins being determined as coproporphyrin. Protein concentration was measured with crystalline bovine serum albumin as standard (Lowry *et al.*, 1951). Mass spectra were obtained by using the field-desorption probe of a Varian CH5D mass spectrometer at the Department of Chemistry, University College, Cardiff, Wales, U.K.

Results

Validation of radiochemical assay for coproporphyrinogen oxidase

The validity of using the rate of $^{14}CO_2$ release from [$^{14}C_2$]coproporphyrinogen III to measure coproporphyrinogen oxidase activity was tested with rat liver homogenates as the source of enzyme. These were used, rather than the mitochondrial or partially purified enzyme fractions used in previous work on the mammalian liver enzyme (Sano & Granick, 1961; Porra & Falk, 1964; Batlle *et al.*, 1965), both because it was hoped that the method would be sensitive enough to measure coproporphyrinogen oxidase activity in very small samples of tissue and to allow easier comparison with the activities of other enzymes of the pathway of haem biosynthesis.

The rate of $^{14}CO_2$ production was identical, and was not altered by repeated freeze–thawing, when the following media were used for homogenization: 0.05M-Tris/HCl buffer, pH7.3; 0.05M-potassium phosphate buffer, pH7.4; 0.25M-sucrose/0.01M-Tris, adjusted to pH7.4 with HCl; 0.22M-D-mannitol/0.07M-sucrose/2mM-Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid], containing 0.5mg of bovine serum albumin/ml and adjusted to pH7.4 with KOH. There was no change in enzyme activity when 10% (w/v) homogenates in 0.05M-Tris/HCl buffer, pH7.3, were stored at −20°C for up to 10 weeks.

Stoicheiometry of the reaction catalysed by coproporphyrinogen oxidase $^{14}CO_2$ was released from [$^{14}C_2$]coproporphyrinogen only under conditions that allowed the formation of protoporphyrinogen. Thus $^{14}CO_2$ was not produced when liver homogenate was omitted from the incubation mixture, or replaced by heat-inactivated homogenate (80°C for 10min), or when the reaction vessel was gassed with N_2. No $^{14}CO_2$ was formed when the [$^{14}C_2$] coproporphyrin III solution used for reduction was incubated with liver homogenate, showing that any radioactive impurity in this solution, which might be a source of $^{14}CO_2$, required treatment with sodium amalgam before it could be decarboxylated.

Fig. 1 shows the relationship between the production of $^{14}CO_2$ and protoporphyrin when [$^{14}C_2$]-coproporphyrinogen III was incubated with rat liver homogenates for periods of up to 90min. Harderoporphyrin was also formed and accounted for about

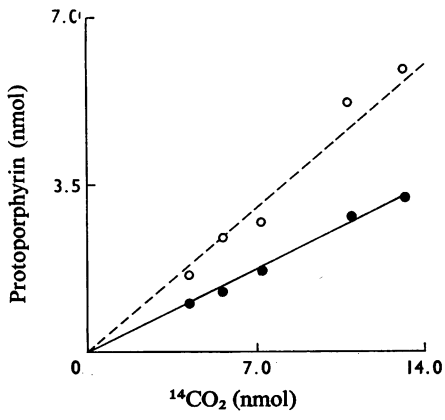

Fig. 1. *Relationship between formation of $^{14}CO_2$ and protoporphyrin IX*

Points (●) were obtained from means of duplicate measurements of $^{14}CO_2$ and protoporphyrin formation and each was corrected for recovery of total porphyrin (○). The distribution of harderoporphyrin between fractions (see the text) was taken into account when calculating the line (----) for the theoretical agreement between the two methods.

25% of the total products. The specific radioactivity of the harderoporphyrin was close to half (52±1%; mean and range for two experiments) that of the substrate, whereas the protoporphyrin was not radioactive. In these experiments protoporphyrin was separated from coproporphyrin by the solvent-partition method described by Tait (1972). T.l.c. of the methyl ester derivatives of the porphyrins in the two fractions obtained showed that more than 85% of the harderoporphyrin was in the coproporphyrin fraction. When the theoretical relationship between $^{14}CO_2$ and protoporphyrin formation is compared with that found by experiment (Fig. 1) it is clear that either more $^{14}CO_2$ or less protoporphyrin is produced experimentally than is predicted from the stoicheiometry of the reaction catalysed by coproporphyrinogen oxidase. However, in these experiments the recovery of total porphyrin by solvent extraction ranged from 51 to 62% (mean 56%) of the porphyrinogen added. The percentage recovery varied little over a range of substrate consumption from 0 to 40%, suggesting that losses of substrate and products were similar. Close agreement with the theoretical relationship between the formation of $^{14}CO_2$ and protoporphyrin is obtained when these values are used to correct the amounts of protoporphyrin for losses during the oxidation, extraction and partition stages (Fig. 1).

Additional evidence that the decarboxylation of [$^{14}C_2$]coproporphyrinogen III was the sole source of $^{14}CO_2$ was provided by two other findings. First, the specific radioactivity of [$^{14}C_2$]coproporphyrin III isolated after incubation with rat liver homogenates under conditions in which up to 20% of the radioactivity was recovered as $^{14}CO_2$ did not differ from that of the [$^{14}C_2$]coproporphyrin III used for preparation of the substrate. Secondly, the rate of release of CO_2 was unaltered when the same homogenate was incubated with substrates of different specific radioactivities (0.66–2.40mCi/mmol).

Factors affecting the release of $^{14}CO_2$ from [$^{14}C_2$]coproporphyrinogen III. The production of $^{14}CO_2$ was independent of the concentration of [$^{14}C_2$]-coproporphyrinogen III, and linear with time for at least 60min, at substrate concentrations from 50 to 360μM when 14% or less of the substrate was consumed. At substrate concentrations of 76–200μM the rate of $^{14}CO_2$ production was the same in the absence as in the presence of sodium thioglycollate (10–20mM) for up to 30min, but after this time the rate decreased progressively in the absence of the reducing agent. At lower substrate concentrations the rate fell off more rapidly when sodium thioglycollate was absent. Thiol-group-containing compounds are thought to stabilize the substrate by slowing the rate of oxidation to coproporphyrin III and similar effects have been reported by others (Sano & Granick, 1961; Porra & Falk, 1964).

The rate of $^{14}CO_2$ production varied linearly with the concentration of homogenate over the range 0.2–5.0mg of liver (0.03–0.80mg of protein) per 0.1ml of final incubation mixture at a substrate specific radioactivity of 2.4mCi/mmol. The specific activity of the enzyme in rat liver homogenates was 1.97±0.20nmol of CO_2/h per mg of protein (mean±S.E.M. for measurements on 12 animals).

The rate of $^{14}CO_2$ formation varied little between pH7.1 and 7.5 and was within 10% of the maximum value over the pH range 6.90–7.65 in either 0.1M-Tris/HCl or 0.1M-Hepes/HCl buffers at 37°C. The highest rate was reached at pH7.3 at 37°C, which agrees well with the pH optimum of 7.4 reported for partially purified coproporphyrinogen oxidase from rat liver mitochondria by Batlle *et al.* (1965).

Fig. 2 shows double-reciprocal plots of the rate of $^{14}CO_2$ formation against [$^{14}C_2$]coproporphyrinogen III concentration. Initial velocities were determined by making duplicate measurements (coefficient of variation 9.4%) of $^{14}CO_2$ at time intervals from 5 to 30min after the start of the reaction. At substrate concentrations of 2.0μM and above, the rate of $^{14}CO_2$ production was constant for at least 10min. At lower concentrations, the rate measured by sampling at zero time and at 5min was assumed to represent the initial velocity. The K_m values obtained with homogenate as the source of enzyme agree closely with those found for mitochondrial fractions, the mean±S.E.M.

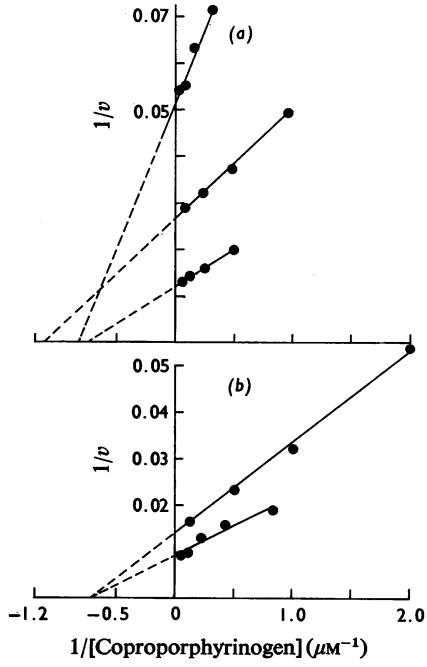

Fig. 2. *Effect of substrate concentration on $^{14}CO_2$ formation* The reaction mixture contained either 65–130 μg of mitochondrial protein/0.1 ml (*a*) or homogenate equivalent to 2.5 mg wet wt. of rat liver/0.1 ml (*b*). Other experimental details are given in the Experimental and Results sections. *v* is expressed as c.p.m. of $^{14}CO_2$/min. The specific radioactivity of the substrate (3.6–4.8 mCi/mmol) was not the same for all measurements.

for the five measurements being 1.2±0.1 μM. Previously reported values for the K_m of partially purified coproporphyrinogen oxidase from mammalian liver have been much higher (20–30 μM) (Sano & Granick, 1961; Batlle *et al.*, 1965). However, at these concentrations and above, initial rates were independent of substrate concentration in our experiments.

The rate of $^{14}CO_2$ production was decreased by coproporphyrin III, the product of oxidation of the substrate. Measurements of initial velocities in the presence of different concentrations of coproporphyrin III when plotted as described by Cornish-Bowden (1974) showed that this compound was a competitive inhibitor (Fig. 3*a*) with an apparent K_i of 7.6 μM (Fig. 3*b*). Three other porphyrins corresponding to known or putative substrates also inhibited the reaction (Table 1) but a fourth, mesoporphyrin VI, when tested under identical conditions, did not. Inhibition was not restricted to porphyrins corresponding to substrates, for coproporphyrins I and II were the most effective inhibitors found (Table 1). However, other porphyrins, less closely related in structure to substrates, were not inhibitors (uroporphyrins I and III; hepta- and hexa-carboxylate porphyrins III; mesoporphyrin IX). Coproporphyrinogen I was as effective an inhibitor as coproporphyrin I, which, together with the structural relationships described above, suggests that it is the nature and arrangement of the side chains that determines the inhibitory properties of porphyrins in this reaction.

The end product of the reaction, protoporphyrinogen IX, inhibited the formation of $^{14}CO_2$ by 36% at a relative concentration (40 μM-inhibitor, 5 μM-substrate) at which protoporphyrin IX had no effect, even when albumin was omitted from the incubation mixture. Protohaem (44–77 μM), either in the presence or absence of albumin, had no effect on $^{14}CO_2$ release.

Substrate specificity of rat liver coproporphyrinogen oxidase

Oxidative decarboxylation of porphyrinogens by rat liver homogenates and mitochondrial fractions. Table 2 shows that, in addition to coproporphyrinogen III,

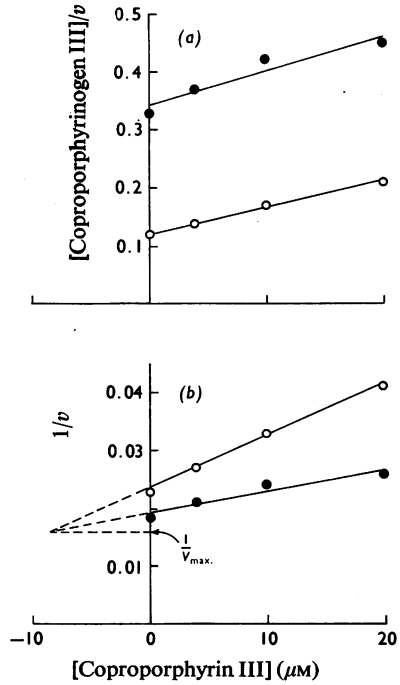

Fig. 3. *Effect of coproporphyrin III on $^{14}CO_2$ formation* Measurements were made at two concentrations (5.1 μM, ○; 17.7 μM, ●) of coproporphyrinogen III, and values of *s*/*v* (*a*) and 1/*v* (*b*) were plotted against the concentration of coproporphyrin III. $V_{max.}$ for the homogenate was determined in the same experiment. *v* is expressed as c.p.m. of $^{14}CO_2$ trapped/min.

rat liver homogenates or mitochondrial fractions metabolize coproporphyrinogen IV, harderoporphyrinogen and pentacarboxylate porphyrinogen III. At high substrate concentrations the rates of formation of dehydroisocoproporphyrinogen and protoporphyrinogen are similar. Previous reports have shown that harderoporphyrinogen (Cavaleiro *et al.*, 1973; Games *et al.*, 1976) and coproporphyrinogen IV (Porra & Falk, 1964; Granick & Levere, 1964; Frydman & Frydman, 1975; Al-Hazimi *et al.*, 1976; Mombelli *et al.*, 1976) are converted into protoporphyrinogen IX and protoporphyrinogen XIII respectively in various tissues from other species, but oxidative decarboxylation of a propionate group of pentacarboxylate porphyrinogen III has not been demonstrated before.

The product of the reaction with pentacarboxylate porphyrinogen as substrate was identified as dehydroisocoproporphyrinogen by the following techniques. After oxidation and conversion into its methyl ester derivative, the major product isolated from the incubation mixture migrated on t.l.c. in chloroform/kerosene/methanol (100:50:3, by vol.) with the same mobility as isocoproporphyrin tetramethyl ester. Field-desorption mass spectrometry showed a molecular ion at *m/e* 708, consistent with replacement of one of the propionic acid substituents of pentacarboxylate porphyrin pentamethyl ester (mol.wt. 768) by a vinyl group. The presence of an acetate group in the corresponding porphyrin acid was demonstrated by showing that one of the four acid substituents was decarboxylated by heating in 0.3M-HCl at 175°C *in vacuo* for 3h (Edmondson & Schwartz, 1953). Light-absorption maxima in chloroform were (with relative intensities in parentheses): 628 (0.25), 572 (0.50), 542 (0.91), 504 (1.0) and 405nm. This spectrum is similar to that reported for crude preparations of dehydroisocoproporphyrin tetramethyl ester (Elder, 1972). When compared with the spectrum of isocoproporphyrin tetramethyl ester, with maxima in chloroform at 623 (0.33), 569 (0.44), 535 (0.67), 500 (1.0) and 402nm, it shows a bathochromic shift of all maxima and a change in relative intensities, which is again consistent with the presence of a vinyl group. Variable amounts of two minor products were also isolated after incubation of pentacarboxylate porphyrinogen with mitochondrial fractions. They were tentatively identified from the mass spectra and chromatographic mobility of their methyl ester derivatives as the methanol and water adducts of dehydroisocoproporphyrin and were probably formed during the extraction procedure.

Thus incubation of pentacarboxylate porphyrinogen III with mitochondrial fractions, which contain

Table 1. *Inhibition of release of $^{14}CO_2$ from [$^{14}C_2$]coproporphyrinogen III by porphyrins*

Values for percentage inhibition are means of duplicate or quadruplicate measurements made at a [$^{14}CO_2$]coproporphyrinogen III concentration of 2.0μM. Porphyrins were added to the standard reaction mixture (see the Experimental section) in 0.01M-KOH before the addition of substrate.

Porphyrin	Inhibitor (μM)	Inhibition (%)
Coproporphyrin I	19.5	70
Coproporphyrin II	20.6	61
Coproporphyrin III	19.4	52
Coproporphyrin IV	19.6	30
Harderoporphyrin	20.0	27
Pentacarboxylate porphyrin III	19.8	10

Table 2. *Substrate specificity of coproporphyrinogen oxidase*

Porphyrinogens were incubated with rat liver homogenate (equivalent to 0.05g wet wt. of liver) or rat liver mitochondrial fraction (7–11mg of protein) for 1h under the conditions for coproporphyrinogen oxidase measurements (see the Experimental section) but in a total volume of either 0.5 or 1.0ml. In the experiments with homogenates $^{14}CO_2$ production from [$^{14}C_2$]coproporphyrinogen III was linear with time under the same conditions. With mitochondria, mean consumption of substrate was 45% (coproporphyrinogen III, 25μM), 15% (pentacarboxylate porphyrinogen III, 25μM) and 28% (pentacarboxylate porphyrinogen III, 93μM). The reaction was stopped and the porphyrins were extracted, converted into methyl esters and fractionated as described in the Experimental section. Means and ranges for duplicate measurements or * the mean and range for five measurements are shown.

Enzyme preparation	Substrate (μM)	Products (nmol/h per mg of protein)			Mean recovery (%)
		Dehydroisocoproporphyrin	Tricarboxylate porphyrin	Protoporphyrin	
Homogenate	Coproporphyrinogen III (25)		0.07±0.00	0.19±0.01	34
	Coproporphyrinogen IV (25)		0.16±0.01	0.26±0.02	30
	Harderoporphyrinogen (26)			0.14±0.02	36
Mitochondria	Coproporphyrinogen III (25)		0.09±0.00	0.35±0.02	27
	Pentacarboxylate porphyrinogen III (25)	0.20 (0.13–0.26)*			37
	(93)	0.37±0.07			39

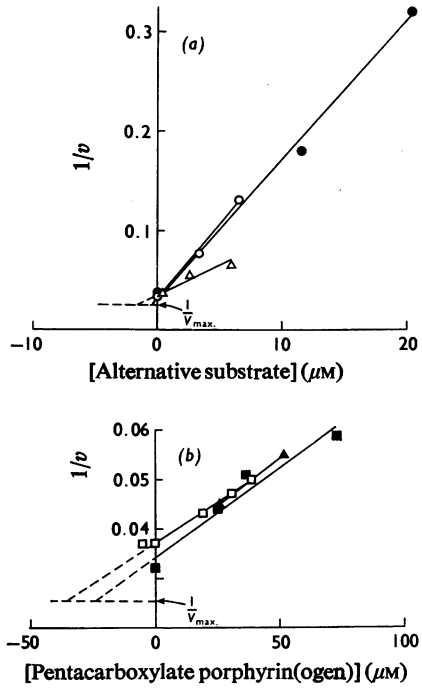

Fig. 4. *Effect of alternative substrates on $^{14}CO_2$ formation from [$^{14}C_2$]coproporphyrinogen III*

Substrate mixtures were prepared and added to the standard reaction mixture (containing 65–130 μg of mitochondrial protein/0.1 ml) as described in the Experimental section. All measurements were made at a [$^{14}CO_2$]coproporphyrinogen III concentration of 2.0 μM. The results of separate experiments are shown: ○ and ●, coproporphyrinogen IV; △, harderoporphyrinogen; □ and ■, pentacarboxylate porphyrinogen III. ▲, Effect of pentacarboxylate porphyrin III. The same mitochondrial fraction was used in all experiments. $V_{max.}$ is the mean of three measurements. v is expressed as c.p.m. of $^{14}CO_2$ trapped/min.

little uroporphyrinogen decarboxylase (EC 4.1.1.37) (Elder *et al.*, 1976), gave only dehydroisocoproporphyrinogen. This finding strongly suggests that this product is not a substrate for coproporphyrinogen oxidase. Although insufficient dehydroisocoproporphyrinogen was available to confirm this directly, its ethyl analogue, isocoproporphyrinogen, was not metabolized by the mitochondrial fractions used in these experiments.

Mixed-substrate experiments. Mixed-substrate experiments with harderoporphyrinogen, coproporphyrinogen IV and pentacarboxylate porphyrinogen III showed that each of these compounds inhibits the release of $^{14}CO_2$ from [$^{14}C_2$]coproporphyrinogen III (Fig. 4), suggesting that they are metabolized at the same active centre. In these experiments the initial rate of $^{14}CO_2$ formation was measured at one concentration of coproporphyrinogen III in the presence of different concentrations of alternative substrate, with a mitochondrial fraction for which the K_m and $V_{max.}$ values for [^{14}C]coproporphyrinogen III were known. The point on the plot of $1/v$ against [alternative substrate] that lies at a height of $1/V_{max.}$ then gives the apparent K_m for the alternative substrate, since the equations for the rate of product formation in the presence of either an alternative substrate (Dixon & Webb, 1966*a*) or a competitive inhibitor (Dixon & Webb, 1966*b*) are the same. Thus the K_m values of coproporphyrinogen oxidase for different substrates can be compared, even though it is possible to measure initial rates for only one of them.

Coproporphyrinogen IV and harderoporphyrinogen markedly decrease the rate of production of $^{14}CO_2$ (Fig. 4) at concentration ratios at which the corresponding porphyrins are much less effective inhibitors (Table 1). The apparent K_m values for coproporphyrinogen IV (0.8 and 1.0 μM) and harderoporphyrinogen (1.6 μM) are close to the K_m for coproporphyrinogen III (1.2 μM, Fig. 2). $V_{max.}$ cannot be found by this method, but rate measurements at saturating substrate concentrations (Table 2) show that these substrates are converted into protoporphyrin at comparable rates, that for coproporphyrinogen IV being the greatest. The rapid rate of metabolism of coproporphyrinogen IV conflicts with previous observations that this substrate is converted into protoporphyrin by mammalian liver mitochondria more slowly than is coproporphyrinogen III (Porra & Falk, 1964; Mombelli *et al.*, 1976), but agrees with findings from experiments with avian haemolysates (Frydman & Frydman, 1975; Al-Hazimi *et al.*, 1976). Pentacarboxylate porphyrinogen III is a much less effective inhibitor than either coproporphyrinogen IV or harderoporphyrinogen, the mean value of the apparent K_m from two experiments being 29 μM (Fig. 4). In this case the corresponding porphyrin is an equally effective inhibitor of $^{14}CO_2$ formation at the same concentration ratios (Fig. 4). However, the inhibition found with pentacarboxylate porphyrinogen III was not due to oxidation of this putative substrate to porphyrin, since possible preferential oxidation of one substrate was avoided by mixing the corresponding porphyrins before reduction. Table 2 shows that the maximum rates of formation of protoporphyrin and dehydroisocoproporphyrin are similar, in spite of the 20-fold difference between the apparent K_m values of the enzyme for their respective precursors. However, the rate of decarboxylation of coproporphyrinogen III is about twice that of pentacarboxylate porphyrinogen III, since only 1 mol of CO_2 is formed for each mol of dehydroisocoproporphyrinogen produced.

Discussion

Few measurements of the activity of coproporphyrinogen oxidase in mammalian liver have been reported. The activity found with the specific radiochemical method described here (330nmol of CO_2/h per g wet wt. of liver, 1.97nmol of CO_2/h per mg of protein) agrees well with the activity of 144–148nmol of protoporphyrin/h per g wet wt. found by Sano & Granick (1961) for rabbit and guinea-pig liver, but is much higher than the value of 0.12nmol of protoporphyrin/h per mg of protein obtained by Batlle *et al.* (1965) for rat liver. Both these groups measured protoporphyrin production by methods based on solvent extraction and partition, and it is probable that at least part of the difference between their results reflects the difficulty of obtaining high and reproducible recoveries of oxidized protoporphyrinogen with these techniques. The radiochemical method avoids this problem and is thus likely to give a more accurate measurement of enzyme activity. The rate of protoporphyrinogen formation cannot be calculated from measurements of $^{14}CO_2$ because the rate of production of tricarboxylate porphyrinogen is unknown. In theory, measurement of $^{14}CO_2$ production will not be an accurate method for measuring coproporphyrinogen oxidase activity unless the ratio of the rates of production of these two porphyrinogens is the same in all assays. Recent experiments in which the rates of decarboxylation of the 2- and 4-propionate groups of coproporphyrinogen III have been measured separately suggest that this ratio will be constant provided that initial rates are measured (Elder *et al.*, 1978).

Several of the authors who have found higher values (20–40μM) for the K_m of coproporphyrinogen oxidase for coproporphyrinogen III than that reported here (1.2μM) have pointed out that the K_m value is difficult to determine accurately owing to oxidation of the substrate during the course of the reaction (Sano & Granick, 1961; Batlle *et al.*, 1965; Hsu & Miller, 1970; Tait, 1972; Poulson & Polglase, 1974). Thus at substrate concentrations approaching the K_m the reaction rate may decrease before there has been sufficient conversion of substrate into product to measure accurately by solvent-partition methods, an effect that will be enhanced by competitive inhibition by oxidized substrate (Fig. 3). By measuring the formation of $^{14}CO_2$ from a substrate of high enough specific radioactivity (about 4.0mCi/mmol) initial rates can be determined at low substrate concentrations. This advantage may explain the lower K_m found with this method. *In vivo*, the concentration of substrate is probably well below 1.2μM, since De Matteis *et al.* (1973) found the total porphyrin concentration of rat liver to be 0.14nmol/g wet wt., using an extraction procedure that converted any porphyrinogen present into porphyrin.

Coproporphyrinogen oxidase has not been purified to homogeneity, and all investigations of its substrate specificity have been carried out with crude preparations. Evidence from experiments with [$^{14}C_1$]coproporphyrinogens, ^{14}C-labelled in the carboxyl carbon atom of either the 2- or 4-propionate substituents, indicates that both these groups may be oxidatively decarboxylated at a single active centre (Elder *et al.*, 1978). The mixed-substrate experiments were designed both to show that substrates other than coproporphyrinogen III were also decarboxylated at this site and to compare the apparent K_m values for different substrates. The unexpected finding that coproporphyrinogen I, which is not a substrate for the enzyme, inhibited the release of $^{14}CO_2$ indicated that the decreased rate of $^{14}CO_2$ formation in these experiments did not necessarily show that the alternative substrate and coproporphyrinogen III were metabolized at the same active centre. Thus it is possible that each alternative substrate inhibited coproporphyrinogen oxidase, yet was decarboxylated by a separate enzyme. This seems an unlikely explanation for our findings with harderoporphyrinogen and coproporphyrinogen IV. Unlike coproporphyrinogen I, both these compounds are much more effective inhibitors of $^{14}CO_2$ production than the corresponding porphyrins, and their K_m values and rates of metabolism are similar to those for coproporphyrinogen III. On the other hand, pentacarboxylate porphyrinogen III was a relatively poor inhibitor and, furthermore, did not differ from the corresponding porphyrin in this respect. However, it seems unlikely that a separate enzyme should exist to convert this compound into dehydroisocoproporphyrinogen when an identical side-chain degradation in other substrates appears to be catalysed by one enzyme. Study of the substrate specificity of the purified enzyme should solve this problem. The failure of coproporphyrinogen oxidase to decarboxylate the 4-propionate substituent of dehydroisocoproporphyrinogen may be explained by the presence of a neighbouring acetate group in this compound, since all propionate groups known to be attacked by this enzyme are flanked by methyl groups.

The experiments with pentacarboxylate porphyrinogen III described here provide the first direct evidence that a natural porphyrinogen, other than those directly involved in the reaction (coproporphyrinogen III and harderoporphyrinogen), is a substrate for coproporphyrinogen oxidase. In porphyria cutanea tarda in man and in porphyria caused by hexachlorobenzene in the rat, a marked increase in the production of dehydroisocoproporphyrinogen occurs when the formation of pentacarboxylate porphyrinogen III is increased (Elder, 1975; Elder *et al.*, 1976). The latter appears to be a compensatory change that follows a decrease in the activity of uroporphyrinogen decarboxylase (Kushner *et al.*, 1976;

Elder, 1977), the enzyme that catalyses the formation of coproporphyrinogen III from uroporphyrinogen III by sequential decarboxylation (Jackson *et al.*, 1976*b*). The circumstances under which increased formation of dehydroisocoproporphyrinogen is observed are therefore those under which the intrahepatic pentacarboxylate porphyrinogen III/coproporphyrinogen III concentration ratio would be expected to increase. Presumably this ratio determines the relative rates at which coproporphyrinogen oxidase decarboxylates these two substrates, so that very little dehydroisocoproporphyrinogen is formed under normal circumstances when the ratio is low. As the activity of uroporphyrinogen decarboxylase decreases and pentacarboxylate porphyrinogen III accumulates, the ratio increases until, when it reaches 20–30, the rates of production of dehydroisocoproporphyrinogen and protoporphyrinogen IX become similar. Both substrates are formed in the cytosol, whereas coproporphyrinogen oxidase is a mitochondrial enzyme (Sano & Granick, 1961). However, the enzyme appears to be in the intermembrane space of the mitochondrion (G. H. Elder & J. O. Evans, unpublished work) and therefore would be equally accessible to each compound.

Isocoproporphyrinogen, although not metabolized by coproporphyrinogen oxidase, is converted into the ethyl analogue of harderoporphyrinogen by uroporphyrinogen decarboxylase (Elder *et al.*, 1976). If the same is true for dehydroisocoproporphyrinogen, an alternative pathway for haem synthesis, which by-passes coproporphyrinogen III, exists (Elder, 1972). Whether this route is ever quantitatively important is at present unclear, not least because the formation of dehydroisocoproporphyrinogen in any quantity appears to be secondary to a decrease in the activity of the enzyme responsible for its further metabolism.

We thank Dr. J. R. Jackson for carrying out part of the synthesis of [$^{14}C_2$]coproporphyrin III, Professor A. H. Jackson for much helpful discussion, and Dr. D. E. Games for the mass spectra (all of the Department of Chemistry, University College, Cardiff, U.K.). We also thank Professor G. W. Kenner (University of Liverpool) and Professor A. H. Jackson for kindly providing samples of synthetic porphyrins. Financial support came from a Medical Research Council project grant.

References

Al-Hazimi, H. M. G., Jackson, A. H., Ryder, D. J., Elder, G. H. & Smith, S. G. (1976) *J. Chem. Soc. Chem. Commun.* 188–189

Baptista de Almeida, J. A. P., Kenner, G. W., Smith, K. M. & Sutton, M. J. (1975) *J. Chem. Soc. Chem. Commun.* 111–112

Batlle, A. M. del C., Benson, A. & Rimington, C. (1965) *Biochem. J.* **97**, 731–740

Cavaleiro, J. A. S., Kenner, G. W. & Smith, K. M. (1973) *J. Chem. Soc. Chem. Commun.* 183–184

Cornish-Bowden, A. (1974) *Biochem. J.* **137**, 143–144

De Matteis, F., Abritti, G. & Gibbs, A. H. (1973) *Biochem. J.* **134**, 717–727

Dixon, M. & Webb, E. C. (1966*a*) *Enzymes*, 3rd edn., pp. 84–87, Longmans Green, London

Dixon, M. & Webb, E. C. (1966*b*) *Enzymes*, 3rd edn., pp. 327–331, Longmans Green, London

Doss, M. (1974) in *Clinical Biochemistry, Principles and Methods* (Curtins, H. C. & Roth, W., eds.), vol. 2, pp. 1323–1371, Gruyter, Berlin

Edmondson, P. R. & Schwartz, S. (1953) *J. Biol. Chem.* **205**, 605–609

Elder, G. H. (1972) *Biochem. J.* **126**, 877–891

Elder, G. H. (1974) *Enzyme* **17**, 61–68

Elder, G. H. (1975) *J. Clin. Pathol.* **28**, 601–607

Elder, G. H. (1977) *Semin. Hematol.* **14**, 227–242

Elder, G. H., Evans, J. O. & Matlin, S. (1976) *Clin. Sci. Mol. Med.* **51**, 71–80

Elder, G. H., Evans, J. O., Jackson, J. R. & Jackson, A. H. (1978) *Biochem. J.* **169**, 215–223

Falk, J. E. (1964) *Porphyrins and Metalloporphyrins*, pp. 231–246, Elsevier, Amsterdam, London and New York

Frydman, R. B. & Frydman, B. (1975) *FEBS Lett.* **52**, 317–322

Games, D. E., Jackson, A. H., Jackson, J. R., Belcher, R. V. & Smith, S. G. (1976) *J. Chem. Soc. Chem. Commun.* 187–188

Granick, S. & Levere, R. D. (1964) *Prog. Hematol.* **4**, 1–47

Grinstein, M. (1947) *J. Biol. Chem.* **167**, 515–519

Hsu, W. P. & Miller, G. W. (1970) *Biochem. J.* **117**, 215–220

Jackson, A. H., Jones, D. M., Smith, S. G. & Elder, G. H. (1976*a*) *J. Chem. Soc. Chem. Commun.* 322–323

Jackson, A. H., Sancovich, H. A., Ferramola, A. M., Evans, N., Games, D. E., Matlin, S. A., Elder, G. H. & Smith, S. G. (1976*b*) *Philos. Trans. R. Soc. London Ser. B* **273**, 191–206

Kennedy, G. Y., Jackson, A. H., Kenner, G. W. & Suckling, C. J. (1970) *FEBS Lett.* **6**, 9–12

Kushner, J. F., Barbuto, A. J. & Lee, G. R. (1976) *J. Clin. Invest.* **58**, 1088–1097

Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275

Mombelli, L., McDonald, E. & Battersby, A. R. (1976) *Tetrahedron Lett.* **13**, 1037–1040

Porra, R. J. & Falk, J. E. (1964) *Biochem. J.* **90**, 69–75

Poulson, R. & Polglase, W. J. (1974) *J. Biol. Chem.* **249**, 6367–6371

Sano, S. (1966) *J. Biol. Chem.* **241**, 5276–5283

Sano, S. & Granick, S. (1961) *J. Biol. Chem.* **236**, 1173–1180

Sparatore, F. & Mauzerall, D. (1960) *J. Org. Chem.* **25**, 1073–1076

Stoll, M. S., Elder, G. H., Games, D. E., O'Hanlon, F., Millington, D. S. & Jackson, A. H. (1973) *Biochem. J.* **131**, 429–432

Suckling, C. J. (1970) Ph.D. Thesis, University of Liverpool

Tait, G. H. (1972) *Biochem. J.* **128**, 1159–1169

Watson, C. J., Cardinal, R. A., Bossenmaier, I. & Petryka, Z. J. (1975) *Proc. Natl. Acad. Sci. U.S.A.* **72**, 5126–5129

With, T. K. (1957) *Nature (London)* **179**, 824