Studies on Haem α -Methenyl Oxygenase

ISOMERIC STRUCTURE OF FORMYLBILIVERDIN, A POSSIBLE PRECURSOR OF BILIVERDIN

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1. The enzyme from guinea-pig liver that degrades pyridine-haemochromogen into formylbiliverdin, a possible precursor of biliverdin, breaks the porphyrin ring exclusively at the α -methene bridge. The enzyme is therefore appropriately named haem α -methenyl oxygenase. 2. It is deduced that this enzyme plays an essential role in haemoglobin catabolism *in vivo*.

Haemoglobin is transformed *in vivo* into bile pigments that are wholly IX α in structure (Gray, Nicholson & Nicolaus, 1958), i.e. they have been formed by removal of the α -methene bridge of protoporphyrin IX, the ferrous complex of which is the prosthetic group of haemoglobin. However, the exact mechanism of this process still remains to be elucidated.

Since the report on the transformation *in vitro* of haem into 'verdohaemochrome' (Lemberg & Legge, 1949) or 'green haemin' (Warburg & Negelein, 1930), numerous instances of haemin degradation under oxidative conditions have been attributed to the formation of peroxides. Lemberg & Legge (1949) presumed that haemoglobin could be converted into biliverdin in the presence of oxygen and ascorbic acid. Verdohaemochrome is a mixture of several degradation products (Nakajima, 1963), and studies on the bile pigments obtained from haem under these conditions showed that their formation involved loss of methene bridges other than that at the α -position (Petryka, Nicholson & Gray, 1962). Thus the degradation of the protoporphyrin of haem *in vitro* under the conditions described by Lemberg & Legge (1949) is unlikely to be of physiological significance in the metabolism of haemoglobin. The fission of pyridine-haemochromogen and of the haemoglobin-haptoglobin complex into formylbiliverdin, a possible precursor of biliverdin, by homogenates of ox and guinea-pig liver is an enzymic reaction (Nakajima, Takemura, Nakajima & Yamaoka, 1963). However, the product is different from that which is formed as a consequence of peroxide formation (Nakajima, 1963).

The present paper describes an investigation of the isomeric structure of enzymically produced formylbiliverdin.

EXPERIMENTAL

Chemicals. Haemin (iron content 8.2%) and 'silicic acid for lipid chromatography' were purchased from British Drug Houses Ltd. (Poole, Dorset). Prednisolone 21-phosphate was obtained from Merck, Sharp and Dohme Ltd. (Hoddesden, Herts.). 4-Carboxyethyl-3-methylpyrrole-2,5-dicarboxylic acid (I) was kindly given by Professor R. A. Nicolaus, and 3-methylpyrrole-2,4,5-tricarboxylic acid (II) by Miss G. A. Lemmon. The former had been prepared by the method of Fischer & Orth (1934) and the latter by condensing the oxime of ethyl acetoacetate with the sodium salt of ethylxaloacetate followed by alkaline hydrolysis (Nicolaus & Scarpati, 1962). All other chemicals were reagent grade.

Spectrophotometry. All spectra were determined with a Unicam SP.800 recording spectrophotometer.

Preparation of crude haem α -methenyl oxygenase. Male guinea pigs weighing 450–500 g. were injected intraperitoneally with 1.0 mg. of prednisolone 21-phosphate and 50 mg. of L-ascorbic acid/100 g. body wt. This treatment has been shown to induce the enzyme (O. Nakajima, unpublished work). The guinea pigs were killed by decapitation 4 hr. after injection and the livers were removed immediately. After coarse mincing with scissors, the livers were washed exhaustively with ice-cold 0.1 M-phosphate buffer, pH 7.5, to remove blood and other soluble substances. The organs were then homogenized in 4 vol. of the same buffer with a Potter-Elvehjem homogenizer. After standing at 4° for 1 hr. the homogenate was centrifuged at 1500 g for 15 min. and the supernatant was used as enzyme. All these procedures were performed below 5°. The protein concentration of the preparation was 40–50 mg./ml. as determined by the biuret method of Robinson & Hodgen (1940a,b).

Preparation of formylbiliverdin. Haemin (80 μ moles) was dissolved in 250 ml. of pure pyridine and to this was added 250 ml. of enzyme solution and 500 ml. of 0.5 M-veronal buffer, pH 8.3. The mixture was incubated aerobically in a 2 l. flask at 38° with gentle mechanical shaking for 40 min. The reaction was stopped by the addition of 400 ml. of acetic acid to the cooled reaction mixture and all subsequent

procedures were performed below 5°. The mixture was saturated with solid sodium acetate, and the green product and remaining substrate were quantitatively extracted with chloroform. The chloroform layer was collected by centrifugation, washed with an equal volume of cooled 0.01N-HCl and with water (to remove excess of pyridine), and then concentrated *in vacuo* to about 5 ml. The solution was left overnight at -10°, when 35-40 mg. of the product was obtained as green prisms. The product was further purified by partition chromatography on a 50 cm. $\times$ 2.5 cm. silicic acid column and recrystallization (Nakajima, 1963). In chloroform it showed four bands in the visible spectrum at 397, 498, 528 and 657 m μ . The product is thus spectroscopically identical with formylbiliverdin (Nakajima, 1963).

Hydrolysis of formylbiliverdin. Formylbiliverdin (20 mg.) was hydrolysed in 50 ml. of 0.16N-HCl for 6 hr. at 35°, to yield a bluish-green solution of biliverdin with absorption maxima at 665 and 375 m μ .

Oxidative degradation of the biliverdin. The bluish-green solution was diluted with 30 ml. of water, to give a solution 0.1N with respect to HCl, and treated dropwise with saturated KMnO₄ solution (about 2 ml.) until the permanganate colour persisted in the supernatant for 10 min. Excess of oxidant was destroyed with Na₂S solution, and MnO₂ was filtered off after brief boiling. The pH of the filtrate was adjusted to 4-4.5 with 2N-NaOH. Ten drops of 6M-CaCl₂ solution were added and, after standing 1 hr., any precipitate was removed by filtration. After ensuring that a portion of the solution afforded a precipitate with saturated ammonium oxalate solution, it was made strongly acid to Congo red by the addition of 12N-HCl. The resulting pyrrolic acid was extracted with peroxide-free diethyl ether, and the ethereal solution was dried over MgSO₄ and evaporated to 2 ml. *in vacuo*. Final evaporation to dryness was effected in a small test tube at 60-70°. Ethanol (1.0 ml.) was added to the residue and 12 hr. later the yellow solution was filtered and used for paper chromatography.

Paper chromatography of pyrrolic acids. Pyrrolic acids were separated by descending paper chromatography on Whatman no. 1 filter paper at about 20°. The solvent systems were butan-1-ol-acetic acid-water (4:1:5, by vol.), and ethanol-aq. ammonia (sp.gr. 0.88)-water (20:1:4, by vol.). The authentic pyrrolic acids (I) and (II) were used as markers. After the solvent front had advanced about 40 cm., the chromatograms were dried and sprayed successively with a freshly prepared solution of diazotized sulphanilic acid and 2N-NaOH. The pyrrolic acids appear as reddish-violet spots (Nicolaus, 1960).

Identification of the oxidation product of biliverdin. Only one pyrrolic acid was detected. The R_F values of the spot in the two solvent systems were identical with those of pyrrolic acid (I) (Table 1). After spraying with diazotized sulphanilic acid one or two additional yellow spots were observed, but these have not been identified.

Table 1. R_F values of pyrrolic acids

Substance	R_F values	
	Butan-1-ol-acetic acid-water (4:1:5, by vol.)	Ethanol-aq. ammonia (sp.gr. 0.88)-water (20:1:4, by vol.)
Pyrrolic acid prepared by oxidation of the formylbiliverdin-iron complex	0.82-0.85	0.06-0.09
Pyrrolic acid (I)	0.82-0.84	0.05-0.07
Pyrrolic acid (II)	0.45-0.47	0.22-0.24

Scheme 1. Isomeric verdins theoretically formed by fission of protoporphyrin IX, and their oxidation products the pyrrolic acids (I) and (II). M=CH₃; V=CH=CH₂; P=CH₂·CH₂·CO₂H.

Scheme 2. Proposed structure of compounds formed enzymically from pyridine-haemochromogen.

CONCLUSIONS

Partition chromatography on silicic acid has separated verdohaemochrome into five to seven components; on the other hand, formylbiliverdin behaves as a single component (Nakajima, 1963). Theoretically bile pigments IX α , IX β , IX γ and IX δ could be formed from haem depending on whether the α -, β -, γ - or δ -methene bridge is lost (Scheme 1). The α - and γ -isomers may be differentiated by oxidation with potassium permanganate, the former giving the acid (I) and the latter the acid (II). The other two isomers must give rise to mixtures of these two acids. The sole formation of pyrrolic acid (I) in our experiments shows that the biliverdin obtained from formylbiliverdin consists solely of the IX α isomer. Thus the degradation of protoporphyrin of haem *in vitro* by the enzyme from guinea-pig liver must involve the loss of the α -methene bridge (Scheme 2). The enzyme catalysing the degradation of haem into formylbiliverdin is therefore appropriately named 'haem α -methenyl oxygenase'. We conclude that the main pathway of haemoglobin metabolism *in vivo* must involve this oxygenase, since the naturally occurring tetra-pyrrolic bile pigments have only the IX α configuration (Gray *et al.* 1958). The physiological substrates of this enzyme are the haemoglobin-haptoglobin complex (Nakajima, 1962*a,b*), myoglobin (Nakajima, 1964) and foetal haemoglobin-haptoglobin complex (Kuma, 1966).

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