Urinary excretion of isomers of biliverdin after destruction *in vivo* of haemoproteins and haemin

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The amount and isomeric composition of urinary biliverdin in rabbits were analysed by h.p.l.c. Physiological values were maintained after the injection of haemin. On the other hand, when haemoglobins from several mammalian species were injected into rabbits, the excretion of biliverdin-IX α and biliverdin-IX β were increased 6–18-fold and 32–66-fold respectively over physiological excretion. Injection of myoglobin resulted in a 44-fold increase in excretion of the IX α -isomer. Coupled oxidation with ascorbate of haemoglobin and myoglobin by oxygen produced mainly the IX α - and IX β -isomers from haemoglobin and the IX α -isomer from myoglobin. The destruction of part of the haem from injected haemoproteins by non-enzymic chemical degradation would account for the observed respective increases in the excretion of biliverdin isomers. The excretion of biliverdin isomers after the injection of phenylhydrazine into rabbits was similar to that after the injection of haemoglobin.

In the physiological degradation of haem, protoporphyrin-IX is converted by oxidative cleavage at its α -meso position into biliverdin, and biliverdin is reduced to bilirubin. Three enzymes, haem oxygenase (EC 1.14.99.3), NADPH:cytochrome *c* reductase (EC 1.6.2.4), and biliverdin reductase (EC 1.3.1.24), and an oxidant and a reductant, O₂ and NADPH, are required to complete the process (Yoshida & Kikuchi, 1978). In a non-enzymic reaction *in vitro* known as coupled oxidation, O₂ and a reductant such as ascorbate or phenylhydrazine convert haem into biliverdin (Lemberg, 1956). Coupled oxidation of haem or haemoproteins with reducing agents is thought to involve the same chemical reactions as the physiological conversion of haem into biliverdin (Schmid & McDonagh, 1975). The two processes differ from each other, however, in the specificity of the cleavage site on the porphyrin ring of haem. Whereas physiological cleavage has been assumed to be specific for the α -meso position of protoporphyrin-IX, coupled oxidation of haem in the presence of pyridine results in cleavage at all four meso positions (Petryka *et al.*, 1962). In haemoproteins, the apoprotein must influence the site of cleavage, because coupled oxidation cleaves

only the α -meso position in myoglobin, and the α -meso and β -meso positions in haemoglobin (O'Carra & Colleran, 1969). Reports of the presence of bilirubin IX β in normal bile (Blumenthal *et al.*, 1977; Fevery *et al.*, 1983; O'Carra & Colleran, 1970) are in conflict with the assumed α -meso specificity of protoporphyrin-IX cleavage in physiological haem degradation.

Pigments excreted in the urine in Heinz-body haemolytic anaemia differ from bilirubin, but they have not been obtained in amounts and purity sufficient to permit their exact identification. Goldstein *et al.* (1968) found that Heinz bodies formed by exposure of erythrocytes to phenylhydrazine were catabolized to abnormal products and postulated an alternate pathway to physiological degradation that would account for the degradation of haem in Heinz bodies. Rokugo (1962) reported that biliverdin was one of the products found in urine after the administration of oxyhaemoglobin to rabbits. The isomeric composition of biliverdin depends on the type of degradative process and source of haem, and separation and quantification of the isomers of biliverdin has become feasible in recent years with the use of h.p.l.c. In the present work we have injected haemoglobin from different animals, myoglobin and phenylhydrazine into rabbits and have determined the identity and quantity of biliverdin isomers excreted in urine.

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Experimental procedures

Reagents and apparatus

Myoglobin (horse heart, primarily in the ferric form), haemin (bovine, type I), and biliverdin were purchased from Sigma (St. Louis, MO, U.S.A.). Haemolysates prepared by lysis of fresh erythrocytes with water were centrifuged to remove insoluble material, and the supernatant solutions were used for injection. The four isomers of biliverdin dimethyl ester were prepared from haemin (Bonnett & McDonagh, 1973). Concentrations of solutions of these isomers in methanol were calculated from the molar absorption coefficients of the isomers (Petrier *et al.*, 1979). These solutions were used for measurements of recorder responses to the isomers in h.p.l.c. analysis. Other reagents and solvents were of the best commercially available grades.

H.p.l.c.

H.p.l.c. was carried out on a Model 3A Shimadzu liquid chromatograph with a u.v. detector (Shimadzu SPD-2A). The detector was modified to detect biliverdin dimethyl esters at 656 nm, the wavelength of a strong line emitted by the deuterium lamp. The second-order emission was removed by a coloured glass filter (Toshiba) between the lamp and the detector cell. For analyses of the isomers of biliverdin dimethyl ester, use was made of a Zorbax ODS column (15 cm $\times$ 0.46 cm) from du Pont (Wilmington, DE, U.S.A.) with a mobile phase consisting of equal volumes of acetonitrile, methanol, and sodium acetate buffer (0.01 M, pH 3.65). The flow rate was 1.0 ml/min at a column temperature of 44°C.

Determination of urinary biliverdin

All operations were carried out in subdued light. To prepare samples for the standard curve, 0–0.5 μ mol of biliverdin-IX α (Sigma; 60–75% purity according to our analysis) in acetone/water (1:1, v/v; 1.5 ml) was added to normal rabbit urine (20 ml). The normal urine with added biliverdin and the sample (20 ml) from an injected animal were diluted with an equal volume of water and acidified to pH 3.8 with acetic acid. Talc (10 g) was added, and the suspension was shaken for 1 h at room temperature to adsorb biliverdin. The talc was filtered off, washed twice with water (25 ml), and dried over silica gel in a vacuum. A suspension of the dried talc in a solution (10 ml) of 5% (v/v) H₂SO₄ acid in methanol was left overnight at 4°C to esterify biliverdin. The talc was filtered off and washed twice with 5 ml and then twice with 3 ml of methanol. The combined filtrate and washings (about 17 ml) were diluted with 70 ml of water and extracted with three 5 ml portions of chloroform.

The extract was washed with four 10 ml portions of water and dried over anhydrous Na₂SO₄ (5 g). The sulphate was filtered off and washed twice with chloroform (3 ml). The filtrate and washings were combined and evaporated *in vacuo*. A solution of the resulting residue of biliverdin dimethyl ester in methanol (3 ml) was diluted with water (7 ml), and passed through a Sep-Pak C-18 cartridge (Waters Associates) that had been regenerated by the successive passing of 2 ml of methanol and 2 ml of methanol/water (3:7, v/v). The ester, which had been retained on the cartridge, was eluted with methanol (3 ml) and the eluate was evaporated. The residue was dissolved in the mobile phase for h.p.l.c. and injected on the h.p.l.c. column. Usually esters stored at –10°C for a few days were injected.

The elution sequence of the isomers of biliverdin dimethyl ester was IX α , IX β , IX δ , and IX γ , and the respective relative absorbances at 656 nm were 1.0, 1.25, 1.02 and 0.90. The peak corresponding to each isomer was cut out from the h.p.l.c. recording chart and weighed. Isomer proportion was calculated from this weight and the concentration of the eluate corresponding to the peak. The concentration of the IX α -isomer was obtained from a standard curve, which was determined for each analysis. Concentrations of the other isomers were obtained from the same standard curve with corrections for differences in their relative absorbances.

Results

Retention times by h.p.l.c. of the isomers of biliverdin dimethyl ester from samples were measured, and the isomers were identified by comparison with the retention times of known samples of the four isomers prepared by the coupled oxidation of haemin (Fig. 1). Isomers from samples except those described below were also identified by their relative positions after t.l.c. (Bonnett & McDonagh, 1973; Yoshinaga *et al.*, 1982). These identifications were confirmed by comparison of absorption spectra with published spectra of the isomers (Blanckaert *et al.*, 1976; Bonnett & McDonagh, 1973; Rasmussen *et al.*, 1980; Yamaguchi *et al.*, 1979). Retention times and spectral parameters of typical samples are listed in Table 1. The IX β -isomer in the urine of non-treated and myoglobin-treated rabbits, and the IX δ -isomer in urinary samples and in the product of coupled oxidation of haemoglobin were present in amounts too small to permit similar confirmation. These isomers were identified by h.p.l.c. with an independent system in which known isomers prepared by the coupled oxidation of haemin were eluted in a different sequence.

Fig. 1. *H.p.l.c. chromatograms of biliverdin dimethyl esters*

The ester was analysed on a reversed-phase column with a mobile phase consisting of equal volumes of acetonitrile, methanol and sodium acetate buffer (0.01M, pH 3.65). The esters were detected at 656nm. Peaks labelled I, II, III, and IV are those of the IX α -, IX β -, IX δ - and IX γ -isomers respectively. Biliverdin in the urine was adsorbed on talc and was esterified as described in the Experimental procedures section. (a) Four isomers prepared by the coupled oxidation of haemin (Bonnett & McDonagh, 1973). (b) Control urine. (c) Day-5 urine of phenylhydrazine-treated rabbit (cf. Fig. 2).

H.p.l.c. with use of a mobile phase consisting of acetate buffer (0.01M, pH 3.65)/acetonitrile (5:6, v/v) resulted in the isomer elution sequence: IX α -, IX δ -, IX β -, IX γ -isomer (cf. Fig. 1).

The effect of various procedures on the results of isomer analysis of biliverdin dimethyl ester was examined. The ester was passed through a Sep-Pak cartridge; without this passage the back pressure increased abnormally in the course of h.p.l.c. analyses and interfered with the continuous analysis of a large number of samples. This procedure did not change isomer proportions. Neither storage in methanol nor adsorption on talc significantly altered isomer proportions.

Isomeric compositions of biliverdin dimethyl ester obtained by coupled oxidation and esterification of haemin, horse heart myoglobin and several mammalian haemoglobins are shown in Table 2. O'Carra & Collieran (1969) observed only the IX α -isomer from myoglobin and the IX α - and IX β -

isomers from haemoglobin by t.l.c. The detection of small fractions of other isomers in the present work may have been due to the greater sensitivity of the detection system in h.p.l.c.

The urinary biliverdin of control rabbits was determined after esterification, and the results are shown in Table 3. Amounts excreted differed between the animals, but isomer proportions were relatively constant, with 94–96% IX α -isomer and 4–6% IX β -isomer. Of the estimated total physiological haem degradation, about 0.3% and 0.01–0.02% were recovered as urinary biliverdin-IX α and biliverdin-IX β respectively.

Haemoproteins were injected intravenously into rabbits, and the subsequent urinary excretion of biliverdin isomers was determined as shown in Table 4. Increased excretion of biliverdin was found in urine samples collected for up to 24 and 48 h respectively after the injection of haemoglobin and myoglobin. Later samples showed normal levels of biliverdin.

Increases over the physiological excretion of biliverdin isomers that followed the injection of haemoproteins and the isomer composition of the excess amounts excreted are shown in Table 5. After the injection of haemoglobin, excretion of both the IX α - and the IX β -isomer was increased, but the proportionate increases in the excretion of the IX β -isomer was 3–7 times that of the IX α -isomer. A small fraction of IX δ -isomer, which is absent from normal urine, was excreted after the injection of haemoglobin. Injection of myoglobin resulted in a greater increase in biliverdin excretion than did injection of haemoglobin. Most of this increase was due to biliverdin-IX α . When haemin was injected into a rabbit in the same dosage, neither the amount nor the isomer proportion of urinary biliverdin was altered from that of control urine (results not shown).

Daily subcutaneous injections of phenylhydrazine were administered to a rabbit, and 24 h urine samples were collected for 5 days. The isomer content of urinary biliverdin in each sample was determined, and the results are shown on Fig. 2. Increase in the excretion of the IX α -isomer commenced immediately after the injection, whereas that of the IX β -isomer was not detected until day 4. The IX δ -isomer, which was absent in a control urine, was detected after 5 days. At this time, the proportions of these isomers were: IX α , 80%; IX β , 17%; and IX δ , 3%. The IX γ -isomer was not detected.

Discussion

Owing to the asymmetrical arrangement of the substituents of protoporphyrin-IX, the oxidative degradation of haem can result in one or more of

Table 1. *Chromatographic and spectral parameters of biliverdin dimethyl esters*

Esters were obtained by coupled oxidation and esterification of haemin (Bonnett & McDonagh, 1973) and haemoglobin (O'Carra & Colleran, 1969), and isomers were separated by t.l.c. Urinary biliverdin was adsorbed on talc and esterified as described in the Experimental procedures section. The isomers were separated by t.l.c. and h.p.l.c. Abbreviation used: HbO₂, oxyhaemoglobin.

Source of ester	Isomer	R _F *	Retention time (min)	λ _{max} (nm)†	A ₃₈₀ /A ₆₅₀
Coupled oxidation of haemin	IXα	0.20	20.1	379–380, 650–663	3.5
	IXβ	0.25	24.8	381–382, 639–650	3.3
	IXγ	0.18	39.0	376–377, 640–643	2.7
	IXδ	0.15	25.8	377–378, 642–661	2.9
Coupled oxidation of human HbO ₂	IXα	0.20	20.1	378–380, 646–665	3.7
	IXβ	0.25	24.8	381–382, 640–657	3.4
Human HbO ₂ -injected rabbit urine‡	IXα	0.20	20.1	379–380, 645–663	3.7
	IXβ	0.25	24.8	381–382, 640–656	3.3
Phenylhydrazine-injected rabbit urine§	IXα	0.20	20.1	379–380, 650–663	3.7
	IXβ	0.25	24.8	381–382, 640–650	3.7
Non-treated rabbit urine	IXα	0.20	20.1	377–381, 648–665	3.7
	IXβ	0.25	24.8	Not determined	

* A glass plate precoated with silica gel 60 (0.25mm) (Merck); solvent, 3% (v/v) acetone in chloroform.

† In chloroform.

‡ 24h-urine after the injection of haemoglobin (cf. Table 4).

§ Day-5 urine of phenylhydrazine-treated rabbit.

Table 2. *Proportions of biliverdin isomers produced by coupled oxidation in vitro*

Haemoproteins (O'Carra & Colleran, 1969) and haemin (Bonnett & McDonagh, 1973) were subjected to oxidation and esterification. The resulting esters were analysed by h.p.l.c. Abbreviations used: HbO₂, oxyhaemoglobin; Mb, myoglobin.

Material	Isomer	...	Amount produced (%)			
			IXα	IXβ	IXγ	IXδ
Rabbit HbO ₂ †			56	41	0	3
Rat HbO ₂ †			65	35	0	1
Guinea pig HbO ₂ †			76	24	0	0
Human HbO ₂ ‡			56	43	0	0
Horse heart Mb‡			96	4	0	0
Haemin‡			33	17	4	36

† Means for two experiments.

‡ Means for four experiments.

four structural isomers of biliverdin. Cleavage controlled by haem oxygenase in physiological degradation is specific for the α-meso position and produces only biliverdin-IXα (Noguchi *et al.*, 1982; Yoshinaga *et al.*, 1982). Coupled oxidation, on the other hand, results in random cleavage at all four meso positions in haemin and denatured haemoproteins and in preferred cleavage at one or two of the four positions in native haemoproteins (O'Carra & Colleran, 1969, 1976; O'Carra, 1975). Accordingly the mode of degradation and the source of haem together determine the isomeric composition of biliverdin produced in the non-enzymic oxidative cleavage of haem. In the present work, rabbits were injected with several haemo-

proteins, and the resulting amount and isomeric composition of urinary biliverdin were determined. Urinary biliverdin was used because its yield can be quantified and because it is obtainable non-surgically.

Coupled oxidation of haemoglobin has been reported to produce the IXα- and IXβ-isomers of biliverdin (O'Carra & Colleran, 1969). Small fractions of products with the elution behaviour of the IXδ-isomer in two h.p.l.c. systems were also observed in the present study. Rabbit and human haemoglobins produced more of the IXβ-isomer than did guinea pig and rat haemoglobin. Previously only the IXα-isomer was found in the coupled oxidation of myoglobin (O'Carra &

Table 3. *Biliverdin in the urine of control rabbits*

Urine samples (24h) were collected and their biliverdin contents (C) were determined as described in the Experimental procedures section. Physiological degradation of 9 μmol of haem/day per kg of rabbit wt. was estimated on the basis of a 68-day mean life span for erythrocytes (Brown & Eadie, 1953; Neuberger & Niven, 1951), 58 ml of whole blood per kg of rabbit wt. (Courtice & Gunton, 1949), 11.9 g of haemoglobin per 100 ml of whole blood (Albritton, 1952), and 70% as the proportion of haem derived from haemoglobin (Berthelot *et al.*, 1982).

Rabbit	Rabbit body wt. (kg) (A)	Haem physiologically degraded ($\mu\text{mol/day}$) (B*)	Urinary excretion ($\mu\text{mol/day}$) (C)	Proportion of physiologically degraded haem excreted (%) (D†)
1	3.57	32.1	IX α , 0.080 (94‡) IX β , 0.005 (6)	0.249 0.016
2	3.78	34.0	IX α , 0.065 (96) IX β , 0.003 (4)	0.191 0.009
3	3.17	28.5	IX α , 0.060 (95) IX β , 0.003 (5)	0.211 0.011
4	3.46	31.1	IX α , 0.140 (96) IX β , 0.006 (4)	0.450 0.019
Mean $\pm$ s.d.	3.50 $\pm$ 0.25	31.4 $\pm$ 2.3	IX α , 0.086 $\pm$ 0.037 (95.3 $\pm$ 1.0) IX β , 0.004 $\pm$ 0.002 (4.8 $\pm$ 1.0)	0.275 $\pm$ 0.119 0.014 $\pm$ 0.005

* $B = A \times 9$.

† $D = (C/B) \times 100$.

‡ Isomer proportion (%).

Table 4. *Biliverdin in the urine of haemoprotein-treated rabbits*

To solutions of haemoglobin and myoglobin, NaCl was added to a concentration of 0.9%. These solutions (10 μmol as haem per kg of rabbit wt.) were injected into the rabbit ear vein at a concentration of 10 mM. The injected animals were the same as those in Table 3; 24 h urine samples after the injection of oxyhaemoglobin (HbO₂) and 48 h urine samples after the injection of myoglobin (Mb) were collected, and the biliverdin contents (F) and isomer proportions were determined. Results are means for two experiments.

Rabbit	Injection		Biliverdin after injection	
	Haemoprotein source	Amount injected as haem (μmol) (E)	Urinary excretion (μmol) (F)	Proportion of total degraded haem excreted (%) (G*)
1	Rabbit HbO ₂	35.7	IX α , 0.570 (74‡) IX β , 0.167 (22) IX δ , 0.030 (4)	0.841 0.246 0.044
2	Guinea-pig HbO ₂	37.8	IX α , 1.230 (87) IX β , 0.170 (12) IX δ , 0.014 (1)	1.713 0.237 0.019
3	Human HbO ₂	31.7	IX α , 0.630 (72) IX β , 0.201 (23) IX δ , 0.044 (5)	1.047 0.334 0.073
4	Horse Mb	34.6	IX α , 6.370 (98) IX β , 0.101 (2)	9.700 0.154

* $G = [F/(B+E)] \times 100$ (For the significance of quantity B, see Table 3).

† Isomer proportion (%).

Colleran, 1969), but small amounts of a product that chromatographed in the same way as the IX β -isomer were also observed in the present work.

The amount of haem injected into rabbits in the form of free haemin or haemoglobin was nearly the

same as the amount destroyed physiologically each day in the normal animal (Brown & Eadie, 1953; Neuberger & Niven, 1951; Courtice & Gunton, 1949; Albritton, 1952; Berthelot *et al.*, 1982; cf. the legend to Table 3). The injection of haemin did not alter the normal urinary excretion pattern of the

Table 5. *Effect of haemoprotein injection on biliverdin excretion*
The animals are the ones in Tables 3 and 4.

Rabbit	Biliverdin	Increase in excretion after injection (μmol) (H^*)	Proportion of injected haem excreted (%) ($I^\dagger$)	H/C
1	IX α	0.490 (71.8 $\dagger$)	1.4	6.1
	IX β	0.162 (23.8)	0.5	32.4
	IX δ	0.030 (4.4)	0.1	—
2	IX α	1.165 (86.6)	3.1	17.9
	IX β	0.167 (12.4)	0.4	55.7
	IX δ	0.014 (1.0)	0.04	—
3	IX α	0.570 (70.2)	1.8	9.5
	IX β	0.198 (24.4)	0.6	66.0
	IX δ	0.044 (5.4)	0.1	—
4	IX α	6.090 (98.6)	17.6	43.5
	IX β	0.089 (1.4)	0.3	14.8

* $H = F - C$ for the injection of oxyhaemoglobin; $H = F - 2C$ for the injection of myoglobin.

$\dagger I = (H/E) \times 100$; see Tables 3 and 4 for C, E and F.

$\ddagger$ Isomer proportion (%).

Fig. 2. *Amounts of biliverdin isomers excreted in the urine of phenylhydrazine-treated rabbit*

Phenylhydrazine hydrochloride dissolved in water was neutralized with 10% (w/v) NaOH. NaCl was added to obtain a concentration of 0.9%. The solution (2.0 ml) was divided into three equal parts, and each solution was injected subcutaneously daily (at arrows) into three points at least 2 cm apart on the back of the rabbit (2.7 kg body wt.). The daily amount was 75 μmol of phenylhydrazine per kg of rabbit wt. Biliverdin in 24 h urine was determined as described in the Experimental procedures section. H.p.l.c. conditions were the same as in Fig. 1. ●, IX α -isomer; ○, IX β -isomer; ▲, IX δ -isomer. IX γ -isomer was not detectable.

rabbit. This result suggested that exogenous free haemin was degraded by the physiological pathway, which is believed to have a capacity far in excess of physiological turnover (Schmid &

McDonagh, 1978). In contrast, injection of a haemoprotein was followed by multifold increase in the excretion of biliverdin isomers.

When haemoglobin was injected, the excretion of both the IX α - and IX β -isomers was increased. Compared with physiological levels, the absolute increase was greater for the IX α -isomer, but the relative increase was greater for the IX β -isomer. The increase in excretion after myoglobin injection was largely due to the IX α -isomer. The large increase in the excretion of the IX β -isomer by animals injected with haemoglobin is evidence of a non-physiological process, because physiological degradation produces only the IX α -isomer. The large increase in IX α -isomer excretion after myoglobin injection likewise indicates a non-physiological process, since biliverdin-IX α produced enzymically is rapidly converted into bilirubin (Barrowman *et al.*, 1976; Berthelot *et al.*, 1982). Coupled oxidation is a non-enzymic chemical reaction, the products of which would account both for the increased formation of IX β -isomer from haemoglobin and the preponderant formation of IX α -isomer from myoglobin. The biliverdin excreted in urine is, however, equivalent to but a small fraction of the haem from injected haemoprotein. The bulk of the haem from exogenous haemoprotein may be released intact from its apoprotein and be degraded physiologically, or else most of the biliverdin from coupled oxidation may be reduced to bilirubin. Biliverdin is not the only product of abnormal haem catabolism. Other pigments that are produced and are excreted in bile and urine (Goldstein *et al.*, 1968) are, however, not quantifiable because their chemical structures are not known.

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