Biochem. J. (1984) **221**, 717–721
Printed in Great Britain

Biliary and urinary excretion rates and serum concentration changes of four bilirubin photoproducts in Gunn rats during total darkness and low or high illumination

Shoju ONISHI,*§ Takatoshi OGINO,† Takao YOKOYAMA,† Kenichi ISOBE,* Susumu ITOH,* Takeshi YAMAKAWA* and Takashi HASHIMOTO‡

**Department of Pediatrics, Kagawa Medical School, Miki, Kitagun, Kagawa 761-07, Japan, and †Department of Pediatrics and‡ First Department of Surgery, Nagoya City University Medical School, Mizuhoku, Nagoya 467, Japan*

(*Received 9 February 1984/Accepted 17 April 1984*)

On cycled exposure of Gunn rats to total darkness and low and high illumination, biliary excretion rates of (*EZ*)- and (*ZE*)-bilirubin and (*EZ*)-cyclobilirubin increased up to approx. 10-fold from the mean basal values of 1.2 and 0.2 μg/h to the mean maximum values of 25.2 and 4.2 μg/h respectively, and at the same time those of (*EE*)-bilirubin and (*EE*)-cyclobilirubin also increased, but at very much lower rates than those of the first-mentioned two. During the low illumination only (*EZ*)- and (*ZE*)-bilirubin and (*EZ*)-cyclobilirubin appeared in the urine; during the high illumination (*EE*)-bilirubin and (*EE*)-cyclobilirubin also appeared, showing a similar excretion pattern to that observed in the bile, but the total urinary excretion rates were lower than the total biliary excretion rates. The serum bilirubin concentrations fell gradually to lower values, accompanied by an increment in (*EZ*)- and (*ZE*)-bilirubin, but (*EZ*)-cyclobilirubin was not detected. It is concluded that during phototherapy the predominant pathway for the removal of bilirubin from the body in the Gunn rat is by biliary excretion of the geometric photoisomers (*EZ*)- and (*ZE*)-bilirubin, derived from $Z \rightarrow E$ isomerization, and the structural photoisomer (*EZ*)-cyclobilirubin, formed from intramolecular endo-vinyl cyclization.

Since the original report by Cremer *et al.* (1958), phototherapy has been successfully applied to the treatment of hyperbilirubinaemia of the newborn infant, and is now an established procedure in neonatal medicine (Brown & McDonagh, 1980). In 1969, we found an increment of propentdyopent adducts and an appearance of a substance with $\lambda_{max.}$ 415nm in the urine after phototherapy (Onishi *et al.*, 1969, 1971). An observation on the urinary propentdyopent adducts was independently reported by Porto (1970). The major mechanism was initially postulated as the formation of oxidative bilirubin derivatives by photosensitized attack by singlet oxygen (McDonagh, 1971). However, the fact that considerable amounts of unconjugated bilirubin appear in the bile of Gunn rats (Ostrow, 1971) or newborn infants (Lund & Jacobsen, 1972) after irradiation with blue light is not consistent with this mechanism. According to the latest information (Stoll *et al.*, 1982; McDonagh *et al.*, 1982*a*,*b*; Onishi *et al.*, 1980*b*, 1984), the major bile pigments excreted during phototherapy are (*EZ*)- and (*ZE*)-bilirubin IXα, photobilirubin IXα and (*EZ*)-cyclobilirubin IXα, an 'unknown pigment' named by us. It is now established in experiments *in vitro* that bilirubin undergoes rapid reversible configurational photoisomerization to *E* isomers and that the endo-vinyl group of 4*E* isomer is cyclized further photochemically to (*EZ*)-cyclobilirubin IXα, a structural isomer of bilirubin (Onishi *et al.*, 1981*b*, 1984; Stoll *et al.*, 1982; Isobe *et al.*, 1983). Since Onishi *et al.* (1984) established the structures of all the theoretically expected photoproducts of bilirubin IXα, we undertook to determine the proportions of the geometric and structural photoisomers formed in bilirubin meta-

Abbreviation used: h.p.l.c., high-pressure liquid chromatography.

§ To whom correspondence should be addressed.

bolism during phototherapy and how light affects the biliary and urinary excretion pattern of the individual photoproducts in the Gunn rat. A preliminary report of these results has been published (Ogino *et al.*, 1983). Gunn rats, which are a strain of Wistar rats, have non-haemolytic jaundice as a result of UDP-glucuronyltransferase deficiency, a condition that corresponds to Criglar–Najjar syndrome in humans. The predominant natural bilirubin isomer, (*ZZ*)-bilirubin IXα, is simply referred to as bilirubin. Also, the description of an isomeric form of bilirubin IXα has been omitted.

Materials and methods

Preparation of experimental animals

Five adult female homozygous Gunn rats weighing 230–288g were used. Before the experiment, the animals were shaved on their backs, and residual fur was removed with a depilating cream. Under diethyl ether anaesthesia, cannulas were placed into a common bile duct, a femoral vein and the urethra of Gunn rats. The animals were placed in restraining cages and kept under an i.r. heating lamp. A solution containing glucose (2.6g/100ml), NaCl (70mequiv./l) and sodium lactate (20mequiv./l) was infused through the femoral cannula at 2.5ml/h throughout the experiments.

Illumination of experimental animals and collection of bile, urine and serum

After the recovery period from anaesthesia and operation, there then followed two successive periods of illumination of each animal. Each illumination consisted of three experimental periods: a control period with the animal in the dark, a light period during which the rat was exposed to light provided by four 20 blue–white fluorescent lamps (Matsushita FL 20 BW), and a recovery period in which the canopy lights were turned off. The light-intensity of each illumination, determined with a Minolta Air-Shields Fluoro-litemeter, was as follows: the mean irradiances of the first and the second experiments were 1.4μW/cm^2 per nm (low illumination) and 22.5μW/cm^2 per nm (high illumination) respectively, and their durations are shown in the Figures. Bile and urine were collected by the external bile fistula and urethral canula respectively, in Pyrex tubes shielded from light by aluminium foil, as 1h fractions throughout the experiments. The volume of each specimen was measured. During the experiments blood was drawn periodically from an incised tail vein for the determination of bilirubin and its photoproducts and haematocrit values. All samples were frozen immediately at −70°C and kept in the dark until analysis by h.p.l.c.

Sample preparation for h.p.l.c.

The deproteinizing reagent was acetonitrile/0.01M-phosphate buffer (pH7.4)/10% (w/v) tetra-n-butylammonium hydroxide (60:40:1, by vol.) was adjusted with H_3PO_4 to pH7.4. For the serum samples, 50μl of dimethyl sulphoxide and 150μl of the deproteinizing reagent were added to 50μl of the serum. For the bile, 4 vol. of the deproteinizing reagent only was added to 1 vol. of the bile. For the urine, an equal volume of the deproteinizing reagent was added. Then these samples were vortex-mixed for 30s and centrifuged for 5min at 1000rev./min. Portions (25–50μl) of supernatant were applied to the chromatograph. All manipulations of the samples were carried out in subdued light. Chromatographic operation, preparation of a calibration curve of peak area and assignment of each peak separated by h.p.l.c. were carried out as described previously (Onishi *et al.*, 1979, 1980*a*). The limit of detection of the h.p.l.c. method was 1ng/h, but values below 10ng/h are plotted as 10ng/h in the Figures.

Pigment and reagents

Bilirubin (E. Merck, Darmstadt, Germany) and tetra-n-butylammonium hydroxide (Wako, Osaka, Japan) were used without further purification. Dimethyl sulphoxide, acetonitrile and all other reagents used were of analytical grade.

Results

Biliary excretion of bilirubin and its photoproducts (Fig. 1 and Table 1)

During the control period the animals produced pale-yellow bile, containing low concentrations of bile pigments, which was excreted at a steady rate. On exposure of animal to low illumination, the bile promptly turned a deep golden-brown colour, and the change was accompanied by an approx. 4–10-fold increase in excretion of bilirubin as (*EZ*)- and (*ZE*)-bilirubin and a concomitant greater rise in the excretion of (*EZ*)-cyclobilirubin. On exposure to high illumination, the biliary excretion rates of these photoproducts were further increased compared with those in low illumination. Biliary excretion rates of photoproducts during high illumination were approx. 2–5 times those during low illumination. Under low illumination biliary excretion of (*EE*)-bilirubin and (*EE*)-cyclobilirubin increased, and during high illumination the biliary excretion rate of (*EE*)-cyclobilirubin approached that of (*EE*)-bilirubin. The relative proportions of the last-mentioned two photoproducts in the total biliary excretion rates of bile pigments were significantly smaller than those of the first-mentioned two. After the light had been

Table 1. *Biliary excretion rates of bilirubin and its photoproducts during cycled exposure of Gunn rats to total darkness and low and high illumination*

For experimental details see the text.

Experiment	Biliary excretion rate (ng/h)												
	Total darkness	Low illumination (1.4 μW/cm² per nm)					Total darkness		High illumination (22.5 μW/cm² per nm)				
Duration of experiment (h)		1	2	3	4	5			1	2	3	4	5
No. of animals	6	6	6	6	6	6	5	3	5	5	5	5	5
(*EZ*)- and (*ZE*)-Bilirubin													
Mean	1158	3418	7199	10070	9079	9703	6520	4882	12486	25187	20986	16469	13964
S.E.M.	309	812	941	1563	1008	1424	970	1098	2902	5337	4424	3509	3499
(*EZ*)-Cyclobilirubin													
Mean	180	378	674	872	814	713	530	230	1749	3549	3652	4194	3446
S.E.M.	87	141	225	281	243	118	142	77	397	758	533	964	738
(*EE*)-Cyclobilirubin													
Mean	2	20	33	51	43	44	16	2	196	375	363	426	405
S.E.M.	1	13	10	17	11	12	5	1	70	161	139	151	172
(*EE*)-Bilirubin													
Mean	18	70	93	141	133	140	101	51	222	352	298	390	313
S.E.M.	13	44	42	54	47	53	43	39	87	172	104	114	114

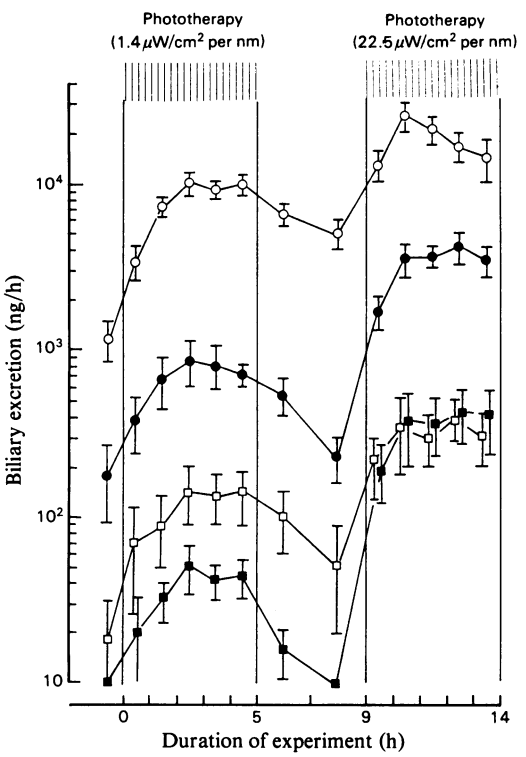

Fig. 1 *Excretion patterns of four bilirubin photoproducts by the five Gunn rats exposed to successive periods of low and high illumination*

For experimental details see the text. ○, (*EZ*)- and (*ZE*)-Bilirubin IXα; ●, (*EZ*)-cyclobilirubin IXα; □, (*EE*)-bilirubin IXα; ■, (*EE*)-cyclobilirubin IXα.

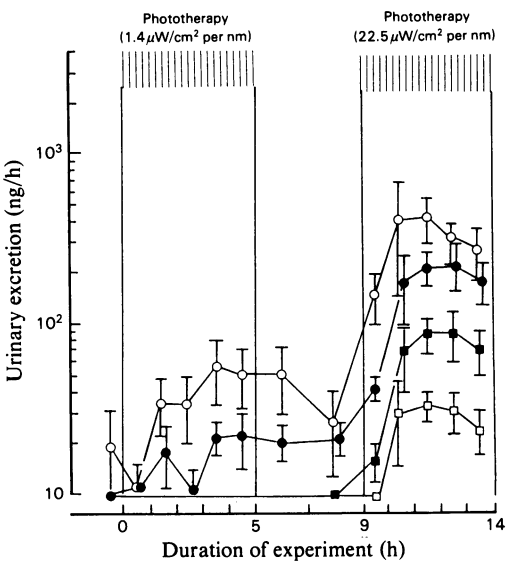

Fig. 2. *Urinary excretion patterns of four bilirubin photoproducts by the five Gunn rats exposed to successive periods of low and high illumination*

For details and explanation of the symbols see the legend to Fig. 1.

extinguished the various excretion rates gradually reverted to control values. The flow rates of all but one animal decreased during high illumination.

Urinary excretion of bilirubin and its photoproducts (Fig. 2 and Table 2)

On exposure of animals to low illumination, urinary excretion rates of bilirubin as (*EZ*)- and

Table 2. *Urinary excretion rates of bilirubin and its photoproducts during cycled exposure of Gunn rats to total darkness and low and high illumination*
For experimental details see the text.

Experiment	Urinary excretion rate (ng/h)												
	Total darkness	Low illumination (1.4 μW/cm^2 per nm)					Total darkness		High illumination (22.5 μW/cm^2 per nm)				
Duration of experiment (h)		1	2	3	4	5			1	2	3	4	5
No. of animals	6	5	5	5	6	6	6	3	6	6	5	6	6
(*EZ*)- and (*ZE*)-Bilirubin													
Mean	19	11	35	35	58	52	52	27	151	425	430	329	276
S.E.M.	12	4	13	14	24	19	22	14	46	271	132	102	93
(*EZ*)-Cyclobilirubin													
Mean	13	11	18	11	22	23	21	22	43	187	222	229	181
S.E.M.	4	4	7	3	5	7	5	5	7	81	55	68	48
(*EE*)-Cyclobilirubin													
Mean	0	0	0	1	4	4	3	0	16	72	88	91	73
S.E.M.	0	0	0	1	2	2	2	0	4	31	20	27	20
(*EE*)-Bilirubin													
Mean	1	0	2	3	7	5	5	0	9	31	34	32	24
S.E.M.	1	0	2	1	3	2	2	0	4	16	7	9	7

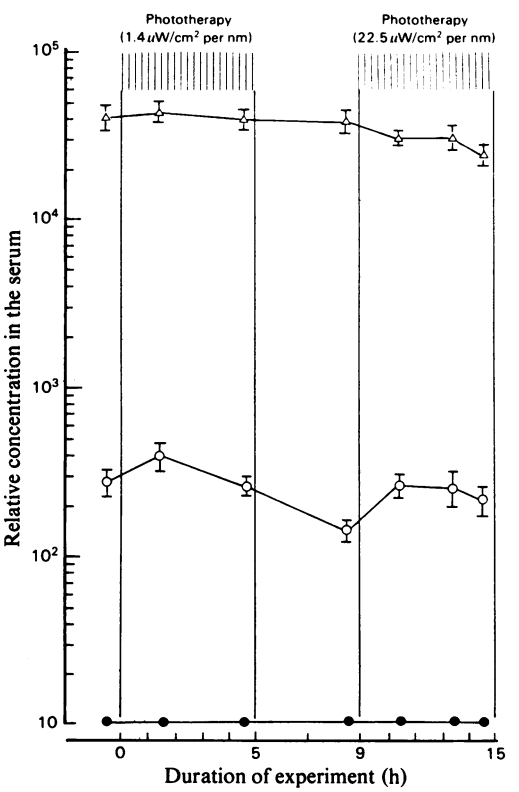

Fig. 3. *Effects of cycled environmental total darkness and phototherapy on serum concentrations of total bilirubin and (EZ)- and (ZE)-bilirubin IXα of five Gunn rats*
△, Total serum bilirubin; ○, (*EZ*)- and (*ZE*)-bilirubin IXα; ●, (*EZ*)-cyclobilirubin.

(*ZE*)-bilirubin and (*EZ*)-cyclobilirubin increased slightly, but (*EE*)-bilirubin and (*EE*)-cyclobilirubin were negligible. On high illumination, those of the first-mentioned two photoproducts increased up to 10-fold, and the last-mentioned two also increased, although the rates were still very low. The total urinary excretion rate was low compared with the total biliary excretion rate. No significant alteration in urine flow was observed under low or high illumination.

Changes in concentrations of plasma bilirubin and its photoproducts (Fig. 3 and Table 3)

During the control period, the rat maintained a stable concentration of serum bilirubin. On exposure to light, the serum bilirubin concentration fell gradually to low values. As found for a hyperbilirubinaemic newborn infant, (*EZ*)- and (*ZE*)-bilirubin in the serum of the Gunn rat was demonstrated before and during phototherapy, but only at a low concentration. (*EZ*)-Cyclobilirubinin remained below the detection limit during phototherapy. During the recovery period, the bilirubin concentration remained unchanged, but (*EZ*)- and (*ZE*)-bilirubin decreased.

Discussion

Concerning the main process responsible for hepatic bile-pigment excretion during phototherapy, Ostrow (1971) described the excretion of considerable amounts of unconjugated bilirubin, which has proved to be excreted in the form of (*EZ*)- and (*ZE*)-bilirubin (Onishi *et al.*, 1981*a*).

Table 3. *Changes in relative serum concentrations of bilirubin and its photoproducts during cycled exposure of Gunn rats to total darkness and low and high illumination*

For experimental details see the text.

Experiment	Relative serum concentration						
	Total darkness	Low illumination (1.4 μW/cm^2 per nm)		Total darkness	High illumination (22.5 μW/cm^2 per nm)		
Duration of experiment (h)		1–2	3–5		1–2	3–4	5
No. of animals	5	4	5	5	6	3	5
(*ZZ*)-Bilirubin							
Mean	4165	4434	3861	3811	2961	3020	2307
S.E.M.	676	565	563	581	336	456	331
(*EZ*)- and (*ZE*)-Bilirubin							
Mean	28	39	26	14	26	25	21
S.E.M.	5	7	3	2	4	6	4
(*EZ*)-Cyclobilirubin							
Mean	0	0	0	0	0	0	0
S.E.M.	0	0	0	0	0	0	0

McDonagh *et al.* (1982*a*, *b*) reported that the main process is single-photon $Z \rightarrow E$ isomerization, predominantly at the 15*Z* bridge, and that intramolecular endovinyl cyclization is a relatively minor contributor. Although the species difference between human and rat should be kept in mind, the relative proportion of biliary excretion of (*EZ*)-cyclobilirubin is much greater than that of (*EZ*)- and (*ZE*)-bilirubin during phototherapy of neonatal hyperbilirubinaemia (Onishi *et al.*, 1980*b*), and also the bilirubin derived from (*EZ*)-and (*ZE*)-bilirubin might undergo significant enterohepatic recirculation, which would diminish its net excretion (Lester & Schmid, 1963). On the basis of these previous observations and the data obtained from the present study, it is concluded that pronounced changes in bile and urine composition, especially the excretion of (*EZ*)- and (*ZE*)-bilirubin, and (*EZ*)- and (*EE*)-cyclobilirubin, are observed in a short time, in contrast with the hours of phototherapy required to produce significant changes in serum bilirubin concentration. Moreover, this fact supports our theory that during phototherapy of neonatal hyperbilirubinaemia the predominant pathway for the removal of bilirubin from the body is by biliary excretion of (*ZE*)-bilirubin and (*EZ*)-cyclobilirubin (Onishi *et al.*, 1980*b*, 1981*a*).

This research was supported by Grant-Aid for Scientific Research nos. 58770675 and 58580426 from the Ministry of Education of Japan, Science and Culture, and by a grant given by the Ministry of Health and Welfare of Japan for Research on Prevention of Physical and Mental Disabilities.

References

Brown, A. K. & McDonagh, A. F. (1980) *Adv. Pediatr.* **27**, 341–389

Cremer, R. J., Perryman, P. W. & Richards, D. H. (1958) *Lancet* **i**, 1094–1097

Isobe, K., Itoh, S., Onishi, S., Ogino, T. & Yokoyama, T. (1983) *Biochem. J.* **209**, 695–700

Lester, R. & Schmid, R. (1963) *J. Clin. Invest.* **42**, 736–746

Lund, H. T. & Jacobsen, J. (1972) *Acta Paediatr. Scand.* **61**, 693–696

McDonagh, A. F. (1971) *Biochem. Biophys. Res. Commun.* **44**, 1306–1311

McDonagh, A. F., Palma, L. A., Trull, F. R. & Lightner, D. A. (1982*a*) *J. Am. Chem. Soc.* **104**, 6865–6867

McDonagh, A. F., Palma, L. A., Trull, F. R. & Lightner, D. A. (1982*b*) *J. Am. Chem. Soc.* **104**, 6867–6869

Ogino, T., Yokoyama, T., Togari, H., Ogawa, Y., Wada, Y., Isobe, K., Itoh, S., Yamakawa, T. & Onishi, S. (1983) *Photomed. Photobiol.* **5**, 63–64

Onishi, S., Shimidzu, K. & Yamakawa, T. (1969) *Shonika Rinsho* **22**, 138–150

Onishi, S., Yamakawa, T. & Ogawa, J. (1971) *Proc. Int. Congr. Pediatr. 13th* vol. 1, pp. 373–379

Onishi, S., Itoh, S., Kawade, N., Isobe, K. & Sugiyama, S. (1979) *Biochem. Biophys. Res. Commun.* **90**, 890–896

Onishi, S., Kawade, N., Itoh, S., Isobe, K. & Sugiyama, S. (1980*a*) *Biochem. J.* **190**, 527–532

Onishi, S., Isobe, K., Itoh, S., Kawade, N. & Sugiyama, S. (1980*b*) *Biochem. J.* **190**, 533–536

Onishi, S., Kawade, N., Itoh, S., Isobe, K., Sugiyama, S., Hashimoto, T. & Narita, H. (1981*a*) *Biochem. J.* **198**, 107–112

Onishi, S., Itoh, S., Isobe, K. & Sugiyama, S. (1981*b*) *Photomed. Photobiol.* **3**, 59–60

Onishi, S., Miura, I., Isobe, K., Itoh, S., Ogino, T., Yokoyama, T. & Yamakawa, T. (1984) *Biochem. J.* **218**, 667–676

Ostrow, J. D. (1971) *J. Clin. Invest.* **50**, 707–718

Porto, S. O. (1970) in *Bilirubin Metabolism in the Newborn* (Bergsma, D., Hsia, D. Y. & Jackson, C., eds.), pp. 83–89, Williams and Wilkins, Baltimore

Stoll, M. S., Vicker, N., Gray, C. H. & Bonnett, R. (1982) *Biochem. J.* **201**, 179–188