J Comp Physiol B (1987) 157:61–66

Journal of Comparative Physiology B Biochemical, Systemic, and Environmental Physiology

The excretion pattern of biliverdin and bilirubin in bile of the small skate (*Raja erinacea*)

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Accepted September 21, 1986

Summary. Bile pigment composition (biliverdin, bilirubin and their conjugates) was analyzed in stored gallbladder bile and newly synthesized hepatic bile from the small skate (*Raja erinacea*). During a five day period of captivity, gallbladder volume remained relatively constant while bilirubin and biliverdin content increased two to three fold. Biliverdin which accounted for 50% of the pigments did not increase as a percentage of tetrapyrroles during this period. The relative proportion of bilirubin and its conjugates also remained constant, averaging 65% for bilirubin monoglucuronide, 30% for bilirubin diglucuronide and 5% for unconjugated bilirubin as measured by HPLC methods. Intravenous administration of biliverdin resulted in significant increases in the biliary excretion of both biliverdin and all bilirubin tetrapyrroles. Insignificant quantities of ^{3}H-biliverdin were detected in hepatic bile following the intravenous administration of ^{3}H-bilirubin. These studies indicate that the small skate excreted both biliverdin and bilirubin conjugates in bile and that the biliverdin was not produced by in vitro oxidation of bilirubin or its metabolites.

Introduction

Biliverdin as well as unconjugated bilirubin and the conjugates of bilirubin have been detected in gallbladder bile of elasmobranchs (Chowdhury et al. 1980; McDonagh and Palma 1982). All of these biliary pigments are the products of heme catabolism. However, the source of the biliverdin detected in elasmobranch bile is of interest since biliverdin could be either an end product of heme metabolism or be formed in the gallbladder by the oxidation of bilirubin after excretion. In some vertebrates, biliverdin is the major pigment detected in the bile but is also found with bilirubin and its conjugates (McDonagh and Palma 1982; Colleran and O'Carra 1977; Lin et al. 1974). The origins of biliverdin in small skate (*Raja erinacea*) bile were investigated in the following studies by comparing the tetrapyrrole composition of freshly secreted bile with that of bile stored in the gallbladder for variable but usually extended periods of time. To determine whether an endogenous mechanism exists for biliverdin excretion, bile pigment composition of skate bile was also studied after the intravenous injection of biliverdin.

Materials and methods

Animals. Forty-five male small skates (*Raja erinacea*) (1–2 kg) were obtained by net in waters off Southwest Harbor, Maine during the summers of 1983 and 1984 and maintained in well-oxygenated holding tanks at Mount Desert Island Biological Laboratories for up to five days until they were used.

Gallbladder bile. Upon removal from the holding tank, the skates were anesthetized with pentobarbital (5 mg/kg). During the surgical procedures, the skates were oxygenated by continuously perfusing the gills with cold sea water at a rate of 1.5 l/min through rubber catheters placed through the opercula. The catheters were temporarily secured in place with minimal trauma to the tissues by inflating balloon tips. A midline abdominal incision was made, the gallbladder exposed and the bile aspirated. The bile volume was measured, biliverdin spectra were taken, and then ascorbic acid (5 mg/ml) added to the samples which were also protected from light and stored frozen until analyzed. Ascorbic acid was used as an antioxidant and no changes were detected in measured bile pigments up to five days after its addition (Lightner et al. 1976).

Hepatic bile. After the gallbladder bile was aspirated as described above, the common bile duct was ligated with two 5-0 silk ties. A small incision was then made in the gallbladder and a 10 cm cannula (PE-240 polyethylene tubing) was inserted

and sewn into place with 5-0 silk. The cannula was brought out through the abdominal wall and the wound closed with 4-0 silk sutures. A rubber gasket formed from the plunger of a 10 ml plastic syringe was placed over the end of the cannula and an opaque rubber balloon was attached to the gasket as previously described (Boyer et al. 1976). The small skates (free-swimming) were placed back in the tank and hepatic bile samples were collected by changing the balloons every 12–24 h.

Bile pigment analysis. Estimates of biliverdin and the total bilirubin tetrapyrrole concentrations were obtained in all bile samples by diluting aliquots of the bile with distilled water and reading the absorbances at 454 and 660 nm. Because a single extinction coefficient cannot reflect the absorbance of all bilirubin tetrapyrrole (bilirubin and its conjugates), the absorbance of bile at 454 nm was used as an estimate of bilirubin tetrapyrroles. The conjugates of bilirubin do not absorb maximally at this wavelength and our calculations therefore underestimate the total bilirubin tetrapyrroles by 5–10% (Gordon, unpublished data). Distilled water was used as a blank. Dilutions ranged from 1:1 to 1:40. The concentrations of biliverdin (biliverdin dihydrochloride, 80%, Sigma Chemical Co.) and total bilirubin tetrapyrroles (Sigma Chemical Co.) were calculated by using their respective apparent extinction coefficients in alkalinized H_2O (pH 8.5): for biliverdin $E_{660} = 10.8 \times 10^3$ cm^2 mmol^{-1}, bilirubin tetrapyrroles $E_{454} = 46.0 \times 10^3$ cm^2 mmol^{-1}. A standard curve was constructed to demonstrate that both pigments conform to the Beer-Lambert law over the concentration range studied.

Extraction of bilirubin and the conjugates of bilirubin from bile. Gallbladder bile and hepatic bile were extracted following a method outlined by Heirwegh et al. (1974). Each aliquot of bile was adjusted to 1.0 ml with water. The following additions were then made: 8 ml glycine-HCl buffer (0.4 M HCl adjusted to pH 2.8 with glycine), 2 ml of freshly prepared ascorbic acid (0.1 g/ml) in saturated NaCl, 2 g NaCl and 8 ml of $CHCl_3$:CH_3CH_2OH [1:1 (v:v)]. The mixture was then vortexed and phases separated by centrifugation at 2,000 rpm for 5 min. The organic phases were concentrated under N_2 and made up to a known volume with $CHCl_3$:CH_3CH_2OH [1:1 (v:v)]. Spectrophotometric analysis indicated a virtually complete recovery of both bilirubin and biliverdin in this extraction system.

Detection of bilirubin and the conjugates of bilirubin. The organic extracts were concentrated under N_2 and spotted on thin layer silica gel H [Whatman (LK 5)] chromatograms. The chromatograms were developed in petroleum ether: diethyl ether [1:1 (v:v)], dried, and then developed in $CHCl_3$:CH_3OH:H_2O [65:25:3 (v:v:v)]. Yellow bands (which were well separated from the green biliverdin bands) were scraped from the plates and the gel was eluted with $CHCl_3$:CH_3OH [65:25 (v:v)]. To prove the identity of the yellow bands, aliquots of the eluted pigments were treated with ethyl anthranilate diazo reagent at pH 2.8 or pH 6.8. The azopigments formed were extracted and separated on thin layer chromatograms which were developed in a solvent system containing $CHCl_3$, CH_3OH, and H_2O [65:25:3 (v:v:v)]. Bilirubin tetrapyrroles extracted from dog and rat bile were treated in the same manner and were used as reference standards to identify the diazo compounds detected in skate bile (Gordon et al. 1976, 1977).

Quantitation HPLC. Bile samples were extracted as described above, dried under N_2 and dissolved in chloroform containing 10 mM tetraheptyl ammonium chloride. The extract was concentrated at room temperature under N_2 so that the samples contained approximately 80–200 nmol of bilirubin tetrapyrroles per 10 µl. Acetonitrile was added so that the proportions were 0.6 parts of acetonitrile to 1 part chloroform/10 mM tetraheptyl ammonium chloride concentrate. The samples were analyzed on a Hewlett Packard 1084 high performance liquid chromatograph with a variable wavelength detector. Separation was achieved with an oven temperature at 37 °C using two 200 × 4.6 mm Hewlett Packard 10 µ RP-18 columns in series. The concentration of bile pigments was measured at 436 nm (Gordon and Goresky 1982). A representative chromatograph is shown in Fig. 1.

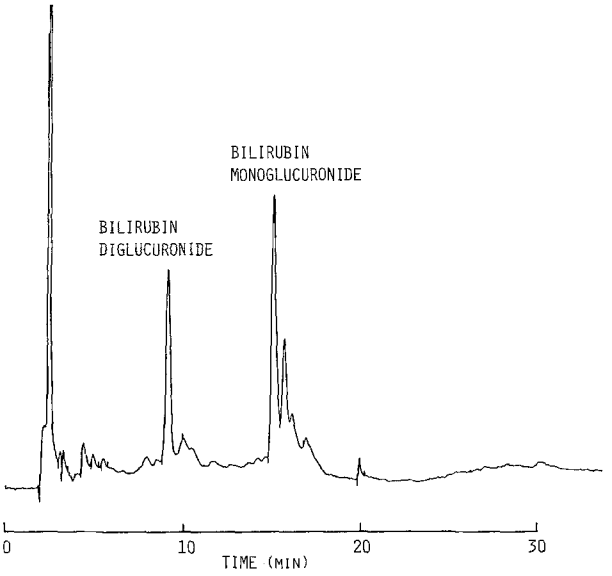

Fig. 1. A HPLC scan illustrating the bilirubin tetrapyrroles detected in the bile of the free-swimming skate. The first major peak at approximately 2 min, is produced by ascorbic acid. Two isomeric forms of bilirubin monoglucuronide were detected, the C-8 isomer being the predominant isomer in almost all instances. Bilirubin diglucoside emerges just after the bilirubin monoglucuronide at 17 min and was detected in some of the samples. Bilirubin emerges at 30 min and was seldom detected

Biliverdin injection. Hepatic bile was collected at 12–24 h intervals for three days in four free swimming skates to determine endogenous rates of tetrapyrrole excretion. Biliverdin in alkalinized elasmobranch Ringer's solution (pH 8.5) was then injected into the caudal vein of each skate (5 mg/kg body weight). Bile samples were subsequently obtained at 12–24 h intervals for three additional days and analyzed as above.

Bilirubin injection. ^{3}H-bilirubin was prepared as described by Ostrow et al. (1961) from delta-aminolevulinic acid hydrochloride and was isolated and crystallized from rat bile. ^{3}H-bilirubin (0.5–0.7 µCi) in alkalinized elasmobranch Ringer's solution (pH 8.5) was then injected into the caudal vein of three free swimming skates. Bile samples were collected at 24 h intervals for three days. Samples were treated as described above and thin layer chromatography was used to separate the bile pigments. Bands identified as biliverdin, bilirubin, bilirubin monoglucuronide and bilirubin diglucuronide were scraped from the plate into separate scintillation vials, and 300 µl of deionized water followed by 6.5 ml of Ready-solv HP were added to the gel. The samples were then counted in a Beckman LS 7000 liquid scintillation counter.

Table 1. The effect of captivity on the biliverdin and bilirubin content of the small skate's gallbladder bile. Means ± SEM

Days in tank	No. of skates	Total volume (ml)	Bilirubin tetrapyrroles (nmol)	Biliverdin (nmol)	Total tetrapyrrole (nmol)
1	11	1.9±0.8	111±78 (45%)	136± 76 (55%)	247±142
2	11	2.1±0.5	132±64 (55%)	109± 72 (45%)	241±100
3	3	2.5±0.5	140±42 (33%)	281±110 (67%)	421± 69
5	4	2.7±1.1	399±98 (64%)	227±194 (36%)	626±287

% Represent % of total tetrapyrroles

Results

Bile pigment content of small skate gallbladder bile

The total volume of bile in the gallbladder of eleven skates averaged 1.9±0.8 ml after one day in captivity. A small but insignificant increase ($P > 0.05$) in the average gallbladder volume was observed during five days of captivity (Table 1). In contrast, the bile pigment content of gallbladder bile appeared to increase with the length of time the skates were maintained in the tank. Despite large variations between fish, a significant two to three fold increase ($P < 0.05$) was detected after three days in captivity. These findings suggested that bile was secreted into the gallbladder while the skates were in the tank but that fluid was continuously being reabsorbed by the gallbladder epithelium.

Both the total amount of bilirubin and the total amount of biliverdin in the gallbladder increased over the five days of captivity. However, biliverdin did not increase as a percentage of the measured tetrapyrroles during this period suggesting that biliverdin was probably not formed exogenously by bilirubin oxidation.

Bile pigment content of hepatic bile

The tetrapyrrole content of hepatic bile was obtained by analyzing freshly secreted bile from the free swimming skate. The rate of secretion of bile pigments in 15 free swimming skates varied markedly from 5.0 nmoles/day to 761.0 nmoles/day. A representative experiment is illustrated in Fig. 2. In every bile sample taken from the 15 skates, the concentration of biliverdin exceeded the concentration of bilirubin. In addition, the relative proportions of biliverdin and bilirubin tetrapyrroles remained fairly constant during the entire collection period which lasted up to five days.

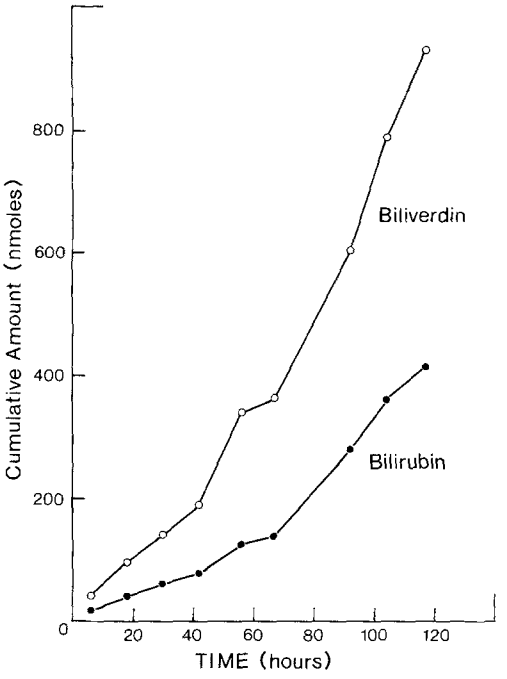

Fig. 2. Representative graph of the cumulative amounts of biliverdin (o) and bilirubin tetrapyrroles (•) excreted into the bile of a free-swimming skate during a five day period

Table 2. The relative proportions of bilirubin and its conjugates in gallbladder bile and hepatic bile of the small skate. Means ± SEM

Source of bile	Number of samples	Relative amount of pigment expressed as a % of total pigments		
		Bilirubin	Bilirubin monoglucuronide	Bilirubin diglucuronide
Gallbladder	16	5±7	69±12	26±10
Hepatic bile[a]	41	2±7	63±16	35±16

[a] Values are derived from all bile samples collected from seven free-swimming skates

The relative proportions of bilirubin and the conjugates of bilirubin detected in both hepatic bile and gallbladder bile of the small skate are summarized in Table 2. Bilirubin monoglucuronide accounted for 65% of the bile pigments excreted, bilirubin diglucuronide for 30% and unconjugated bilirubin for 5% or less. In some of the samples bilirubin diglucoside was detected, but it accounted for less than 1% of total bile pigments. The bile pattern did not change significantly during the five

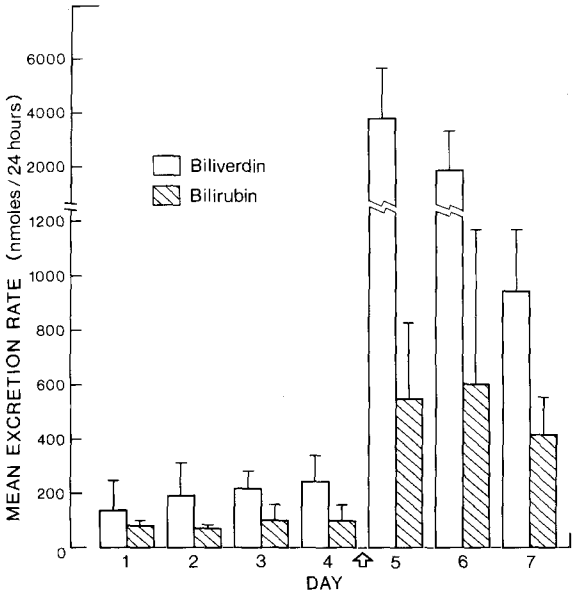

Fig. 3. Mean excretion rate per 24 h of biliverdin and bilirubin tetrapyrroles before and after the intravenous injection of biliverdin. Data were compiled from hepatic bile samples collected from four free-swimming skates. Arrow indicates the time of biliverdin injection. Means ± SEM

day collection period. There was also no significant difference in the pattern obtained from gallbladder bile regardless of the length of captivity prior to sampling.

To determine the ability of the skate to excrete an exogenous load of biliverdin, biliverdin (5 mg/kg) was injected intravenously three to four days after gallbladder cannulation. The average volume of bile produced per day was 1.8 ± 0.8 ml before and 1.6 ± 0.7 ml after the injection of biliverdin. Figure 3 illustrates the effect of a load of biliverdin on the mean excretion rate of biliverdin and bilirubin in four free-swimming skates. The excretion rate of biliverdin exceeded that of bilirubin, and the excretion rates and proportions of biliverdin and bilirubin in hepatic bile remained relatively constant before giving the exogenous load. After the injection of biliverdin, 5–10 fold increases in the excretion rate of both biliverdin and bilirubin occurred in each animal. Biliverdin excretion rose from 245 ± 94 nmoles/day in the interval before injection to 3,797 ± 1,881 nmoles/day in the interval after injection, whereas bilirubin tetrapyrroles increased from 100 ± 57 nmoles/day to 548 ± 277 nmoles/day.

^{3}H-bilirubin was administered intravenously to three free-swimming skates as a control experiment. If biliverdin is excreted directly into small skate bile rather than being formed by the oxidation of bilirubin, then the revocery of ^{3}H-biliverdin from bile should be negligible while the recovery of ^{3}H-bilirubin and ^{3}H-conjugated bilirubins should be significant. 50–60% of the injected label was ultimately recovered in the bile over a 72-h sampling period. Of the recovered label, 93 ± 2.1% was detected within 48 h. In all samples, greater than 60% of the recovered label was associated with bilirubin and its conjugates. In two of the three skates, no significant ($P < 0.05$) label was detected on the thin layer chromatograms in the region of the biliverdin standard. In the third skate at 24 h, 13% of the label was associated with the biliverdin standard and 11% was associated with conjugated biliverdin. Conjugated biliverdins were identified in four of the other eight bile samples, but comprised less than 1% of the overall recovered label in each case.

Discussion

In mammals, the degradation of heme initially results in the formation of biliverdin, which is subsequently reduced to bilirubin. The heme degradation product that is ultimately secreted into bile varies from species to species. Humans, rats, dogs and some fish secrete bilirubin and its conjugates rather than biliverdin into their bile (Chowdhury et al. 1980; Gordon et al. 1976, 1977). However, in some elasmobranchs, like *Torpedo californicus*, unconjugated biliverdin is the predominant bile pigment, while unconjugated bilirubin is absent (McDonagh and Palma 1982). Likewise, biliverdin is the predominant bile pigment identified in chicken bile (Lin et al. 1974).

In this study, both biliverdin and bilirubin tetrapyrroles were detected in gallbladder and hepatic bile of the small skate. Hepatic and gallbladder bile contained only one band corresponding to the biliverdin standard as detected by tetrapyrrole thin layer chromatography. Therefore, we assume that only unconjugated biliverdin was present in bile of this species.

Our results suggest that biliverdin is a primary constituent of small skate bile and not merely a by-product of bilirubin oxidation in the gallbladder. This conclusion is based on the inability to detect increasing percentages of biliverdin in gallbladder bile with time in captivity and the relatively smaller percentage of biliverdin in gallbladder as compared to hepatic bile. Also of significance is the absence of conjugated biliverdins in either hepatic or gallbladder bile. Since conjugated bilirubin accounts for about 95% of the bilirubin tetrapyrroles detected by HPLC, oxidation of these compounds would be expected to yield conjugated

biliverdins. Finally, if biliverdin were formed by bilirubin oxidation, then ^{3}H-biliverdin should be detected in bile samples collected after the injection of ^{3}H-bilirubin in free swimming skates. Of nine bile samples collected from these skates, only a single sample contained labeled biliverdin, while significant radioactive label was associated with bilirubin and its conjugates in all samples. Conjugated biliverdins were also detected in small amounts, but had not been noted elsewhere in our studies or in those of other investigators and were most likely an artifact of pigment oxidation during the chromatographic analysis.

The injection of biliverdin into four free-swimming skates resulted in a dramatic increase in the excretion of both biliverdin and bilirubin tetrapyrroles. This finding demonstrates that an endogenous mechanism exists for the excretion of biliverdin into bile. Since bilirubin tetrapyrrole excretion also increased, biliverdin reductase (the enzyme responsible for the conversion of biliverdin to bilirubin) may be limiting in the skate. This possibility is consistent with Fang and Bada's (1982) results from a study of biliverdin reductase activity in nine species of marine fishes which showed that the biliverdin reductase activity in all of the species was less than that found in the rat or guinea pig, animals that do not excrete biliverdin in their bile. The simultaneous excretion of biliverdin and bilirubin into skate bile might also reflect the existence of different compartments within the skate liver for a polar biliverdin salt and a more hydrophobic bilirubin molecule. The significant increase in the excretion of bilirubin and its conjugates occurring after biliverdin injection suggests that the skate liver may contain a reserve of biliverdin reductase activity which is ordinarily not utilized since biliverdin may not require reduction prior to excretion from its compartment. Further studies of the kinetics of biliverdin reductase in the skate will be necessary to answer these questions.

The total amount of biliverdin and bilirubin in skate bile increased with time in captivity. Because the small skates were not fed while in the holding tank, it is conceivable that the increased tetrapyrrole content observed with prolonged captivity may be partly attributable to the fasting state itself. Such an increase in bilirubin and total tetrapyrrole production has been reported in fasting mammals (Bakken et al. 1972). Although tetrapyrrole content of gallbladder bile increased with time in captivity, gallbladder bile volume did not, suggesting that reabsorption of fluid from the gallbladder occurred in proportion to the rate of hepatic bile secretion. Biliverdin might also be reabsorbed, which would explain the apparently low relative content of biliverdin in the gallbladder as compared with its presence in free-swimming skate hepatic bile. Alternatively, biliverdin reductase activity might diminish in the cannulated free swimming-skate or heme degradation could be enhanced.

HPLC and TLC analysis of both gallbladder bile and hepatic bile samples show that the skate is capable of excreting bilirubin, bilirubin monoglucuronide and bilirubin diglucuronide into its bile. However, the skate excretes these compounds in different relative proportions than previously observed in a variety of mammalian, avian and piscine systems (Cornelius et al. 1975; Fevery et al. 1977). In rat and dog bile, bilirubin diglucuronide is the major bilirubin conjugate. In bile of the small skate, bilirubin monoglucuronide is the predominant conjugate and constitutes 65% of the bilirubin tetrapyrroles. Bilirubin diglucuronide and bilirubin account for 30% and 5% of the bilirubin tetrapyrroles, respectively. These findings are in contrast with those of Chowdhury et al. (1980, 1982) and of Jansen and Arias (1977) who reported that the bile of two elasmobranchs, the dogfish shark (*Squalus acanthias*) and the small skate, contain bilirubin monoglucuronide, bilirubin and bilirubin diglucuronide in decreasing order of abundance. This discrepancy may possibly be accounted for by differences in methodology in quantitating the bile pigments. The previously reported results on small skate bile were obtained by eluting pigments from thin layer silica gel plates, and HPLC was not performed. In our studies, such TLC data were found to be more variable than the data obtained from HPLC and more prone to artifactual findings. For instance, on more than one occasion, the TLC elutions yielded a relatively high spectrophotometric reading for bilirubin yet no yellow pigment was visible to the eye.

The present studies complement those of previous investigators who have studied organic anion excretion in the small skate. It appears not only that biliverdin is excreted into small skate bile, but also that bilirubin may require conjugation prior to its excretion, since unconjugated bilirubin is detected in only small amounts in skate bile. The ability of the skate to excrete predominantly conjugated bilirubin into its bile suggests that the skate's hepatic conjugating system is able to process the load of bilirubin that it ordinarily must handle.

With respect to biliverdin excretion, bile pigment metabolism in the small skate is similar to that in *Torpedo californicus*, another elasmobranch which has been reported to excrete biliverdin into

its bile (McDonagh and Palma 1982). However, unlike that animal, the small skate also secretes significant quantities of bilirubin and its conjugates into bile as has been previously reported in mammalian systems. Thus, the present studies suggest that the skate's biliary tetrapyrrole excretion system can be characterized functionally as an intermediate between *Torpedo californicus* and mammalian systems.

Acknowledgements. This work was supported in part by AM 25636 and AM 32741. M.L. Grossbard was the recipient of an American Liver Foundation Student Fellowship Award.

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