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UDP-glucuronosyltransferase activity and bilirubin conjugation in the bullfrog

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1. Bile pigments of bile and serum of *Rana catesbeiana* were investigated by means of high-pressure liquid chromatography. 2. The major pigment in both bile and serum was bilirubin IXα. 3. Bilirubin UDP-glucuronosyltransferase activity was found in the livers of all animals examined, but no conjugated bilirubin was detectable in the bile. 4. Frog bile was found to contain large amounts of β-glucuronidase. 5. When the β-glucuronidase inhibitor saccharo-1,4-lactone was introduced into the gall bladder followed by an exogenous bilirubin load, bilirubin glucuronide appeared in the bile.

Few reports exist on bile-pigment metabolism in amphibians, and those that are available are incomplete. Lester & Schmid (1961) reported the occurrence of unconjugated bilirubin in the bile of adult frogs and found significant *o*-aminophenol UDP-glucuronosyltransferase activities in amphibian liver, but were unable to detect any conjugated bilirubin in the bile. In contrast, Garay *et al.* (1965) found conjugated bilirubin in several amphibian bile specimens. The view that appears to have been adopted by authorities in the field is that biliverdin is the end product of haem metabolism in amphibians, and bilirubin found in the bile of these animals is an artifact caused by reduction of biliverdin *post mortem* (Colleran & O'Carra, 1977; Schmid & McDonagh, 1978).

It seemed to us that sufficient uncertainty existed concerning bile-pigment metabolism in amphibians to warrant its re-investigation in the bullfrog (*Rana catesbeiana*), in order to determine if that species might provide a valid animal model for developmental studies of the hepatic bilirubin excretory pathway. We have sought to answer the following questions. (1) Which bile pigment (bilirubin or biliverdin) is the end product of haem metabolism in this species? (2) Does the bullfrog have the ability to conjugate bilirubin with glucuronic acid? (3) Is bilirubin glucuronide excreted in the bile of this species? For purposes of comparison some experiments were repeated with mice as experimental animals.

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

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Experimental

Animals

Adult bullfrogs (*Rana catesbeiana*) were obtained from Riverside Biological Center (Somerset, WI, U.S.A.). Adult male mice (C57BL/5J) were obtained from Jackson Laboratories (Bar Harbor, ME, U.S.A.). The bullfrogs were maintained in separate cages and were fed on a diet of a tadpole each once a week.

Chemicals

Saccharo-1,4-lactone was obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.), dissolved in water, neutralized with 5 M-NaOH and freeze-dried. Tricaine (ethyl *m*-aminobenzoate), bovine serum albumin and 4-methylumbelliferyl β-D-glucuronide were obtained from Sigma Chemical Co. Bilirubin and biliverdin used as standards were obtained from Porphyrin Products (Logan, UT, U.S.A.). Other chemicals were reagent grade and were obtained from various suppliers.

Amphibian saline

To 1 litre of distilled water the following were added: NaCl (4.3 g), HCl (0.3 g) and $CaCl_2$ (0.2 g).

Protein determination

Protein concentration of the crude liver homogenates (diluted 1:100 with 1 M-NaOH and stored at −40°C) was determined with the Bio-Rad protein reagent (Bio-Rad Laboratories, Richmond, CA, U.S.A.). Bovine serum albumin (fraction V) served as a protein standard. Diluted homogenates were placed in a 45°C water bath for approx. 15 min to solubilize the protein completely, and the protein

assay was performed in accordance with instructions supplied with the reagent.

Collection of biological samples

Frogs were anaesthetized by an intraperitoneal injection of tricaine (100mg/ml) in the amount of 20μl/g body wt. The thorax was opened, and blood was collected by cutting the conus arteriosus and allowing the blood to flow into a conical centrifuge tube. Gall-bladder bile was collected by aspiration into a hypodermic syringe. The bile was immediately stored on ice and protected from light. The liver was then excised and placed in a beaker with ice-cold 0.25M-sucrose.

Preparations of samples for h.p.l.c. or t.l.c.

Bile and serum samples were divided into two halves, one of which was used as a blank, and diluted to 0.5ml. The method of Van Roy *et al.* (1971) was utilized to prepare *p*-iodoaniline derivatives for chromatography.

H.p.l.c. and t.l.c. analysis of bile pigments

H.p.l.c. and t.l.c. analysis of bile pigments was accomplished as previously described (Cole & Little, 1982). An additional mobile phase (E) was used to separate biliverdin from bilirubin glucuronide. This mobile phase consisted of 30ml of acetonitrile, 33ml of ethyl acetate, 45ml of methanol, tetrabutylammonium hydroxide (final concn. 4.8mM), tetraheptylammonium chloride (final concn. 5.2mM) and water to 350ml. The pH was adjusted to 6.1 with phosphoric acid. Azo-pigments were identified by their characteristic retention times or R_F values when compared with azodipyrroles prepared from dog bile or azodipyrroles of synthetic bilirubin isomers (Cole & Little, 1982).

Assay of bilirubin UDP-glucuronosyltransferase activity

Livers were blotted, weighed and finely minced with a razor blade. The minced livers were placed in a glass/Teflon homogenizer and diluted with 3ml of ice-cold 0.25M-sucrose per g of tissue. The livers were homogenized by six passes of a motor-driven pestle at 1000rev./min.

Incubation mixtures were prepared by adding the following to round-bottomed screw-cap tubes: 0.1ml of 25mM-$MgCl_2$/0.4M-Hepes [4-(2-hydroxyethyl-1-piperazine-ethanesulphonic acid]/NaOH buffer (pH7.15 for the bullfrog and pH7.8 for the mouse), 0.1ml of a bilirubin/albumin solution prepared by dissolving 2.5mg of bilirubin in 0.2ml of 50mM-NaOH and diluting to 10ml with human serum albumin (24mg/ml, dialysed overnight against 1mM-EDTA, pH7.4), 0.03ml of UDP-glucuronic acid (33.3mg/ml), 0.03ml of saccharo-1,4-lactone (128.4mg/ml) and 0.1ml of liver homogenate to begin the reaction. In some experiments UDP-glucose (83.3mg/ml) or UDP-xylose (36.3mg/ml) was substituted for UDP-glucuronic acid. Blanks were prepared by omitting the UDP-glucuronic acid and bilirubin solutions. All tubes were mixed and placed in a shaking water bath at 30°C for 20min. At the end of the incubation period the tubes were removed and placed in ice. Bilirubin standards consisted of 36μl of the bilirubin/albumin solution and 324μl of water in a screw-cap tube. To all tubes 0.35ml of *p*-iodoaniline diazo reagent was added. At the end of 1h, 2.15ml of ascorbic acid solution (10mg/ml) and 1.0ml of butyl acetate were added to all tubes. The tubes were mixed on a mechanical inverter for 15min, then centrifuged at 1470*g* for 20min.

A 0.5ml portion of the organic phase was removed and concentrated to dryness with a gentle stream of N_2 at 30°C. The dried azo-pigments were stored at −80°C protected from light until the reaction products were analysed by h.p.l.c. (mobile phase A) (Cole & Little, 1982).

Assay of β-glucuronidase activity

β-Glucuronidase activity was assayed fluorimetrically with 4-methylumbelliferyl β-D-glucuronide as substrate. The reaction mixture contained 0.5ml of 0.2M-sodium phosphate buffer, pH7.0, 25μl of substrate (0.54mg/ml) and sample plus water to make a total volume of 2ml. Excitation was at 240nm. The increase in total fluorescence was measured.

Hydrolysis of bilirubin glucuronide of dog bile by frog bile

We performed the following experiment to determine the ability of frog bile to hydrolyse bilirubin glucoronide, with dog bile being used as a source of bilirubin glucuronide.

In order to separate hydrolytic activity from bile pigments, frog bile (2.0ml, pooled from several individuals) was applied to a Sephadex G-50–150 column (15mm × 400mm) equilibrated with 0.1M-sodium phosphate buffer, pH7.0. Fractions (100 drops) were collected and assayed for β-glucuronidase activity. The fractions with maximal activity were pooled (total volume 10.0ml) and used as an enzyme source.

In a screw-cap tube, 1.5ml of the pooled fractions was added to 0.04ml of dog bile (previously diluted with 1vol. of citrate/phosphate buffer, pH4.0) (Compernolle *et al.*, 1978) and incubated at 30°C. Samples (0.25ml) were taken at zero time and at 15min intervals for 2h, and placed on ice. All samples were then diazo-coupled with *p*-iodoaniline as described by Van Roy *et al.* (1971), and the bilirubin glucuronide concentration at each time was determined by h.p.l.c.

Demonstration of bilirubin glucuronide in vivo

In order to determine whether bullfrogs are capable of synthesizing bilirubin glucuronide *in vivo*, two animals were injected intraperitoneally with a solution of saccharo-1,4-lactone (333 mg/ml) at a dose of 2 μl/g body wt. Then 90 min later the animals were restrained and a small incision was made in the abdomen to expose the gall-bladder. A hypodermic needle was inserted into the gall-bladder and the bile was aspirated. The gall-bladder was then flushed with 0.5 ml of amphibian saline followed by injection of 150 μl of 200 mM-saccharo-1,4-lactone in amphibian saline. The incision in the abdomen was closed by suturing. The animals were then injected intraperitoneally with a solution of bilirubin (2.5 mg/ml dissolved in dimethyl sulphoxide) in the amount of 2 μl/g body wt. Then 4 h later the animals were restrained, the incision was re-opened and the gall-bladder was injected with 0.5 ml of amphibian saline. The gall-bladder was then aspirated, and the contents were placed on ice protected from light. The gall-bladder was re-injected with 150 μl of 200 mM-saccharo-1,4-lactone in amphibian saline solution. The incision was resutured. The next day (26.5 h after the initial bile sample) the animals were restrained, the incision was re-opened and the gall-bladder was injected with 0.5 ml of amphibian saline. The contents of the gall-bladder were collected as before. The samples were then diazo-coupled with *p*-iodoaniline as described by Van Roy *et al.* (1971) and subjected to h.p.l.c.

Results

Bile pigments of adult bullfrog bile and serum

The major azo-pigments of bullfrog are chromatographically identical with the azodipyrroles of bilirubin IXα. Also present (15% of total bilirubin) were azo-pigments chromatographically identical with the azodipyrroles of bilirubin IXγ. Non-polar azo-pigments corresponding to azo-pigments $\alpha_{F'}$ and α_F (derived from bilirubin IXδ and bilirubin IXβ respectively) (Blanckaert *et al.*, 1976) were observed in some individuals. An azo-pigment that is eluted later than either azo-pigment $\alpha_{F'}$ or azo-pigment α_F was often seen in the bile of bullfrogs. This non-polar azo-pigment was not an ester conjugate, since it is resistant to treatment with alkaline methanol. We consistently failed to demonstrate the presence of ester conjugates in frog bile by transesterification in alkaline methanol. Glucuronic acid-, glucose- or xylose-conjugated azodipyrroles were undetectable in frog bile by means of our h.p.l.c. systems or by the t.l.c. systems of Heirwegh *et al.* (1974).

A green bile pigment with the same R_F (0.71) value as biliverdin IXα was seen when frog bile was subjected to t.l.c. with chloroform/methanol/water (65:25:3, by vol.). This bile pigment had an absorption spectrum very similar to that of biliverdin IXα (absorption maxima at 660 and 375 nm). When bullfrog bile was subjected to h.p.l.c. with mobile phase E, which separates biliverdin from the conjugated azodipyrroles of bilirubin, a non-diazo-positive pigment was seen with the same elution time as biliverdin IXα. This bile pigment in bullfrogs had the same characteristic A_{658}/A_{546} absorption ratio as authentic biliverdin IXα (average ratio about 3.4).

Serum was examined in the same manner as bile. The only bile pigment detectable was unconjugated bilirubin IXα. The concentrations of the various bile pigments in frog serum and bile are summarized in Table 1. Data for the mouse are included for purposes of comparison.

Table 1. *Concentrations of bile pigments in bile and serum of the bullfrog and the mouse*

Bile pigments were diazotized and separated by h.p.l.c. as described in the text. Concentrations were calculated from the areas of individual azo-pigment peaks, with bilirubin and biliverdin as standards. Values are given as means ± S.E.M. for the numbers of determinations indicated in parentheses.

	Concentration (μmol/l)					
Specimen	Bilirubin IXα	Bilirubin IXα glucuronide	Bilirubin IXγ	Bilirubin IXδ	Bilirubin IXβ	Biliverdin
Bullfrog						
Bile						
Group 1	50 ± 18 (12)	Not detected	9 ± 2 (8)	Trace	1.3 ± 0.4 (5)	Not determined
Group 2	62 ± 24 (9)	Not detected	11 ± 4 (9)	Not determined	Not determined	30 ± 10 (9)
Serum						
Group 1	0.26 ± 0.04 (9)	Not detected	Not detected	Not detected	Not detected	Not detected
Group 2	0.19 ± 0.03 (18)	Not detected	Not detected	Not detected	Not detected	Not detected
Mouse						
Bile	94 ± 13 (8)	102 ± 10 (8)	Not determined	Not determined	Not determined	Not determined
Serum	1.46 ± 0.21 (10)	Not detected	Not determined	Not determined	Not determined	Not determined

Table 2. *Hepatic bilirubin UDP-glucuronosyltransferase activity in the bullfrog and the mouse*

The assay was performed as described in the text. Activity is expressed as μmol of bilirubin glucuronide produced/h per g of liver protein at 30°C (means ± S.E.M.). Numbers in parentheses represent the numbers of animals utilized.

Animal	Liver bilirubin UDP-glucuronosyltransferase activity
Bullfrog	
Group 1	0.51 ± 0.04 (11)
Group 2	0.47 ± 0.06 (9)
Mouse	2.25 ± 0.17 (8)

Bullfrog liver bilirubin UDP-glucuronosyltransferase

Bullfrog liver crude homogenates were examined for bilirubin UDP-glucuronosyltransferase activity, and significant amounts were found to be present in all animals (Table 2).

Bilirubin glucuronide synthesis was linear for over 1 h under the assay conditions used. A standard incubation period of 20 min was used. Digitonin, when added to an equal volume of liver homogenate at concentrations of 1, 6, 12 and 18 mg/ml, failed to increase bilirubin UDP-glucuronosyltransferase activities, and at higher concentrations inhibited the activity. When assayed under our conditions, the enzyme had a pH optimum of 6.85. Doubling the amount of UDP-glucuronic acid did not increase activity, indicating saturating conditions for this substrate in the standard assay. The inclusion of 1 mM-UDP-*N*-acetylglucosamine did not increase bilirubin UDP-glucuronosyltransferase activity. No conjugate synthesis was detected when liver homogenates were incubated with UDP-glucose or UDP-xylose.

Hydrolysis of bilirubin glucuronide of dog bile by frog bile

Frog bile is capable of hydrolysing bilirubin glucuronide. At these concentrations of frog bile hydrolytic activity (0.12 μmol of 4-methylumbelliferyl β-D-glucuronide/h per ml) and bilirubin glucuronide (6.25 μM) hydrolysis was virtually complete in 45 min. Frog bile is capable of hydrolysing approx. 14 nmol of bilirubin glucuronide/h per ml of bile. Incubating the enzyme preparation for 15 min in a boiling-water bath completely abolished the hydrolytic activity. Hydrolysis of the glucose and xylose conjugates of bilirubin was insignificant.

Demonstration of bilirubin glucuronide in vivo

At 4 h after injection of the bilirubin, bilirubin glucuronide was found in the bile of both animals at amounts of 24.3% and 12.2% respectively of the total (conjugated and unconjugated) bilirubin IXα in their biles. After 26.5 h one animal had a negligible content of glucuronic acid conjugates and the second animal had glucuronide present at an amount corresponding to 20.2% of the total bilirubin.

Discussion

Our finding that bilirubin IXα is the only detectable bile pigment in serum and the major bile pigment in bile of *Rana catesbeiana* suggests that bilirubin rather than biliverdin is the end product of haem metabolism in this species. If biliverdin occurs in frog serum its concentration must be less than 0.1 μM (the sensitivity of our method), which is less than half the bilirubin concentration. Since biliverdin was found in bile at concentrations of about 40% of the total bilirubin concentration, biliverdin may be present in serum at undetectable concentrations. If this is true, it would indicate that, although frogs are capable of reducing biliverdin to bilirubin, this capacity may not be sufficient to handle the entire bilirubin load. Biliverdin in the bile could also arise from oxidation of bilirubin in the gall-bladder or biliary tree. Clarification of these points will require additional experimentation.

The bulk of haem catabolism in the bullfrog apparently proceeds by cleavage of the haem macrocycle at the α-methylene bridge. Specificity for this position is not so strict as in mammals, since 15% of the total bilirubin of frog bile is bilirubin IXγ and 2% is bilirubin IXβ. The presence of non-α-isomers of bilirubin in bile but not in serum may indicate that the haem oxygenase of liver has different specificity from that of the haem oxygenase of spleen and bone marrow in this species.

Serum bilirubin concentrations in the bullfrog are about 15% of those of the mouse. Bile concentrations of bilirubin were comparable in the frog and the mouse, which may result from low turnover of bile in the frog due to long intervals between meals. The most striking difference in the bile of the two species is the absence of conjugates from the frog bile. There are two possible explanations for this difference. The first possibility is that bilirubin is secreted by the liver in an unconjugated form. The second possibility is that bilirubin is secreted by the liver in a conjugated form that is hydrolysed on entry into the bile. We have presented three lines of evidence that support the latter.

First, we have established that frog liver has bilirubin UDP-glucuronosyltransferase activity. When bilirubin UDP-glucuronosyltransferase activities in the mouse and the bullfrog are both assayed at 30°C, the bullfrog liver had activity corresponding to about 22% of the mouse liver activity. When assayed at 37°C the mouse bilirubin UDP-glucuronosyltransferase activity approximately

doubled. If it is possible to relate the rates of glucuronidation *in vitro* to the situation *in vivo*, frog liver would have approx. 10% of the activity that the mouse possesses. Thus it appears that the bullfrog possesses sufficient liver bilirubin UDP-glucuronosyltransferase to conjugate bilirubin.

Secondly, frog bile contains large amounts of β-glucuronidase activity, which we have demonstrated to be capable of hydrolysing bilirubin glucuronide. If the assumption is made that the bilirubin of frog bile was 100% monoconjugated with glucuronic acid when secreted in the bile, the hydrolytic activity of the bile is sufficient to hydrolyse completely the gall-bladder contents in 1 h. Detection of bilirubin glucuronide in frog bile is thus unlikely under normal circumstances.

We have sought to overcome this difficulty by introducing the specific β-glucuronidase inhibitor saccharo-1,4-lactone into the gall-bladder followed by an exogenous load of bilirubin. Under these conditions significant amounts of bilirubin glucuronide were detectable in the bile, and this provides our third line of evidence supporting bilirubin conjugation in the frogs. Failure to see higher percentages of conjugated bilirubin is probably due to several factors. The initial draining of the gall-bladder may not have removed all of the unconjugated bilirubin, and it probably did not remove any unconjugated bilirubin higher up in the biliary system. Also, saccharo-1,4-lactone must enter the bile in sufficient quantities to inhibit the powerful hydrolytic activity. Any residual activity will result in significant hydrolysis. Akamatsu *et al.* (1961) achieved an approx. 60% inhibition of the liver-type β-glucuronidase 30 min after the oral administration of saccharo-1,4-lactone at the rate of 400 mg/kg body wt. in mice. They found that the inhibition almost completely disappeared in several hours. The critical factors in inhibiting biliary β-glucuronidase are getting sufficient quantities of saccharo-1,4-lactone into the bile and maintaining these quantities long enough to measure glucuronidation of bilirubin *in vivo*. The results obtained by Akamatsu *et al.* (1961) indicate that, for the liver, the inhibition occurs rapidly and that saccharo-1,4-lactone is cleared rapidly. Injection of adult bullfrogs with large amounts of saccharo-1,4-lactone failed to yield any detectable bilirubin glucuronide in their bile overnight. Trace amounts of β-glucuronidase activity can probably efficiently hydrolyse the basal production of bilirubin glucuronide. Thus actual production of bilirubin glucuronide is probably higher than we were able to observe.

We conclude that bile-pigment metabolism in the bullfrog is not greatly different from bile-pigment metabolism in mammals, and that the apparent differences result from the presence of large amounts of β-glucuronidase in frog bile. Our results provide very little support for the contention that biliverdin is the end product of haem metabolism in amphibians.

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References

Akamatsu, S., Kiyomato, A., Harigaya, S. & Ohsima, S. (1961) *Nature* (*London*) **191**, 1298–1299

Blanckaert, N., Heirwegh, K. P. M. & Compernolle, T. (1976) *Biochem. J.* **155**, 405–417

Cole, K. D. & Little, G. H. (1982) *J. Chromatogr.* **227**, 503–509

Colleran, E. & O'Carra, P. (1977) in *Chemistry and Physiology of Bile Pigments*, p. 69, U.S. Department of Health, Education and Welfare, National Institutes of Health, Bethesda

Compernolle, F., Van Hees, G. D., Blanckaert, N. & Heirwegh, K. P. M. (1978) *Biochem. J.* **171**, 185–201

Garay, E. R., Noir, B. & Royer, M. (1965) *Biochim. Biophys. Acta* **100**, 411–417

Heirwegh, K. P. M., Fevery, J., Meuwissen, J. A. T. P. & DeGroot, J. (1974) *Methods Biochem. Anal.* **22**, 205–249

Lester, R. & Schmid, R. (1961) *Nature* (*London*) **190**, 452

Schmid, R. & McDonagh, A. F. (1978) in *The Metabolic Basis of Inherited Disease*, 4th edn. (Stanburg, J. D., Wyngaarden, J. B. & Fredrickson, D. S., eds.), pp. 1221–1257, McGraw-Hill, New York

Van Roy, F. P., Meuwissen, J. A. T. P., De Meuter, F. & Heirwegh, K. P. M. (1971) *Clin. Chim. Acta* **31**, 109–118