PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 170, 172–177 (1982)

Biliary Excretion of Iopanoic Acid in Gunn Rats[1] (41414)

JAMES L. BARNHART,[2] BRENDA L. WITT, AND ROBERT N. BERK

Department of Radiology, University of California, San Diego, La Jolla, California 92093

Abstract. Patients with the Crigler–Najjar syndrome lack hepatic microsomal UDP-glucuronyl transferase activity which is necessary for conjugating bilirubin with glucuronic acid. The gallbladder of these patients opacify normally on oral cholecystography with iopanoic acid (IOP), a contrast agent which like bilirubin, is conjugated with glucuronic acid prior to transport into bile. Experiments were performed with normal (Sprague–Dawley) and Gunn rats (a strain which lacks UDP-glucuronyl transferase activity) to determine the effect of this enzyme deficiency on the hepatic disposition of IOP. Bilirubin excretion in Gunn rats was less than 1% of the Sprague–Dawley animals, while IOP excretion in bile was not statistically different between the two strains whether the contrast agent was infused as IOP or as IOP–glucuronide. HPLC analysis showed that IOP distribution in Gunn rat bile was 92% IOP-G and 8% IOP. The hepatic intracellular distribution of IOP was the same in the two strains. The results indicate that there is no difference in the hepatic disposition of IOP in the Gunn rat compared to the normal, despite the inability of the former to conjugate bilirubin.

Patients with the Crigler–Najjar syndrome fail to transport bilirubin from the liver into bile, because they lack hepatic microsomal UDP-glucuronyl transferase (EC 2.4.1.17) activity to convert bilirubin to its glucuronide conjugate (1, 2). Since the formation of bilirubin glucuronide is required for the excretion of bilirubin, these patients have jaundice due to nonhemolytic unconjugated hyperbilirubinemia.

Despite the fact that the oral cholecystographic contrast agents used in diagnostic radiology for radiographic opacification of the gallbladder are metabolized by the liver to their glucuronide conjugates (3–5), patients with this syndrome have adequate excretion of these compounds by the liver, and radiographic visualization of the gallbladder occurs normally (6–8). It is not known whether the successful cholecystogram is because Crigler–Najjar patients retain the capacity to conjugate the contrast materials despite the absence of glucuronyl transferase activity, if different forms of the enzyme are present, or if sufficient amounts of the parent compound are transported into bile.

Like patients with the Crigler–Najjar syndrome, Gunn rats lack UDP-glucuronyl transferase activity (9, 10) and have chronic, nonhemolytic unconjugated hyperbilirubinemia (2). We used this strain of rats to perform experiments designed to study what effect the absence of this enzyme activity has on the hepatic elimination of iopanoic acid (IOP), a radiographic contrast material which is almost completely conjugated to glucuronide prior to transport into bile.

Methods. *Chemicals.* Bilirubin (Sigma Chemical Co., St. Louis, Mo.) was prepared for injection by dissolving in 0.1 *N* NaOH–0.9% NaCl solution under subdued light and diluting with 0.9% NaCl to obtain a dose volume near 1 ml. IOP was used as obtained from Winthrop Laboratories, New York. The glucuronide conjugate of IOP (IOP-G) was isolated from dog bile according to a procedure published previously (5). IOP-G was greater than 92% pure as determined by chemical iodine determinations (11), elemental analysis (5), thin-layer chromatography, and high-performance liquid chromatography (HPLC). IOP and IOP-G were dissolved in an alkaline solu-

[1] These studies were supported by Grant AM 21620 from the National Institutes of Health, U.S. Public Health Service.

[2] Recipient of a Public Health Service Research Career Development Award AM 00680. To whom correspondence should be addressed.

0037-9727/82/060172-06$01.00/0

tion, diluted to the required concentration with 0.9% NaCl to keep the dose volume near 1 ml, and pH adjusted to 7.5–7.8. Samples for injection were prepared on the day administered.

Experimental procedures. Male Sprague–Dawley rats (Hilltop Lab Animals, Chatsworth, Calif.) weighing 250–350 g and male homozygous Gunn rats (Blue Spruce Farms, Altamont, N.Y.) weighing 200–500 g were allowed free access to water and laboratory rat chow. Rats were anesthetized with pentobarbital (30 mg/kg) and fitted with a bile duct cannula (PE 10 polyethylene tubing) through a midline abdominal incision. PE 50 tubing was placed in a jugular vein. Body temperature was maintained by means of a heating pad placed under the animal. Bile was collected in pretared vials at 10-min time intervals before and after the administration of bilirubin (43 μmole/kg), IOP (300 μmole/kg), and IOP-G (300 μmole/kg). After collecting the third 10-min bile sample, one of the compounds was injected through the jugular cannula over a 3- to 5-min time period. When bilirubin was administered, the studies were carried out in subdued lighting. Bile was collected for an additional 60 min. At the end of the experiment, a sample of blood was taken from the vena cava, allowed to clot, and serum was obtained by centrifugation. The liver was perfused with 20 ml of ice-cold buffer 1 (50 μ*M* Tris–HCl, pH 7.5; 25 m*M* KCl, 250 m*M* sucrose; 5 m*M* $MgCl_2$) through the portal vein, removed, blotted dry, and weighed.

Analytical procedures. Bile volume was determined gravimetrically assuming a density of 1.0 g/ml. Bile salt concentration in bile was determined enzymatically using a 3 α-hydroxysteroid dehydrogenase (Worthington Biochemical Corp., Freehold, N.J.) procedure by a method described previously (12). Concentrations of IOP and IOP-G in serum, bile, and liver fractions were determined by measuring the iodine concentrations by fluorescent excitation analysis (FEA) (13). FEA detection was linearly related to iodine concentration between the range of 1 to 300 μg iodine/ml. The technique does not differentiate between IOP and IOP-G. Total bilirubin was determined by diazotization according to Malloy and Evelyn (14). HPLC analyses of standards and bile were made using a reverse phase column (μBondapak C-18, Waters Associates, Inc.) and a mobile phase of acetonitrile/phosphate buffer, 0.01 *M*, pH 3.0 (50/50, v/v). Bile was prepared for injection by mixing 1 part bile with 5 parts mobile phase. A 20-μl sample of this mixture was administered to the column. Molar absorptives of IOP and IOP-G at 230 nm are equal.

Liver homogenate was obtained by the method of Strange *et al.* (15). The liver (5 g) was homogenized in ice-cold buffer 1 (12 ml). The homogenate was centrifuged at 18,000*g* for 15 min; the pellet was discarded and the supernatant was centrifuged 100,000*g* for 120 min. The supernatant was designated the cytosolic fraction. The pellet was washed by resuspending in 3 ml of sucrose-free buffer 1 and centrifuged at 100,000*g* for 50 min. The washed pellet was designated the microsomal fraction. To obtain the mitochondrial fraction, the homogenate was spun at 800*g* for 10 min. The supernatant was spun at 7000*g* for 10 min with the pellet resuspended in 3 ml of 0.25 *M* sucrose–1 m*M* $MgCl_2$ and spun at 24,000*g* for 10 min. The suspended pellet was designated the mitochondrial fraction. Strange *et al.* (15) assessed by electron microscopy the purity and integrity of the subcellular preparation in this method. The mitochondrial fraction was slightly contaminated with lysosomes, while the microsomal preparation was comprised of typical vesicles which were free from nuclei and mitochondria. IOP concentrations in each fraction were determined by FEA, and protein concentrations were determined by Lowry protein assay. (16).

Results and Discussion. *Biliary excretion of bilirubin.* Because the Gunn rats were obtained from a new commercial source (Blue Spruce Farms), it was essential to confirm that the animals were unable to transport bilirubin into bile as expected. Consequently, the biliary excretion of bilirubin was compared in normal (Sprague–Dawley) and Gunn rats. Maximum bilirubin transport was normal in Sprague–Dawley

rats (1.10 ± 0.31 SD μmole/min/kg), but not in Gunn rats (0.01 ± 0.01 μmole/min/kg). These rates of excretion are similar to those reported by Robinson *et al.* and others for Sprague–Dawley and Gunn rats (1.03 and 0.005 μmole/min/kg, respectively) (10, 17, 18). It is apparent that the Gunn rats used in the present experiments were unable to excrete bilirubin into bile.

Biliary excretion of iopanoate. The rate of iopanoate[3] excretion in bile after IOP administration was slightly less in Gunn rats (0.93 μmole/min/kg) than in Sprague–Dawley rats (1.29 μmole/min/kg) (Table I, Fig. 1). However, the difference was not statistically significant. The rates of bile flow and bile salt excretion both before and after IOP injection in the two strains of rats varied, but no consistent differences were apparent. When IOP-G was administered, biliary excretion of iopanoate was significantly greater in both species than after IOP injection (Table I, Fig. 2). Furthermore, biliary excretion in the Sprague–Dawley strain was higher, but the difference was not significant statistically. Finally, there was no significant difference in the rates of bile flow and bile salt excretion before or after injection of IOP-G (Table I).

Bile from Gunn rats was analyzed for metabolites using HPLC. Under the conditions of the chromatographic study, the retention times of IOP-G and IOP were 3.4 and 12.7 min, respectively (Figs. 3A, B, C). When IOP was injected intravenously, 92% of iopanoate in bile was IOP-G and 8% was the parent compound (Fig. 3E). A similar distribution was found after IOP-G injection (Fig. 3F). A double peak for the IOP-G peak was found (Fig. 3F). The second of the two IOP-G peaks had a retention time of 3.4 min which is the same as the IOP-G standard. This extra peak was not found in the IOP-G infusate prior to injection or in bile from rats injected with IOP-G, and it was not found after IOP administration in either Sprague–Dawley or Gunn rat bile. FEA analysis indicated that both of the peaks

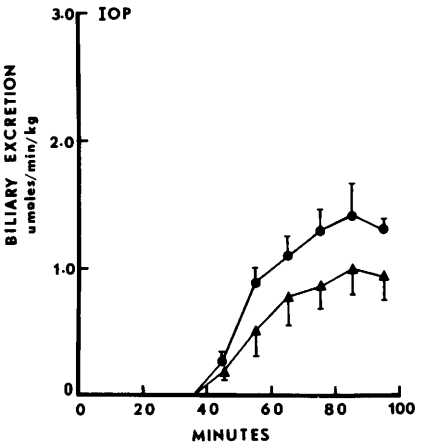

FIG. 1. Biliary excretion of iopanoate in rats administered a 300 μmole/kg dose of IOP after a 30-min control period. Closed circles and closed triangles represent data from Sprague–Dawley and Gunn rats, respectively. Data at each time period are presented as mean ± SD of four studies.

were due to iodine-containing compounds so they may represent epimers of IOP-G. The chromatographic data indicate that the iopanoate in bile of Gunn rats is predominately (92%) in the form of IOP-G. Both peaks of IOP-G were included in the calculations.

Since a fraction of the contrast material appeared in bile of Gunn rats as IOP after it had been administered as IOP-G, it is apparent that deconjugation of IOP-G occurs to a limited degree. This deconjugation was not found in Sprague–Dawley rats.

The molecular basis for the genetic deficiency of UDP-glucuronyl transferase in Gunn rats is not completely understood. Although there is no detectable glucuronyl transferase activity toward bilirubin in the Gunn rat, the rates of glucuronidation of *o*-aminophenol and *o*-aminobenzoic acid are approximately 20% of normal (2, 19, 20), and *p*-nitrophenol glucuronidation is almost the same as in normal rats (20, 21). Evidence from study of the development (22) and the differential inducibility of several substrates of UDP-glucuronyl transferase by phenobarbital and 3-methylcholanthrene (23) and glucocorticoids (24–26) suggests that at least two functional heter-

[3] The term "iopanoate" is used to designate the total concentration of contrast material including both IOP and IOP-G.

TABLE I. BILIARY EXCRETION OF IOPANOATE IN SPRAGUE–DAWLEY AND GUNN RATS

	Compound injected	Sprague–Dawley Pre-injection	Sprague–Dawley Post-injection	Gunn Pre-injection	Gunn Post-injection
Bile flow (μl/min/kg)	IOP	71.0	74.6	69.3	50.3
		6.1	13.4	10.2	25.4
	IOP-G	65.3	65.7	76.9	61.3
		14.9	15.7	11.3	15.7
Bile salt excretion (μmole/min/kg)	IOP	1.76	1.67	1.47	1.07
		0.24	0.43	0.60	0.40
	IOP-G	1.71	1.98	1.95	1.91
		0.23	0.61	0.38	0.51
Biliary excretion (μmole/min/kg)	IOP		1.29		0.92
			0.30		0.37
	IOP-G		2.47		1.85
			0.65		0.46

Note. Four rats in each group; individual samples averaged. Results are means ± SD.

ogeneous forms of the enzyme exist. Furthermore, UDP-glucuronyl transferase activity has been separated into two fractions; one showing activity toward estrone, and the other toward *p*-nitrophenol (27). A third form, which is inducible with pregnenolone-16, α-carbonitrile, has also been recently proposed (28). However, Odell *et al.* (29) report minimal conversion of di-

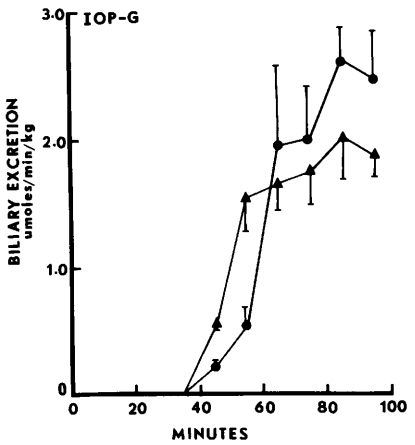

FIG. 2. Biliary excretion of iopanoate in rats administered a 300 μmole/kg dose of IOP-G after a 30-min control period. Closed circles and closed triangles represent data from Sprague–Dawley and Gunn rats, respectively. Data at each time period are presented as mean ± SD of four studies.

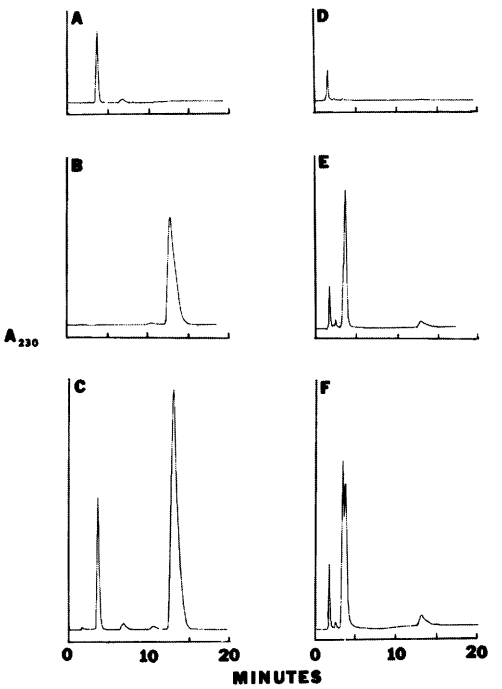

FIG. 3. HPLC chromatograms of bile from Gunn rats administered IOP and IOP-G. Chromatograms in plates A, B, and C represent standards of IOP-G, IOP, and a mixture of both compounds, respectively. Plate D represents a chromatogram of bile sampled prior to injection of any contrast material; plate E is a chromatogram of bile sampled from a Gunn rat administered IOP; plate F is a chromatogram of bile sampled from a Gunn rat administered IOP-G.

TABLE II. HEPATIC CONCENTRATION AND DISTRIBUTION OF IOPANOATE 60 MIN AFTER INJECTION OF IOP OR IOP-G IN SPRAGUE–DAWLEY AND GUNN RATS

Compound injected	Rat strain	Iopanoate (nmole/mg protein)				Liver (μmole/g wet wt)
		Homogenate	Cytosolic fraction	Microsomal fraction	Mitochondrial fraction	
IOP	Sprague–Dawley	10 ± 1	6 ± 1	31 ± 10	13 ± 1	1.40 ± 0.16
IOP	Gunn	10 ± 1	6 ± 1	23 ± 5	14 ± 2	1.62 ± 0.12
IOP-G	Sprague–Dawley	14 ± 3	5 ± 1	38 ± 17	19 ± 2	2.04 ± 0.11
IOP-G	Gunn	16 ± 6	6 ± 1	39 ± 16	21 ± 4	1.71 ± 0.13

Note. Data represent mean ± SD (n = 4).

methyl diester of bilirubin to bilirubin glucuronide in Gunn rats and in the microsomes of Crigler–Najjar patients. These authors propose that a defective microsomal matrix structure may exist at the site where the enzyme is located which interferes with glucuronidation of certain substrates. In addition, glucuronyl transferase-active proteins, isolated from Gunn and Wistar rat liver microsomes, reveal an immunologically identical enzyme which migrates electrophoretically as a single protein (30, 31). However, only the Wistar rat liver preparation conjugates bilirubin.

The results of the present experiments demonstrate the ability of Gunn rats to form glucuronide conjugates with IOP, despite their inability to conjugate bilirubin. Therefore, successful opacification of the gallbladder on cholecystography performed with IOP in patients with Crigler–Najjar syndrome (3–5) would be expected. However, the data do not allow a distinction between the two hypotheses proposed to explain the deficiency in transferase activity for bilirubin, but not for IOP. For IOP either the enzyme form which accepts IOP as a substrate is sufficiently active to account for normal or near normal rates of biliary excretion of IOP, or IOP is accessible to the active site of the enzyme despite the altered microsomal microenvironment.

Hepatic iopanoate distribution. There is no statistical difference in the hepatic concentration and subcellular distribution of iopanoate in liver from Sprague–Dawley and Gunn rats 60 min after the administration of either IOP or IOP-G (Table II). However, biliary excretion of iopanoate during the 10-min period just prior to removal of the liver (60 min after injection of contrast material) was higher after IOP-G injection (2.44 ± SD 0.72 and 1.79 ± 0.21 μmole/min/kg for Sprague–Dawley and Gunn rats, respectively) than after IOP injection (1.27 ± SD 0.17 and 0.92 ± 0.36 μmole/min/kg for Sprague–Dawley and Gunn rats, respectively). Thus, the rate of iopanoate biliary transport after IOP-G was higher than would be predicted from the liver concentration alone. This suggests two possibilities for the augmented biliary excretion: (i) insufficient amount of IOP-G are available intracellularly after IOP injection to establish maximum canalicular transport, and a step prior to canalicular transport is the limiting process in the overall transport; (ii) the presence of IOP in liver inhibits canalicular transfer of IOP-G in a manner similar to that proposed for BSP and its conjugates (32).

1. Crigler JF, Najjar VA. Congenital familial nonhemolytic jaundice with kernicterus. Pediatrics 70:169–179, 1952.
2. Schmid R. Hyperbilirubinemia. In: Stanbury JB, Wyngaarden JB, Frederickson DS, eds. The Metabolic Basis of Inherited Disease. New York, McGraw–Hill, 3rd ed, pp1141–1178, 1972.
3. Barnhart JL, Berk RN, Czuleger PC. Biliary excretion of three cholecystographic contrast agents in dogs: Iocetamic acid acid, iopronic acid and iosumetic acid. Invest Radiol 14:79–87, 1979.
4. McChesney EW, Hoppe JO. Observations on the

metabolism of iopanoic acid. Arch Int Pharmacodyn 99:127–140, 1954.
5. Barnhart JL, Parkhill BJ, Cobo-Frenkel A, Berk RN, Loeb PM. Iopanoate glucuronide: Procedure for its isolation and purification and pharmacokinetics of its biliary excretion. Invest Radiol 13:347–355, 1978.
6. Arias IM, Gartner LM, Cohen M, BenEzzer J, Levi AJ. Chronic non-hemolytic unconjugated hyperbilirubinemia with glucuronyl transferase deficiency. Amer J Med 47:395–409, 1969.
7. Sugar P. Familial non-hemolytic jaundice, congenital with kernicterus. Arch Int Med 108:121–125, 1961.
8. Schmid R, Hammaker L. Metabolism and disposition of C-14 bilirubin in congenital nonhemolytic jaundice. J Clin Invest 42:1720–1734, 1963.
9. Gunn CK. Hereditary acholuric jaundice. J Hered 29:137–139, 1938.
10. Schmid R, Axelrod J, Hammaker L, Swarm RL. Congenital jaundice in rats due to a defect in glucuronide formation. J Clin Invest 37:1123–1130, 1958.
11. Zak B, Boyle AJ. A simple method for the determination of organic iodine. Amer Pharmacol Assoc 41:260–262, 1952.
12. Barnhart JL, Combes B. The effect of theophylline on hepatic excretion function. Amer J Physiol 227:194–199, 1974.
13. Kaufman L, Deconick F, Price DC, Guersry P, Wilson CJ, Hruska B, Swann SJ, Camp DC, Volgele AC, Friesen RD, Nelson JA. An automated fluorescent excitation analysis system for medical applications. Invest Radiol 11:210–213, 1976.
14. Malloy HT, Evelyn KA. The determination of bilirubin with the photoelectric colorimeter. Biol Chem 119:481–484, 1937.
15. Strange RC, Chapman BT, Johnston JD, Nimmo IA, Percy-Robb IW. Partitioning of bile acids into subcellular organelles and the in vivo distribution of bile acids in rat liver. Biochem Biophys Acta 583:535–545, 1979.
16. Lowry DH, Rosenberg NJ, Farr AC, Randall RJ. Protein measurement with the phenol reagent. J Biol Chem 193:265–275, 1951.
17. Robinson SH, Yonnoni C, Nagasawa S. Bilirubin excretion in rats with normal and impaired bilirubin conjugation: Effect of phenobarbital. J Clin Invest 50:2606–2613, 1971.
18. Arias IM, Johnson L, Solson S. Biliary excretion of injected conjugated and unconjugated bilirubin by normal and Gunn rats. Amer J Physiol 200:1091–1094, 1961.
19. Dutton GJ. In: Dutton GJ, ed. Glucuronic Acid: Free and Combined. Academic Press, New York, pp185–299, 1966.
20. Zakim D, Goldenberg J, Vessem DA. Regulation of microsomal enzymes by phospholipids. VI. Abnormal enzyme-lipid interaction in live microsomes from Gunn rats. Biochem Biophys Acta 297:497–502, 1963.
21. Nakata D, Zakim D, Vessey DA. Modification of protein–lipid interactions in the Gunn rat by treatment of microsomal UDP-glucuronyltransferase with diethylnitrosamine. Biochem Pharmacol 24:1823–1825, 1975.
22. Wishart GJ. Functional heterogeneity of UDP-glucuronyl-transferase as indicated by its differential development and inducibility by glucocorticoids. Biochem J 176:485–489, 1978.
23. Bock KW, Josting D, Lilienblum W, Pfeil H. Purification of rat liver microsomal UDP-glucuronyl transferase: Separation of two enzyme forms inducible by 3-methylcholanthrene or phenobarbital. Eur J Biochem 98:19–26, 1979.
24. Bock KW, Frohling W, Remmer H, Rexer B. Effects of phenobarbital and 3-methylcholanthrene on substrate specificity of rat liver microsomal UDP-glucuronyltransferase. Biochem Biophys Acta 327:46–56, 1973.
25. Wishart GJ. Demonstration of functional heterogeneity of hepatic uridine diphosphate glucuronosyl transferase activity after administration of 3-methylcholantherene and phenobarbital to rats. Biochem J 174:671–672, 1978.
26. Burchell B. Substrate specificity and properties of uridine diphosphate glucuronyltransferase purified to apparent homogeneity from phenobarbital treated rat liver. Biochem J 173:749–757, 1978.
27. Tukey RH, Billings RE, Tephly TR. Separation of oestrone UDP-glucuronyltransferase and p-nitrophenol UDP-glucuronyltransferase activities. Biochem J 171:659–663, 1978.
28. Watkins JB, Gregus Z, Thompson TN, Klaassen CD. Induction studies on the functional heterogeneity of rat liver UDP-glucuronyltransferase. Hepatology 1:559, 1981.
29. Odell BG, Cukier JO, Gourley GR. The presence of a microsomal UDP-glucuronyltransferase for bilirubin in homozygous jaundiced Gunn rats and in the Crigler–Najjar syndrome. Hepatology 1:307–315, 1981.
30. Burchell B. Isolation and purification of bilirubin UDP-glucuronyl transferase from rat livers. FEBS Lett 111:131–135, 1980.
31. Weatherill PJ, Kennedy SME, Burchell B. Immunochemical comparison of UDP-glucoronyltransferase from Gunn and Wistar-rat livers. Biochem J 191:155–163, 1980.
32. Barnhart JL, Combes B. Biliary excretion of dye in dogs infused with BSP or its glutathione conjugate. Amer J Physiol 231:399–407, 1976.

Received October 13, 1981. P.S.E.B.M. 1982, Vol. 170.