Proc. Natl. Acad. Sci. USA
Vol. 75, No. 2, pp. 682–685, February 1977
Biochemistry

Stimulation of bilirubin catabolism in jaundiced Gunn rats by an inducer of microsomal mixed-function monooxygenases*

(bile flow/bile acids/bilirubin-UDPglucuronate transferase)

JAIME KAPITULNIK†‡ AND J. DONALD OSTROW§¶

§ Gastrointestinal Section, University of Pennsylvania Medical Division (111-H3), Veterans Administration Hospital, Philadelphia, Pennsylvania 19104; and
† Department of Biochemistry and Drug Metabolism, Hoffmann-LaRoche, Inc., Nutley, New Jersey 07110

Communicated by Rudi Schmid, November 14, 1977

ABSTRACT **The homozygous, jaundiced Gunn rat compensates for its inability to form bilirubin conjugates by production of polar bilirubin metabolites which can be excreted in the bile without conjugation. To assess whether microsomal mixed-function monooxygenases might be involved in formation of these metabolites, a potent inducer of these enzymes, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin, was administered to Gunn rats in a single intraperitoneal dose of 10 μg/kg. Four to 6 days after treatment, plasma bilirubin levels had declined by a mean of 61%, whereas no significant change was observed in control Gunn rats that received only the dioxane vehicle. Use of tracer [^{14}C]bilirubin showed that the decline in plasma bilirubin was the result of a 7-fold increase in fractional bilirubin turnover which reduced the bilirubin pool to an average of 11% of the control values. In the new steady state, total bilirubin turnover was unaltered. The accelerated fractional bilirubin turnover was associated with augmented biliary excretion of polar bilirubin metabolites and a 16-fold increase in hepatic benzo[*a*]pyrene hydroxylase activity. Hepatic bilirubin-UDPglucuronate transferase was not induced, and no bilirubin conjugates appeared in the bile. This chemical simulation of the alternate pathways of bilirubin catabolism by the inducer suggests that microsomal cytochrome P_{448}-dependent monooxygenases may be involved in the formation of the polar bilirubin metabolites excreted by the Gunn rat.**

The homozygous, recessive Gunn rat exhibits severe unconjugated hyperbilirubinemia with kernicterus due to a hereditary total inability to conjugate bilirubin (1). In these animals, bilirubin turnover is maintained at a normal rate by metabolism of unconjugated bilirubin to more polar derivatives which are excreted in the bile without conjugation (2). Some of the metabolities in Gunn rat bile have been identified as dihydroxyl (3) and monohydroxyl (4) derivatives of bilirubin, reminiscent of drug metabolites produced by microsomal mixed-function monooxygenases (5). However, in these animals, and in the comparable Crigler–Najjar syndrome in humans (6–9), administration of the microsomal enzyme inducer, phenobarbital, produced no reduction in serum bilirubin concentrations or miscible unconjugated bilirubin pool, no increase in fractional turnover of unconjugated bilirubin, no induction of bilirubin conjugation, and no augmented excretion of ^{14}C-labeled unconjugated bilirubin or its metabolites.

To explain this lack of response to phenobarbital, it has been assumed that the Gunn rats and the patients lack the genetic information necessary for synthesis of bilirubin-UDPglucuronate transferase (6–9) and that the alternate pathways of bilirubin metabolism are not mediated by the mixed-function monooxygenases that respond to treatment with phenobarbital (9, 10). Because phenobarbital induces preferentially the cytochrome P_{450} system (11, 12), we have treated jaundiced Gunn rats with 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), the most potent known inducer of microsomal cytochrome P_{448} ($P_{1\text{-}450}$) (13), to determine its effect on metabolism of unconjugated bilirubin.

MATERIALS AND METHODS

Animals. Female Gunn rats, 6–8 months of age and weighing 240–340 g, were purchased from the Animal Facility, Skin and Cancer Hospital, Temple University, Philadelphia, PA. The rats were kept in an environment free of contact with insecticides, pine shavings, and other known inducers of microsomal monooxygenases. The diet consisted of Purina rat chow (Ralston Purina Co., St. Louis, MO) and water ad lib.

Experimental Plan. The rats were divided into two groups of 11 each, matched for age, weight, and plasma bilirubin concentrations. On day 1, the treated group received a single intraperitoneal injection of TCDD, 10 μg/kg of body weight in 0.3 ml of dioxane; the group of control rats received dioxane alone. On days 4 and 6, six of the animals in each group underwent repeat determination of plasma bilirubin concentrations, and four of them were also used for studies of bilirubin kinetics and bile pigment excretion (2). On day 7, the five other animals in each group were sacrificed for assay of hepatic bilirubin-UDPglucuronate transferase and benzo[*a*]pyrene hydroxylase activities.

Determination of [^{14}C]Bilirubin Kinetics and Excretion. On days 4 and 6, two pairs of matched rats from each group were anesthetized with pentobarbital (50 mg/kg intraperitoneally), provided with an external biliary fistula and femoral vein catheter, and given (intravenously) 15–85 μg of [^{14}C]bilirubin (specific activity, 20.7×10^3 dpm/μg). Beginning 14–16 hr later, five samples of heparinized blood were drawn from the tail vein at 2- to 2½-hr intervals for determination of plasma bilirubin specific activity and kinetics (2). Simultaneously, two 4-hr bile collections were made for assay of bile pigment, bile acid, and ^{14}C excretion. The rats were then exsanguinated by cardiac puncture into heparinized syringes.

Analysis. Plasma samples were analyzed for diazo reactivity (14) and for ^{14}C (15). Bile samples were assayed for total diazo reactivity (14), for bile acids (16), and for bile pigments reactive with diazotized ethyl anthranilate (17). Another 0.5 ml of bile was subjected to Folch solvent partition at pH 5.8 to separate

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U. S. C. §1734 solely to indicate this fact.

Abbreviation: TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin.

* Presented in part at the annual meeting of the American Association for the Study of Liver Diseases, Chicago, IL, Nov. 6, 1976, and abstracted in *Gastroenterology* (1976) 71, 914.

‡ Present address: Department of Pharmacology, Hebrew University–Hadassah Medical School, Jerusalem, Israel.
¶ To whom reprint requests should be addressed.

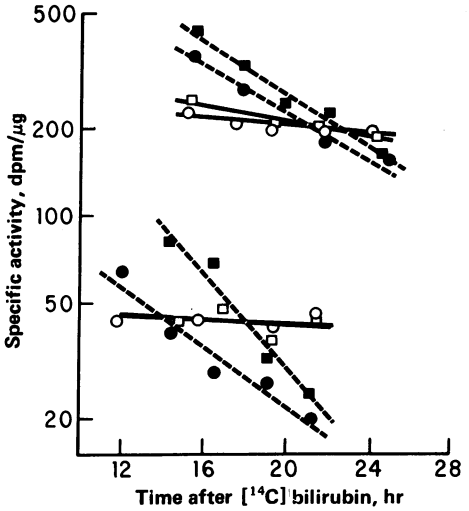

FIG. 1. Specific activity in plasma of Gunn rats after administration of [^{14}C]bilirubin. Rats were studied 4 days (lower group) or 6 days (upper group) after treatment with TCDD (solid symbols and dashed lines) or dioxane (open symbols and solid lines). The 6-day group of four rats received approximately 6 times as much labeled bilirubin as the 4-day group. Lines were calculated by the method of least squares.

unconjugated bilirubin (lower phase) from polar metabolities (upper phase) (18). Each phase was then analyzed for diazo reactivity and radioactivity (15).

Bile samples from the four TCDD-treated and four control rats were pooled, respectively, in proportion to their individual volumes. One portion of each pool was subjected to sequential Folch partition at pH 5.8 and then 3.0, followed by thin-layer chromatography on silica gel of the pigments in each fraction (3, 19). Ethyl anthranilate azopigments were prepared from the other portion and chromatographed (4, 17). The pigment and azopigment bands were eluted individually with 0.06 M HCl in methanol, and their absorbance at 530 nm was determined. For assay of radioactivity, 1 ml of each eluate was blended with 2 ml of ethanol plus 10.0 ml of scintillator solution. Total recovery of absorbance and radioactivity from the eluted bands ranged from 72% to 81%.

Radioassay. Counting was performed to 5000 cpm in a Beckman LS-200B liquid scintillation spectrometer at efficiencies of 0.40–0.65. The scintillation blend consisted of 10 ml of diphenyloxazole (8 g/liter in toluene) plus 3 ml of methanol or ethanol.

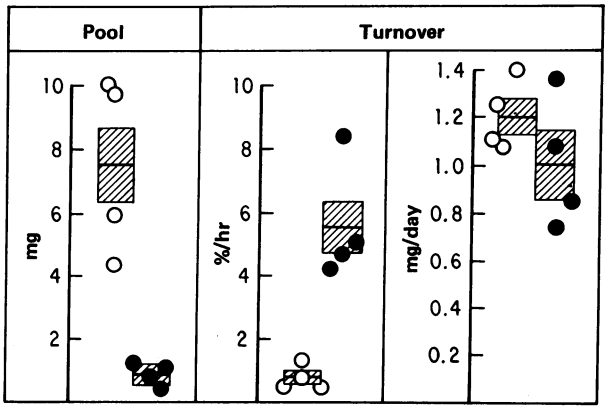

FIG. 2. Effects of TCDD on [^{14}C]bilirubin kinetics in Gunn rats, calculated from the semilogarithmic specific activity curves in Fig. 1. Individual values are given for TCDD-treated rats (solid circles) and dioxane-treated controls (open circles); hatched bars represent the mean ± SEM. Values shown are for total miscible bilirubin pool (*Left*), fractional turnover (*Center*), and absolute daily turnover (*Right*).

Assay of Hepatic Enzyme Activities. A 25% (wt/vol) liver homogenate was prepared in 0.25 M sucrose. For assay of benzo[*a*]pyrene hydroxylase activity, liver homogenate (equivalent to 2 mg wet weight of tissue) was incubated at 37° for 5 min in the presence of NADPH (0.5 μmol), $MgCl_2$ (3 μmol), EDTA (0.1 μmol), benzo[*a*] pyrene (0.1 μmol in 50 μl of acetone), and 0.1 M KH_2PO_4/K_2HPO_4 buffer (pH 7.4) in a total volume of 1 ml. The quantity of fluorescent phenolic metabolites formed was determined (20), with 3-hydroxybenzo[*a*]pyrene as the standard. Bilirubin UDPglucuronate transferase was assayed (21) by using liver homogenate equivalent to 25 mg wet weight of tissue and incubating at 37° for 15 min.

Statistical Analysis. Semilogarithmic declines of plasma specific activity were calculated by the method of least squares (22). Differences between groups were assessed by Student's *t* test. All values were expressed as mean ± SEM.

RESULTS

Plasma Bilirubin Concentrations. Pretreatment plasma unconjugated bilirubin concentrations were matched between the TCDD-treated and control groups (12.4 ± 1.1 versus 12.5 ± 1.2 mg/dl). Although in two of the six control rats, plasma levels decreased by 43% and 30% after dioxane injection, the mean value after dioxane injection, 10.5 ± 1.2 mg/dl, did not differ significantly from the initial mean level. By contrast, TCDD significantly decreased plasma unconjugated bilirubin concentrations by 54%–67%, to a mean of 4.8 ± 0.5 mg/dl ($P < 0.001$). This value also differed significantly from the post-treatment values in the control rats ($P < 0.002$).

Plasma Bilirubin Kinetics. Four to 6 days after treatment, both TCDD and control animals exhibited stable plasma unconjugated bilirubin concentrations and a semilogarithmic decline of plasma [^{14}C]bilirubin specific activity, consistent with a first-order steady state (Fig. 1). Analysis of these specific activity curves indicated that treatment with TCDD had decreased the miscible bilirubin pool by 89% ($P < 0.002$) and caused a 7.2-fold increase in fractional bilirubin turnover ($P < 0.002$) compared with controls (Fig. 2). Total bilirubin turnover in the TCDD-treated animals did not differ significantly from the control values.

Bile Pigment Excretion. Mean output of diazo reactivity in the bile of TCDD-treated rats was 0.92 ± 0.09 OD·ml/hr, compared with 0.26 ± 0.04 in the controls ($P < 0.001$).‖ Likewise, the mean hourly output of radioactivity in bile, expressed equivalent to the μg of parent [^{14}C]bilirubin, increased 3.5-fold with TCDD (96.9 ± 10.3 versus 27.2 ± 3.7 μg/hr, $P < 0.001$). Folch solvent partition revealed that the increased output of labeled pigments represented almost entirely polar bilirubin derivatives with no significant increase in excretion of unconjugated bilirubin.

Thin-layer chromatography of the pigments or azopigments from pooled bile samples of both the TCDD-treated and control animals revealed essentially the same bands, corresponding to the patterns of Gunn rat bile pigments found previously in this laboratory in the same chromatography systems (3, 19). However, the amount of pigment and ^{14}C in each band was signif-

‖ OD units are used because the intensity of the diazo reaction varies among individual bilirubin derivatives excreted by the Gunn rat. With bilirubin as the standard, OD × 5.85 = bile bilirubin concentration in mg/dl.

Table 1. Bile flow and bile acids in Gunn rats*

Treatment group	Bile flow, ml/hr	Bile acid concentration, mM	Bile acid output, μmol/hr
Control	0.72 ± 0.11	1.67 ± 0.22	1.12 ± 0.18
TCDD	1.15 ± 0.07†	1.07 ± 0.15	1.21 ± 0.20

* Mean values ± SEM during the period 14–28 hr after bile duct cannulation, when ^{14}C turnover and excretion studies were performed.
† For difference from control, $P < 0.01$.

icantly greater after TCDD treatment. For each azopigment, the increments in radioactivity and diazo reactivity were roughly proportional, indicating that the parent pigments were derived from [^{14}C]bilirubin. The only exceptions were two faint diazo reactive bands of undetermined composition that appeared after TCDD treatment but were not detected in control biles. Comparison of the ethyl anthranilate azopigments from the treated rats with standards prepared from dog bile (17) indicated no excretion of bilirubin conjugates after TCDD administration.

Bile Flow and Bile Acid Excretion. Hourly bile acid outputs were unaffected by TCDD (Table 1). The significant increase in bile flow was balanced by a not quite significant trend to lower bile acid concentrations in the TCDD-treated animals.

Liver Enzyme Activities. Treatment with TCDD caused a 16-fold increase in benzo[*a*]pyrene hydroxylase activity, from 0.5 ± 0.1 to 8.2 ± 0.9 μmol of phenols formed per g of liver per hr ($P < 0.001$). By contrast, no bilirubin-UDPglucuronate transferase activity was detected in either control or TCDD-treated animals (1, 9).

DISCUSSION

This paper reports successful chemical stimulation of the alternate pathways of bilirubin catabolism in the jaundiced Gunn rat. Within 4 to 6 days after a single injection of TCDD, plasma bilirubin levels decreased by more than 60% and the mean total bilirubin pool contracted by almost 90%. This indicates that unconjugated bilirubin was removed from the body and not simply displaced by TCDD from binding sites on plasma albumin into the tissues. Rather, the decline in plasma unconjugated bilirubin was related to a striking acceleration of fractional bilirubin turnover, but total bilirubin turnover and, by inference, bilirubin production in the new steady state were not significantly altered. This increased fractional turnover caused by TCDD resulted entirely from augmented conversion of unconjugated bilirubin to polar derivatives that were excreted in the bile. Analysis of the excreted pigments and assay of bilirubin-UDPglucuronate transferase activity in the livers indicated no stimulation of bilirubin conjugation by TCDD. Thus, TCDD stimulated a catabolic process that enables the Gunn rat to dispose of bilirubin in the absence of the glucuronidation pathway.

The augmented biliary excretion of unconjugated bilirubin metabolites could not be attributed to an increased bile acid output (23) because this did not occur in the TCDD-treated rats. Neither could it be related solely to enhanced bile flow because flow increased by only about 50% whereas excretion of the pigments increased 3½-fold. The increase in bile volume, without an increase in bile acid output, further suggests that TCDD stimulated the bile acid-independent component of bile flow (24). The lack of an effect on the nadir of bile acid output that occurred in both treated and control rats during the 14–28 hr after duct cannulation (19) also indicates that TCDD, like phenobarbital (25), may not stimulate bile acid synthesis in the rat.

Although the serum bilirubin concentration declined in two of our six control rats, the complete separation of final serum bilirubin values between the control and TCDD-treated animals strongly indicates that the TCDD effect was genuine as well as statistically significant. Our Gunn rats, purchased from an animal facility that does not use insecticides or pine bedding, usually exhibit initial plasma bilirubin concentrations of 10–16 mg/dl, which are higher than the values generally reported for Gunn rats (1, 2, 7–9, 15). Despite our precautions to exclude inducing agents, "spontaneous" declines in plasma bilirubin concentrations, *never exceeding 45%*, usually are seen in a few animals from each batch during the 3–4 weeks after their transfer to our laboratory. This phenomenon, which first suggested to us that bilirubin metabolism might be inducible and prompted us to perform the present experiments, is a well-known problem in studies involving drug metabolism (5). It is the reason why concomitant controls and initially uninduced animals are essential in such studies and were used by us.

Arias *et al.* (6), DeLeon *et al.* (7), and Robinson *et al.* (8, 9) did not detect any effect of phenobarbital, an inducer of microsomal cytochrome P_{450}, on bilirubin metabolism in the Gunn rat. However, the concomitant enhancement, by TCDD treatment, of both hepatic benzo[*a*]pyrene hydroxylase and of biliary excretion of polar unconjugated bilirubin derivatives, some of which are hydroxylated (3, 4), strongly suggests that a microsomal mixed-function monoxygenase system is involved in the oxidative catabolism of unconjugated bilirubin in these jaundiced animals. Microsomal cytochrome P_{448}, which is preferentially induced by TCDD (13), may therefore be the enzyme that hydroxylates unconjugated bilirubin.

Remaining to be clarified also are the organ(s) involved in the effect of TCDD on bilirubin catabolism and whether subcellular oxidases other than microsomal mixed-function monooxygenases can convert unconjugated bilirubin to polar metabolities. Nonetheless, the present report strongly suggests that microsomal cytochrome P_{448} is involved in this process. If nontoxic inducers of cytochrome P_{448} can be developed, pharmacological reversal of hyperbilirubinemia in totally transferase-deficient (Crigler–Najjar) patients may eventually replace the cumbersome phototherapy method that is the sole effective treatment currently available (10).

The authors are grateful to Ms. Eileen Hercker and Heidemarie Cheney for their expert technical assistance and to Dr. Allan H. Conney for his helpful advice and suggestions. This work was supported by a Medical Investigatorship from the U.S. Veterans Administration (MRIS-6680) and by Research Grant 5-R01-AM-14543 from the National Institute of Arthritis and Metabolic and Digestive Diseases, National Institutes of Health, Bethesda, MD.

1. Schmid, R., Axelrod, J., Hammaker, L. & Swarm, R. L. (1958) *J. Clin. Invest.* **37**, 1123–1130.
2. Schmid, R. & Hammaker, L. (1963) *J. Clin. Invest.* **42**, 1720–1734.
3. Berry, C. S., Zarembo, J. E. & Ostrow, J. D. (1972) *Biochem. Biophys. Res. Commun.* **49**, 1366–1375.
4. Blanckaert, N., Fevery, J., Heirwegh, K. P. M. & Compernolle, F. (1977) *Biochem. J.* **164**, 237–249.
5. Conney, A. H. (1967) *Pharmacol. Rev.* **19**, 317–366.
6. Arias, I. M., Gartner, L. M., Cohen, M., BenEzzer, J. & Levi, A. J. (1969) *Am. J. Med.* **47**, 395–409.
7. DeLeon, A., Gartner, L. M. & Arias, I. M. (1967) *J. Lab. Clin. Med.* **70**, 273–278.
8. Robinson, S. H. (1971) *Proc. Soc. Exp. Biol. Med.* **138**, 281–285.

9. Robinson, S. H., Yannoni, C. & Nagasawa S. (1971) *J. Clin. Invest.* **50,** 2606–2613.
10. Ostrow, J. D. (1972) in *Progress in Liver Diseases,* eds. Popper, H. & Schaffner, F. (Grune & Stratton, New York), Vol. 4, pp. 447–462.
11. Sladek, N. E., & Mannering, G. J. (1966) *Biochem. Biophys. Res. Commun.* **24,** 668–674.
12. Alvares, A. P., Schilling, G., Levin, W. & Kuntzman, R. (1967) *Biochem. Biophys. Res. Commun.* **29,** 521–526.
13. Poland, A. P., Glover, E., Robinson, J. R. & Nebert, D. W. (1974) *J. Biol. Chem.* **249,** 5599–5606.
14. Michaelsson, M. (1961) *Scand. J. Clin. Lab. Invest. Suppl. 56* **13,** 1–80.
15. Ostrow, J. D. (1971) *J. Clin. Invest.* **50,** 707–718.
16. Iwata, T. & Yamasaki, K. (1964) *J. Biochem. Tokyo* **56,** 425–431.
17. Heirwegh, K. P. M., Fevery, J., Meuwissen, J. A. T. P., DeGroote, J., Compernolle, F., Desmet, V. & Van Roy, F. P. (1974) *Methods Biochem. Anal.* **22,** 205–250.
18. Ostrow, J. D., & Murphy, N. H. (1970) *Biochem. J.* **120,** 311–327.
19. Ostrow J. D., Berry, C. S. & Zarembo, J. E. (1974) in *Phototherapy in the Newborn: An Overview,* eds. Odell, G. B., Schaffer, R. & Simopoulos, A. P. (National Academy of Sciences, Washington, DC), pp. 74–92.
20. Nebert, D. W. & Gelboin, H. V. (1968) *J. Biol. Chem.* **243,** 6242–6249.
21. Heirwegh K. P. M., Van de Vijver, M. & Fevery, J. (1972) *Biochem. J.* **129,** 605–618.
22. Edwards, A. L. (1954) *Statistical Methods for the Behavorial Sciences.* (Rinehart and Co., New York).
23. Ashkin, J. R., Hercker, E. S. & Ostrow, J. D. (1976) *Gastroenterology* **70,** 977 (Abstr.).
24. Erlinger, S. & Dhumeaux, D. (1974) *Gastroenterology* **66,** 281–304.
25. Shefer, S., Hauser, S. & Mosbach, E. H. (1972) *J. Lipid Res.* **13,** 69–70.