Hepatic excretion of circulating bilirubin photoproducts in the Gunn rat.

A F McDonagh, L A Palma

J Clin Invest. 1980;66(5):1182-1185. https://doi.org/10.1172/JCI109951.

Research Article

To investigate the origin and metabolism of the intermediates that occur in blood during phototherapy of neonatal jaundice, serum from irradiated homozygous Gunn rats was injected intravenously into other homozygous Gunn rats fitted with bile fistulas, and the excretion of pigment in the bile of the recipient rats was studied. In some experiments the donor rats were labeled with [14C]bilirubin; in others the recipient rats were labeled. Injection of donor serum from irradiated rats caused a transient burst of pigment excretion in the bile of the recipient rats. However, simultaneous bursts of pigment and 14C excretion were observed only when the donor rat was labeled and the recipient rat was not, and not when the donor rat was unlabeled and the recipient rat was labeled. In addition, there was simultaneous transient enhanced excretion of pigment and label when labeled recipient rats were exposed briefly to blue light. We conclude that (a) the phototherapy intermediates previously detected spectroscopically in serum are formed from bilirubin and are excreted in bile independently of bilirubin; (b) the enhanced excretion of pigment in bile during phototherapy is not caused by complex formation between bilirubin and photoproducts, or by liver damage produced by photoproducts or light.

Hepatic Excretion of Circulating Bilirubin Photoproducts in the Gunn Rat

A. F. McDonagh and L. A. Palma, *Department of Medicine and Liver Center, University of California, San Francisco, California 94143*

ABSTRACT To investigate the origin and metabolism of the intermediates that occur in blood during phototherapy of neonatal jaundice, serum from irradiated homozygous Gunn rats was injected intravenously into other homozygous Gunn rats fitted with bile fistulas, and the excretion of pigment in the bile of the recipient rats was studied. In some experiments the donor rats were labeled with [^{14}C]bilirubin; in others the recipient rats were labeled. Injection of donor serum from irradiated rats caused a transient burst of pigment excretion in the bile of the recipient rats. However, simultaneous bursts of pigment and ^{14}C excretion were observed only when the donor rat was labeled and the recipient rat was not, and not when the donor rat was unlabeled and the recipient rat was labeled. In addition, there was simultaneous transient enhanced excretion of pigment and label when labeled recipient rats were exposed briefly to blue light. We conclude that (*a*) the phototherapy intermediates previously detected spectroscopically in serum are formed from bilirubin and are excreted in bile independently of bilirubin; (*b*) the enhanced excretion of pigment in bile during phototherapy is not caused by complex formation between bilirubin and photoproducts, or by liver damage produced by photoproducts or light.

INTRODUCTION

When rats with hereditary unconjugated hyperbilirubinemia (Gunn rats) are exposed to blue or broad-spectrum white light there is an apparent increase in the amount of unconjugated bilirubin excreted from the liver into bile (1). A similar effect occurs in jaundiced babies (2) and is believed to be crucially important for the successful treatment of neonatal jaundice by phototherapy. Several theories have been proposed to explain this effect. It has been suggested, for example, that photoproducts formed in peripheral tissues from bilirubin or other metabolites might interact with unreacted bilirubin to form reversible complexes that can be excreted by the liver (complex hypothesis) (3–5). Another suggestion is that light or photoproducts might damage the liver, making it in some way leaky and allowing unconjugated bilirubin to be excreted more readily into bile (leaky hypothesis) (1, 2, 4–7). A third, more recent, proposal is that light converts bilirubin in peripheral tissues to less stable configurational isomers that are readily excretable by the liver and that revert spontaneously to the normal isomer in bile or during analysis of the bile (isomer hypothesis) (For leading references see McDonagh et al. [8]).

We recently reported evidence that strongly supports the isomer hypothesis (8). We showed that unstable substances that behave like synthetic bilirubin photoisomers occur in the blood of jaundiced rats during irradiation with blue light. The concentration of these substances in serum was low, but increased when the bile duct was obstructed. When injected intravenously into recipient jaundiced rats in the dark, these substances caused a pronounced increase in hepatic bile-pigment excretion. Not surprisingly, similar substances also appear to be generated in vivo during phototherapy of jaundiced human neonates (9, 10). Thus far, however, there is no direct evidence that these therapeutically important intermediates, which occur in the circulation during phototherapy, are actually derived from bilirubin, or that they are the molecular precursors of the increased amount of unconjugated pigment that is excreted in bile. Furthermore, although our recent studies strongly supported the isomer hypothesis, they did not rule out other possible mechanisms, particularly the complex and leaky hypotheses noted above. To address these points, we have repeated our earlier studies using jaundiced Gunn rats whose endogenous bilirubin was uniformly labeled with [^{14}C]bilirubin. Two series of experiments were done. In one, serum from irradiated unlabeled donor rats was injected in the dark into labeled recipient rats. In the other, serum from irradiated labeled donor rats was injected into unlabeled recipient rats. In both

Received for publication 16 July 1980 and in revised form 25 August 1980.

series, the excretion of pigment and radioactivity in the bile of the recipient rats was measured.

The results provide the first direct proof that the intermediates detectable in vivo in the circulation during light exposure are derived from bilirubin and that these intermediates themselves are excreted by the liver into bile. Moreover, the results specifically exclude the complex and leaky hypotheses and indicate that the apparent excretion of unconjugated bilirubin during phototherapy is not due to a direct effect of light on the liver.

METHODS

Adult male homozygous Gunn rats, bred at the University of California, San Francisco, were used. They were labeled by intravenous injection of [^{14}C]bilirubin (40–125 μg [2.0 $\times$ 10^5–3.0 $\times$ 10^6 dpm] dissolved in 1 ml Sprague-Dawley rat serum, Simonsen Labs., Gilroy, Calif.) 15–24 h before use. Serum from irradiated homozygous Gunn rats (referred to in the figures as 'light serum') was obtained as described (8). Briefly, restrained shaved rats with cannulated bile ducts were irradiated with 40-W Westinghouse Special Blue tubes (Westinghouse Electric Corp., Pittsburgh, Pa.) for 3.5 h, with the bile flow interrupted during the last 3 h of irradiation by blocking the cannula. The animals were then exsanguinated, and serum was collected under a safelight. Control animals were treated similarly, except that they were not shaved and were exposed only to an infrared heating lamp during the 3.5-h phototherapy period. (Serum from these animals is referred to as 'dark serum' in the figures.) Serum samples (2 ml) were stored overnight at 4°C before being injected intravenously into recipient homozygous Gunn rats in the dark. The recipient rats were kept in restraining cages in a darkroom and infused intravenously at 2 ml/h with a bile-salt–lipid-replacement mixture containing sodium cholate, lecithin, taurine, and cholesterol (11). An infrared lamp was used as necessary to maintain body temperature, and 20-W Westinghouse Special Blue lights placed ~5 cm above the animal were used for visible-light irradiation periods. Pigment excretion by recipient rats was monitored photometrically at 470 nm using a flow cell (0.5-mm pathlength, 2-μl vol) connected by a short cannula to the bile duct (8, 11). Effluent bile from the flow cell was collected continuously at 5-min intervals directly into tared counting vials in a fraction collector. The vials were reweighed to determine the bile flow rate, and the contents were prepared for scintillation counting as follows: protosol/ethanol (1:2 [vol/vol], 1 ml) was added, and the mixture was incubated in a shaking water bath at 60°C for 1 h. The mixture was cooled and 30% (vol/vol) hydrogen peroxide (0.5 ml) was added in drops. After 30 min, Biofluor (15 ml) and 0.5 M HCl (0.5 ml) were added in sequence with vigorous mixing, and the samples were counted twice for 10 min each. Background counts (~14 cpm/5 min interval of bile) were determined using aliquots of bile (100–150 μl) from unlabeled homozygous Gunn rats. Excretion of radioactivity is given in the figures as counts per minute above background, expressed per gram of bile excreted. The ^{14}C excretion curves were not corrected for the hold-up time (6–10 min) caused by the length of connecting tube between the flow cell and the fraction collector. All observations reported were confirmed by duplicate experiments in separate animals, which gave similar results. For the 40-W light fixture the spectral irradiance per nanometer at 450 nm, measured at the level of the rat's back, was 40 J/m^2 per s, corresponding to an incident photon flux of 9.1 $\times$ 10^{19} quanta/m^2 per s; for the 20-W light fixture the corresponding values were 27 J/m^2 per s and 6.1 $\times$ 10^{19} quanta/m^2 per s. Light intensity measurements were made with a calibrated leveled spectroradiometer (model 111D, United Detector Technology, Inc., Santa Monica, Calif.).

Crystalline [^{14}C]bilirubin (0.51–2.5 $\times$ 10^4 dpm/μg sp act) was prepared biosynthetically (12) from 5-amino-[4-^{14}C]levulinic acid (New England Nuclear, Boston, Mass.). Solutions of [^{14}C]bilirubin for intravenous injection were prepared by dissolving 100–310 μg of the solid in 0.1 M NaOH (0.3 ml) and mixing this solution without delay with 2.2 ml rat serum. Serum bilirubin concentrations were measured by diazotization (12). Biofluor and Protosol were obtained from New England Nuclear.

RESULTS

When serum from an unirradiated, unlabeled, bile-obstructed Gunn rat was injected in the dark into a Gunn rat labeled with [^{14}C]bilirubin, there was a very slight and transient increase in pigment excretion and no detectable increase in ^{14}C excretion in the bile of the recipient rat (Figs. 1 and 2). When serum from an irradiated, unlabeled bile-obstructed Gunn rat was injected into the same recipient rat in the dark, there was a marked transient increase in pigment excretion in the bile of the recipient rat, but no corresponding increase in ^{14}C excretion (Figs. 1 and 2). Yet, when the labeled recipient rat was irradiated briefly with blue light, there was an increase in pigment excretion in the bile, and a corresponding increase in ^{14}C excretion (Fig. 2), as anticipated.

In a corollary of the above experiments, an unlabeled Gunn rat was first irradiated briefly with blue light, and

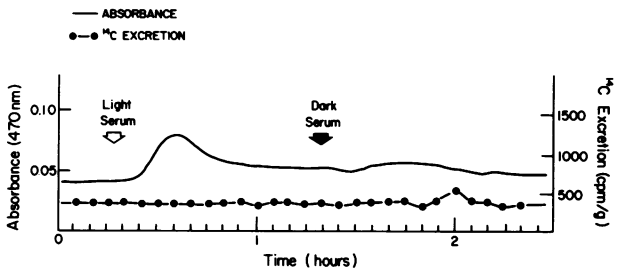

FIGURE 1 Biliary excretion of pigment and ^{14}C by a homozygous Gunn rat labeled with [^{14}C]bilirubin. The Gunn rat was labeled by intravenous injection of [^{14}C]bilirubin. Approximately 15 h later its bile duct was connected to a flow cell and the absorbance of bile at 470 nm was monitored photometrically. Effluent bile from the flow cell was collected at 5 min intervals, weighed, and counted. At the points indicated the rat was injected intravenously with serum (2 ml) from donor homozygous Gunn rats. 'Light serum' was obtained from a Gunn rat that had been irradiated with blue light for 3 h with its bile duct occluded; 'dark serum' was from an unirradiated control. The serum bilirubin concentrations in the light and dark serums were 5.4 and 5.1 mg/100 ml, respectively. The mean bile flow rate of the recipient rat during the experiment was 1.7±0.28 ml/h SD.

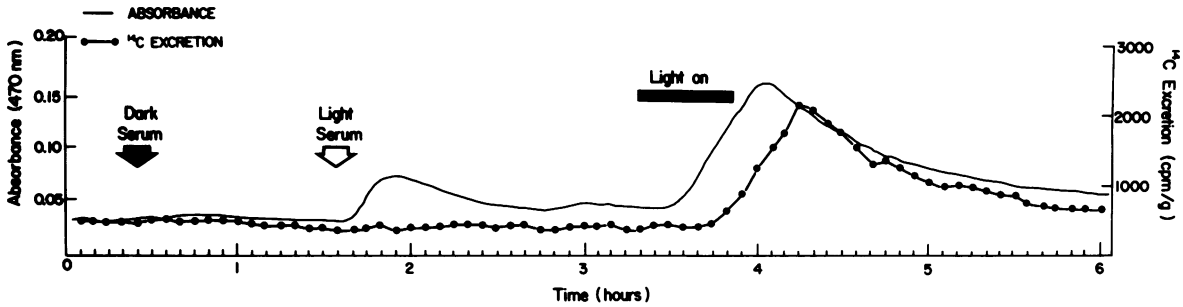

FIGURE 2 Biliary excretion of pigment and ^{14}C by a homozygous Gunn rat labeled with [^{14}C]-bilirubin. The general procedure was the same as in Fig. 1. 1.73 h after injection of the light serum, the recipient rat was irradiated for 35 min with blue light. The serum bilirubin concentrations of the dark (unirradiated) and light (irradiated) serums were 4.4 and 5.1 mg/100 ml, respectively, and the mean bile flow rate of the recipient rat during the experiment was 1.7±0.42 ml/h SD. The delay between the ^{14}C peak and the absorbance peak is due to the brief hold-up time (6–10 min) of the apparatus.

the resulting increase in pigment excretion was recorded (Fig. 3). The rat was then injected with serum from an unirradiated bile-obstructed rat that had been prelabeled with [^{14}C]bilirubin. This led to slight transient increases in both ^{14}C and pigment excretion by the recipient rat. Finally, the recipient rat was injected with serum from an irradiated, prelabeled bile-obstructed rat; there was a marked transient increase in both ^{14}C and pigment excretion in the bile of the recipient rat.

DISCUSSION

Markedly enhanced pigment excretion by the recipient rats in the dark occurred only after injection of serum from irradiated rats, as previously reported (8). The peak of pigment excretion was accompanied by a congruent excretion of radioactivity only when the donor rat was prelabeled with [^{14}C]bilirubin, and not when the recipient rat was prelabeled. Therefore the phototherapy intermediates in the serum from the irradiated rats, which have previously been characterized by absorbance-difference spectroscopy (8), are formed from bilirubin and are themselves excreted in bile. Since there was no detectable increase in ^{14}C excretion when labeled recipient rats were injected with serum from irradiated unlabeled donor rats, it is evident that the intermediates are excreted independently of endogenous bilirubin and that they do not displace endogenous bilirubin from the plasma or liver into the bile. In addition, the experiments show that there is no need to invoke a phototoxic effect of light on

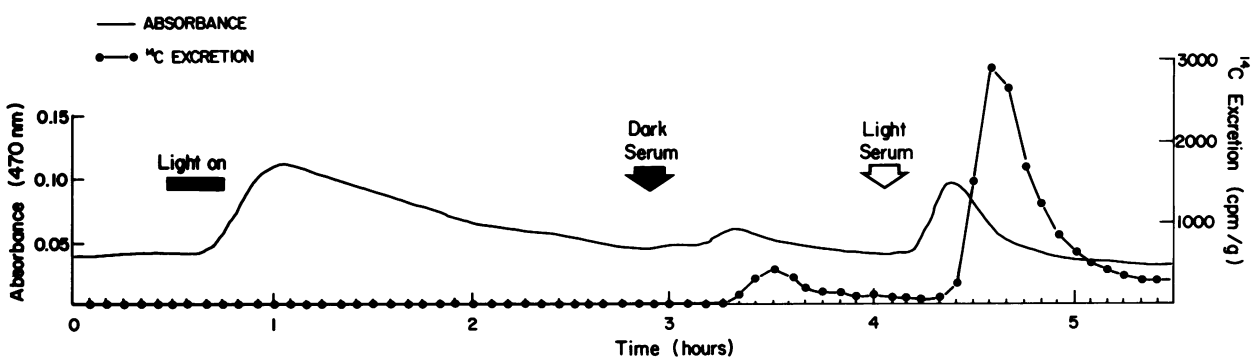

FIGURE 3 Biliary excretion of pigment and ^{14}C by an unlabeled homozygous Gunn rat. Pigment and ^{14}C excretion were measured as in Fig. 1. The rat was irradiated for 15 min with blue light and subsequently injected with serum samples (2 ml) obtained, as in Fig. 1, from irradiated (light serum) and unirradiated (dark serum) homozygous Gunn rats that had been labeled previously by injection of [^{14}C]bilirubin. The [^{14}C]bilirubin concentrations of the dark and light serum were 6.1 and 5.1 mg/100 ml, respectively, and the mean bile flow rate of the recipient rat during the experiment was 1.4±0.20 ml/h SD. The delay between the ^{14}C and absorbance peaks is due to the brief hold-up time (6–10 min) of the apparatus.

the liver as a cause of the enhanced pigment excretion during phototherapy, and they show that circulating photoproducts do not make the liver leaky to unconjugated bilirubin. This last conclusion is consistent with earlier reports that phototherapy does not alter the permeability of the liver to Evans blue, albumin, bile salts, phospholipids, and cholesterol (4, 5), or influence disappearance of bromosulfophthalein from the plasma (13).

Inasmuch as injection of serum from irradiated unlabeled rats did not augment the excretion of ^{14}C in rats prelabeled with [^{14}C]bilirubin, it is apparent that photoproducts in the serum do not form excretable complexes with bilirubin. The complex hypothesis had previously been ruled out by Ostrow and coworkers (4, 5) on the basis of experiments in which extracts of bile from irradiated Gunn rats were injected into donor rats. However, their conclusion was based on the implicit and probably erroneous assumption that phototherapy intermediates in the circulation remain unaltered during their passage through the liver and subsequent extraction from bile. This methodological flaw was avoided in the present work by injecting unadulterated serum obtained directly from light-exposed animals.

We do not know the reason for the slight transient increase in pigment and isotope excretion observed when unlabeled recipient rats were injected with serum from unirradiated labeled donors (Fig. 3). Most likely it was caused by small amounts of excretable colored derivatives of bilirubin in the donor serum. Such derivatives occur normally in Gunn rat bile (14) and they may have accumulated in the blood of the donor rats when the bile flow was restricted.

The present studies provide no new information on the identity of the yellow intermediates that are detectable spectroscopically in the serum of irradiated Gunn rats. However, the results are consistent with previous evidence that the intermediates are geometric isomers of bilirubin (8) and with the theory that photoisomerization of bilirubin plays an important role in phototherapy of neonatal jaundice.

ACKNOWLEDGMENTS

We thank Ms. Lydia Hammaker and Professor R. Schmid for helpful comments on the manuscript, and Mr. Michael Karasik for typing it.

These studies were supported by U. S. Public Health Service grants AM-11275, AM-18220, AM-18520, and AM-26307.

REFERENCES

1. Ostrow, J. D. 1971. Photocatabolism of labeled bilirubin in the congenitally jaundiced (Gunn) rat. *J. Clin. Invest.* **50:** 707–718.
2. Lund, H. T., and J. Jacobsen. 1974. Influence of phototherapy on the biliary bilirubin excretion pattern in newborn infants with hyperbilirubinemia. *J. Pediatr.* **85:** 262–267.
3. Ostrow, J. D., R. G. Knodell, H. C. Cheney, and C. S. Berry. 1974. Effects of phototherapy on hepatic excretory function in man and the rat. *In* Phototherapy for Neonatal Hyperbilirubinemia. A. K. Brown and J. Showacre, editors. Department of Health, Education, and Welfare Publication No. (National Institutes of Health) 76-1075. 151–165.
4. Ostrow, J. D., C. S. Berry, and J. E. Zarembo. 1974. Studies on the mechanism of phototherapy in the congenitally jaundiced rat. *In* Phototherapy in the Newborn: An Overview. G. B. Odell, R. Schaffer, and A. P. Simopoulos, editors. National Academy of Sciences, Washington, D. C. 74–92.
5. Ostrow, J. D., C. S. Berry, R. G. Knodell, and J. E. Zarembo. 1976. Effect of phototherapy on bilirubin excretion in man and the rat. *In* Bilirubin Metabolism in the Newborn. D. Bergsma and S. H. Blondheim, editors. Excerpta Medica, Amsterdam. **2:** 81–90.
6. Ostrow, J. D. 1972. Photochemical and biochemical basis of the treatment of neonatal jaundice. *Prog. Liver Dis.* **4:** 447–462.
7. Ostrow, J. D. 1972. Mechanisms of bilirubin photodegradation. *Semin. Hematol.* **9:** 113–125.
8. McDonagh, A. F., L. A. Palma, and D. A. Lightner. 1980. Blue light and bilirubin excretion. *Science (Wash., D. C.).* **208:** 145–151.
9. Blumberg, W. E., J. Eisinger, A. A. Lamola, R. McLead, A. Fanaroff, and A. F. McDonagh. 1979. Evidence for photobilirubin in blood of infants undergoing phototherapy for hyperbilirubinemia. *Am. Soc. Photobiol. Abstracts.* **7:** 143–144. (Abstr.)
10. Lamola, A. A., W. E. Blumberg, J. Eisinger, J. Flores, F. H. Doleiden, R. McLead, A. Fanaroff, and J. B. Watkins. 1980. Photobilirubin in the blood of neonates undergoing phototherapy. *Am. Soc. Photobiol. Abstracts.* **8:** 102. (Abstr.)
11. McDonagh, A. F., and L. M. Ramonas. 1978. Jaundice phototherapy: micro flow-cell photometry reveals rapid biliary response of Gunn rats to light. *Science (Wash., D. C.).* **201:** 829–831.
12. McDonagh, A. F. 1979. Bile pigments: bilatrienes and 5,15-biladienes. *In* The Porphyrins. D. Dolphin, editor. Academic Press, Inc., New York. **6:** 293–491.
13. Rubaltelli, F. F., F. Donzelli, and G. Largajolli. 1975. Effect of phototherapy on hepatic excretory function in newborns as measured by bromosulphalein clearance. *Biol. Neonate.* **26:** 333–336.
14. Blanckaert, N., J. Fevery, K. P. M. Heirwegh, and F. Compernolle. 1977. Characterization of the major diazo-positive pigments in bile of homozygous Gunn rats. *Biochem. J.* **164:** 237–249.