Photocatabolism of Labeled Bilirubin in the Congenitally Jaundiced (Gunn) Rat

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ABSTRACT To elucidate the mechanism by which phototherapy reduces serum bilirubin, studies were performed on the catabolism of labeled bilirubin in homozygous jaundiced Gunn rats before, during, and after a period of exposure to 1700 foot candles of daylight fluorescent light. Following equilibration with the body pool of an intravenously administered tracer dose of ^{3}H- or ^{14}C-bilirubin, radioactive and diazo reactive compounds were excreted in the bile at a slow, steady rate and plasma specific activity declined semilogarithmically. Subsequent exposure to light caused a marked increase in the biliary excretion of radioactive and diazoreactive compounds. Fecal and urinary radioactivity increased also but remained minor fractions of the total excreted radioactivity. After extinguishing the lights, these variables reverted gradually to control values. Spectral and chromotographic analysis of the excreted pigments and their azopigments demonstrated that the increased biliary radioactivity during phototherapy consisted of two roughly equal fractions: (*a*) unconjugated bilirubin, excreted at rates comparable to the output of conjugated bilirubin in the bile of normal nonjaundiced rats; and (*b*) water–soluble bilirubin derivatives, chromatographically identical with those found in Gunn rat bile under control lighting conditions but different from the products of photodecomposition of bilirubin in vitro. In some animals, phototherapy produced little decline in plasma bilirubin despite comparable acceleration of bilirubin catabolism. This was attributed tentatively to increased synthesis of early labeled bilirubin in these animals.

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INTRODUCTION

Phototherapy is commonly utilized to treat neonatal jaundice (3), but it is not known how illumination of the intact subject reduces the concentration of serum bilirubin. Until now, this problem has been approached by study of the photodecomposition of bilirubin in vitro, either in albumin solution (4) or in icteric serum (5). From such systems, a series of bilirubin photoderivatives have been separated and partially characterized (2, 4, 6), and it has been demonstrated that these products are excreted readily (6), and penetrate the central nervous system poorly (7) after intravenous administration to jaundiced animals. However, such studies are relevant only if these products are the same as the bilirubin derivatives formed during phototherapy of icteric infants, yet several observations suggest that the in vivo and in vitro processes may be different (2). Therefore, to elucidate the mechanism of phototherapy, experiments must be performed with jaundiced subjects.

In the present work, the metabolism of labeled bilirubin was studied in homozygous jaundiced Gunn rats exposed to intense fluorescent light. These animals, with an hereditary deficiency of bilirubin glucuronyl transferase (8) were used as models of the infant with unconjugated hyperbilirubinemia (9).

METHODS

Labeled bilirubin. ^{14}C-bilirubin, 42.5×10^{3} dpm/μg, or ^{3}H-bilirubin, 14.1×10^{3} dpm/μg, were prepared biosynthetically from the corresponding labeled δ-amino-levulinic acid[1] by the method of Lester and Klein (10), then crystalized and radioassayed by the methods of Ostrow, Hammaker, and Schmid (11).

Animal studies. Adult male Gunn rats[2] weighing 370–465 g were anesthetized with intraperitoneal pentobarbital and their torsos were fully shaved. They were then provided

[1] New England Nuclear Corp., Boston, Mass.

[2] Graciously provided by Drs. N. Javitt, New York, L. Johnson, Philadelphia, Pa., R. Schmid, San Francisco, Calif., and S. Schenker, Dallas, Texas.

TABLE I

Ascending Chromatographic Systems for Separation of Pigments and Azopigments

System	Supporting medium	Developing solvent (v/v)	Duration	Reference
A	Paper, S and S 598*	*n*-butanol, ethanol, water. (6:2:3)	6 hr	(15)
B	Paper, S and S 598*	Pyridine, ethyl acetate, water. (1:2:1)	3½ hr	
C	Thin-layer, silica gel‡	*n*-butanol, ethanol, water. (6:2:3)	5 hr	
D	Thin-layer, cellulose§	*n*-butanol, ethanol, acetic acid, water. (6:2:1:2)	5 hr	
E	Thin-layer, silica gel‡	Chloroform, methanol, acetic acid. (85:15:3)	25 min	(21)
F	Thin-layer, silica gel‡	Butan-2-one, propionic acid, water. (4:1:1)	45 min	(20)

* Carl Schleicher and Schuell, Keene, New Hampshire.

‡ Precoated on glass plates, A. G. Merck & Company, Darmstadt, Germany.

§ Precoated on aluminum foil, A. G. Merck & Company, Darmstadt, Germany.

with an external bile fistula (9), and a plastic square was sutured to the perineum to assure separate collection of urine and stools (9). Throughout the experiment, isotonic saline-dextrose was infused through a femoral venous catheter at 1.5–2.0 ml/hr. After recovery from anesthesia (3 hr), 170–400 μg labeled bilirubin, dissolved in 1.5 ml normal rat serum, was administered intravenously and allowed to equilibrate with the endogenous bilirubin pool for 10–12 hr (9). There then followed three 16–20-hr experimental periods: a control period with the animal under the usual laboratory illumination of 85 foot candles,[3] a light period during which the rat was exposed to 1700 foot candles provided by six 15-w daylight fluorescent lamps in a fan-cooled reflecting canopy (4), and a recovery period in which the canopy lights were turned off again. Rectal temperature remained at 35–37°C throughout the three experimental periods.

Before injection of the labeled bilirubin and at 4-hr intervals thereafter, complete collections were obtained of bile (on ice, in the dark), urine, and stool, and 0.3 ml of blood was drawn from an incised tail vein into a heparinized pipette. Plasma was separated at 900 g for 30 min and the hematocrit determined. Diazo reactive compounds in bile (0.1 ml) and plasma (0.02 ml) were measured by a micromodification of the method of Michaelsson (12). Bile and urine samples were tested also for the pentydopent reaction (11).

For radioassay, bile (0.1 ml) or plasma (0.04 ml) was warmed with 0.2 ml of 1.0 M Hyamine[4] and then blended with 10 ml of dioxane-based scintillator (13). Urine (0.5 ml) was added directly to 10 ml scintillator. Filtered alkaline extracts of dried stools were prepared and counted as described by Schmid and Hammaker (9). All samples were counted for 10^4 counts in a Nuclear-Chicago (Des Plaines, Ill.) 720-B liquid scintillation spectrometer, and corrected for quench with counting efficiencies determined after addition of ^{14}C- or ^{3}H-toluene as internal standard. To correct for spurious counts due to spontaneous fluorescence, background rates were determined with unlabeled samples similarly prepared. For bile and plasma, background counts never exceeded 10% and the spurious counts never exceeded 2% of total sample cpm. For urine, the corresponding values were 30 and 15% respectively.

[3] Measured with foot-candle meter type DO-91, General Electric Company, Schenectady, N. Y.

[4] Rohm and Haas Co., Philadelphia, Pa.

The apparent specific activity of plasma bilirubin was calculated as the total plasma radioactivity divided by total plasma diazo reactivity, assuming that all diazo reactivity was bilirubin (9). Where steady-state conditions prevailed, as in the control period, pool sizes and half-lives for bilirubin, were calculated by standard methods (14). In calculating excretion of labeled compounds, radioactivity passed in the feces during each experimental period was arbitrarily assigned to the preceding period, and label found in the intestinal contents at sacrifice was assigned to the recovery period. This compensated for the known delay of 16–24 hr between excretion of radioactivity into the intestine and its passage in the stool (9).

Studies of pigments in bile and plasma. Biles from light and control periods were pooled separately. Large volumes of plasma were obtained from four animals which expired suddenly halfway through the light or control periods and were immediately exsanguinated by cardiopuncture into heparinized syringes.

Extracts of water-soluble bile pigments were prepared by the method of Noir, Garay, and Royer (15), modified by adjustment of pH values to 6.5 before precipitation of proteins with ethanol, to 4.5 before extraction of pigments into *n*-butanol, and to 6.0 before addition of petroleum ether. Using a Beckman DB Spectrophotometer (Beckman Instruments, Inc., Fullerton, Calif.), absorption spectra from 350 to 700 mμ were obtained on appropriately diluted aliquots from each bile pool, the petroleum ether phase, and the aqueous extract. This extract (0.05–0.20 ml) was also radioassayed in Bray's scintillator (13) and chromatographed by the ascending technique in systems A through D (Table I). After drying, spots were identified by color and by fluorescence under ultraviolet light. Ehrlich aldehyde, diazo, and Gmelin reactivity, and zinc-salt fluorescence of each spot were determined with spray reagents according to Garay, Flock, and Owen (16). For radioassay, spots were eluted with 1.0 ml 50% (v/v) ethanol and 10 ml of Bray's scintillator added.

Plasma and bile samples were also subjected to solvent partition by the method of Folch, Lees, and Stanley (17) as modified by Ostrow and Murphy (18). The button at the interphase was discarded and absorption spectroscopy and radioassay performed on each phase. Aliquots of 0.5 ml were evaporated under warm air to 0.1 ml vol and heated with 0.2 ml Hyamine. Bray's scintillator, 10 ml, was added to the upper phase samples while 15 ml toluene-based

scintillator (11) was used for lower phases. Another aliquot of evaporated lower phase was subjected to thin-layer chromatography in systems D and E (Table I). Spots were identified as above and eluted for counting into 0.5 ml of Hyamine plus 15 ml toluene scintillator. To a third portion of lower phase, a measured quantity of unlabeled crystalline bilirubin[5] was added and the bilirubin was then crystallized and its specific activity determined (11), permitting calculation of the proportion of bile radioactivity present as bilirubin (9).

Studies of azo pigments in bile and plasma. Sulfanilic acid azo pigments were prepared and separated: (*a*) By the solvent partition method of Weber and Schalm (19), and (*b*) By thin-layer chromatography on silica gel using the method of Tenhunen (20) (system F, Table I). Azo pigments prepared from normal rat bile served as reference standards. Radioassay of the upper and lower phases from the Weber-Schalm partition was performed as described above for the Folch partition. The chromatograms were examined only qualitatively since the azo pigments faded rapidly and recoveries of diazo reactivity and radioactivity were low.

Ethyl anthranilate azo pigments were prepared and chromatographed by a modification of the method of Van Roy and Heirwegh (21). Nonpolar pigments were diazotized first in 0.05 M phosphate buffer, pH 5.8, for 30 min. At least 90% of the unconjugated bilirubin was diazotized under these conditions. Glycine buffer, 0.4 M, pH 2.5, and fresh diazoreagent were then added and allowed to react for 30 min at pH 2.7 to complete coupling of the more polar pigments. The reaction was then arrested with ascorbic acid and the azo pigments extracted completely into a small volume of pentan-2-one: butyl acetate (17:3, v/v). With bile, this extract was utilized directly for chromatography. With plasma, the extract contained a gelatinous precipitate and was therefore shaken with 2 vol chloroform, 1 vol methanol, and 0.5 vol water, and centrifuged. The clear lower phase, which contained all the color, was evaporated to a small volume and utilized for chromatography. On each pigment extract, absorbance was determined at 530 mμ, 0.5 ml aliquots were counted in 0.2 ml Hyamine plus 15 ml toluene-based scintillator, and 0.1–0.2 ml was subjected to thin-layer chromatography in system E (Table I). Visible spots were eluted for 30 min into 1.0 ml of 0.06 M HCl in methanol. After centrifugation, the absorbance of each eluate was determined at 530 mμ, and 0.5 ml was added to 1.0 ml methanol plus 15 ml toluene scintillator for radioassay.

RESULTS

Only two rats, A and C, were carried completely through the recovery period. Two others, B and D, suddenly developed obstruction of their biliary catheters after 12 hr of the light period, but were otherwise satisfactory.

Excretion of radioactivity and diazo reactive compounds. (Fig. 1 and Table II). During the control period, all four animals produced pale yellow bile which contained low concentrations of diazo reactive material excreted at a steady rate. Biliary radioactivity declined gradually in keeping with the semilogarithmic decline in plasma specific activity (Fig. 2). Of the label lost

[5] The Pfanstiehl Chemical Corp., Waukegan, Ill.

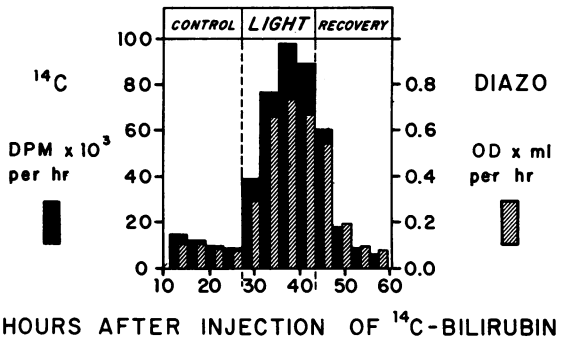

FIGURE 1 Excretion of ^{14}C-radioactivity and of diazo-reactive products in the bile of Gunn rat C before, during and after exposure to 1700 foot candles of daylight fluorescent light. The 465 g homozygous jaundiced male was shaved and given intravenously 226 μg (4.2 μc) of unconjugated ^{14}C-bilirubin at time 0, which was allowed to equilibrate for 11 hr with the endogenous pigment pool. The other three animals showed similar patterns.

from the miscible bilirubin pool, 9–27% was recovered in bile, 2–6% in urine, and 15–16% in stool. The remainder was not accounted for. The pentyopent reaction was faint in bile and weakly positive in urine.

Upon exposure of each animal to light, the bile promptly turned a deep golden-brown color. This was accompanied by a peak 3–7-fold increase in excretion of diazo reactivity and a concomitant even greater rise in excretion of radioactivity without alteration in bile flow. Urinary and fecal radioactivity increased also (up to 3-fold), but contained relatively minor proportions of the total excreted label. The pentydopent reaction intensified slightly in the bile and strongly in the urine. After extinguishing the lights (recovery period), all variables returned slowly to control values.

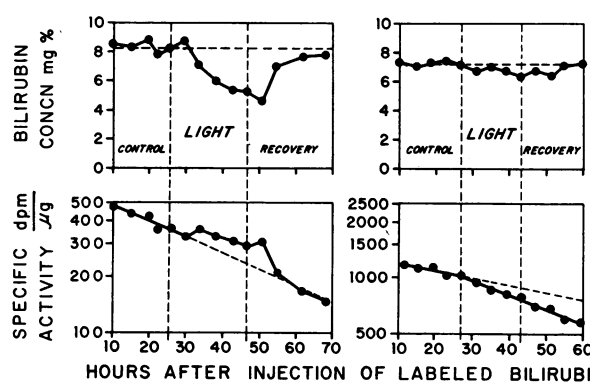

FIGURE 2 Changes in concentration (upper graphs) and apparent specific activity (lower graphs) of plasma bilirubin in Gunn rats A (left) and C (right) before, during and after exposure to Light. Dashed lines represent extrapolations of the mean Control curves. Rat B behaved like A and rat D behaved like C.

TABLE II

Metabolism of Labeled Bilirubin in Gunn Rats before and during Phototherapy

Rat	A		B		C		D	
Source	Javitt		Johnson		Schmid		Schenker	
Color and body weight, *g*	Albino, 415		Albino, 420		Hooded, 465		Hooded, 370	
Isotopic bilirubin used and dose, *μg*	^{3}H, 395		^{14}C, 177		^{14}C, 226		^{3}H, 304	
Experimental period	Control	Light	Control	Light	Control	Light	Control	Light
Plasma bilirubin,* *mg per 100 ml*	8.2	4.6	7.7	4.7	7.2	6.4	9.7	8.8
Bile bilirubin, maximal, *mg per 100 ml*§	0.5	4.1	0.6	3.8	0.6	6.2	0.5	7.6
$t_{\frac{1}{2}}$ of plasma specific activity curve, *hr*‡	34.7	48.5	47.7	39.6	79.0	38.7	59.8	44.2
Total miscible bilirubin pool, *mg**	5.7	—	6.2	—	7.2	6.4	5.7	—
Average losses from bilirubin pool‖								
Pigment, *mg/hr*	0.13	—	0.08	—	0.08	0.14	0.07	—
Radioactivity, 10^3 *dpm/hr*	58.9	—	84.3	—	92.0	127.0	26.3	—
Excretion of radioactivity, 10^3 *dpm/hr*								
Bile	8.6	30.8	7.9**	31.9	10.3	87.8	7.1	20.6
Urine	2.1	2.7	1.8**	4.6	5.8	16.4	1.5	2.8
Stool¶	9.3	12.5	—**	—	14.0	31.8	3.9	6.0

* Mean values for control period; minimal values for light period.

‡ During light period, half-life calculated only from interval during which the new semilogarithmic decay of plasma-specific activity was attained.

§ True bilirubin concentrations, obtained from total diazo-reactivity × proportion of diazo-reactivity in bilirubin, as determined from Michaelsson diazo method (12) and chromatography of ethyl anthranilate azopigments (21, 22) respectively.

‖ Loss of radioactivity calculated from difference between total radioactivity in the pool at start and end of experimental period ÷ duration of period in hours. Loss of pigment is an approximation derived by dividing the radioactive loss by the logarithmic mean specific activity of plasma bilirubin for that period.

¶ Stool losses in each period taken from radioactivity which appeared in the stool during subsequent experimental period. For reasoning, see text.

** Rat B had low bile and urine flow throughout and passed no stools.

Changes in plasma bilirubin concentration and specific activity. (Fig. 2 and Table II). During the control period, all four rats maintained a stable concentration of plasma bilirubin (7–10 mg/100 ml) and a semilogarithmic decay of plasma specific activity with half-lives of 34–79 hr. Upon exposure to light, however, two types of responses were seen (Fig. 2). In rats A and B, as illustrated on the left, plasma bilirubin concentration fell markedly (by 44 and 39% respectively). The apparent plasma specific activity rose initially above the control decay curve, and then declined again semilogarithmically with a half-life similar to that of the control period. During the recovery period, bilirubin concentration rose and plasma specific activity declined gradually toward control values. In rats C and D, by contrast, exposure to light caused little change in plasma bilirubin concentration despite a similar increase in bilirubin catabolism (Table II). The decline in plasma specific activity accelerated promptly to a more rapid semilogarithmic decay curve, which continued into the recovery period for rat C (Fig. 2, right).

Characteristics of pigments in bile. Absorption spectra (Fig. 3) from control biles showed a broad maximum at 408 mμ (9), whereas biles from the light period exhibited a more intense maximum at 425 mμ. The difference spectrum between the two revealed a sharp, symmetrical peak at 437 mμ, similar to that of unconjugated bilirubin added to control bile.

During extraction of pigments by the method of Noir et al. (15) only about half of the bile radioactivity was recovered in the final aqueous extract. About one-fourth of the label remained in the yellow petroleum ether phase, which gave a strong indirect diazo reaction, turned deep green when treated with alkali or acid ferric chloride (Gmelin reaction), and showed a symmetrical absorption maximum at 438 mμ, suggesting that this label was present as unconjugated bilirubin.

Absorption maxima were found at 403 mμ in control and 413 mμ in light bile extracts. Paper chromatography of these water-soluble pigments (Fig. 4) yielded identical patterns, with similar distribution of radioactivity among the spots, from both control and light biles. However, each spot from the light bile contained 4–12 times (mean = 5 times) as much total radioactivity as the corresponding spot from the control bile of the same animal (Fig. 4). In all samples, most of the label appeared in

the two most rapidly migrating derivatives whereas only 11–14% was detected in the yellow fluorescent and weakly diazo positive spot centered 8–9 cm from the origin. Radiochromatography in systems B, C, and D (Table I) produced less distinct separations but similarly yielded almost identical patterns from light and control bile extracts.

The Folch solvent partition of bile recovered over 96% of the spectral absorbance and radioactivity in the two liquid phases. With control biles, the more polar upper phase contained roughly two-thirds of the yellow color and radioactivity (Fig. 5). Following exposure to light, almost all of the additional label and color which appeared in the bile partitioned with the lower (chloroform) phase (Fig. 5). This phase gave strong indirect diazo and Gmelin reactions, and its absorption maximum remained at 450 mμ, identical with that of unconjugated bilirubin.

Thin-layer chromatography in systems A and C (Table I) of the Folch lower phases, or of the petroleum ether phases from the Noir extraction, yielded two radioactive spots at R_f 0.00 and 0.85–0.94. These were characterized by their colors and reactions with spray reagents (15) as unconjugated bilirubin and unconjugated biliverdin respectively (15). The bilirubin spot at the origin contained only 3% of the radioactivity from control bile, but 54% of the total radioactivity and 74% of the label in the Folch lower phase from light bile. Correspondingly, 70–76% of the lower phase radioactivity from light bile cocrystallized with added carrier bilirubin. From this data, it was calculated that the maximal excretion of unconjugated bilirubin in bile during phototherapy ranged from 0.22 to 0.31 μg per min/100 g body weight in the four rats.

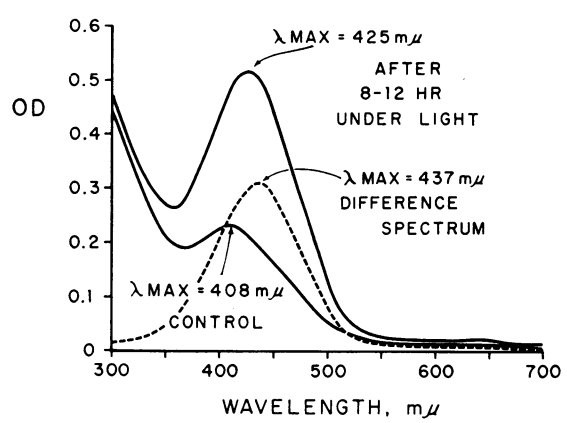

FIGURE 3 Absorption spectra of bile samples from Gunn rat C, diluted 1:10 and read against a distilled water blank. Difference spectrum (dashed line) obtained by subtracting control spectrum from spectrum of bile passed during the light period. Bile flow was identical in both periods. Similar spectra were obtained from bile passed by rats A, B, and D.

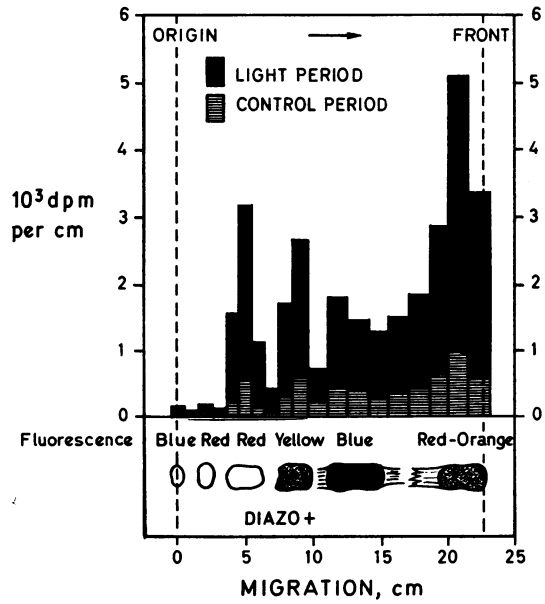

FIGURE 4 Ascending paper chromatogram in butanol-ethanol-water (6:2:3, v/v) of water-soluble pigments extracted by method of Noir et al. (15) from Gunn rat biles of control and light periods. For details, see text. Abscissa shows distance migrated in centimeters, with origin at the left. Depicted below are the spots detected, with shading to indicate their relative fluorescence intensities. Ordinate represents radioactivity in cut segments, averaged for the four animals. Before averaging, values were adjusted proportionally so that total radioactivity on each chromatogram equaled the mean hourly excretion of water-soluble radioactivity in the initial bile sample from which the aqueous extract was prepared.

Characteristics of azo pigments from bile. In the Weber-Schalm partition (19) (Fig. 6), 74% of the radioactivity and 59% of the diazo reactivity from control bile appeared in the upper phase (polar azo pigments). After exposure to light all additional azo pigment and two-thirds of the additional label extracted into the lower phase (Fig. 6). The additional undiazotized labeled compounds partitioned principally with the upper phase.

Sulfanilic acid azo pigments from control bile were orange-red with an absorption maximum of 520 mμ (9), while in light bile they were purple with a maximum at 535 mμ. On chromatography (Fig. 7), both biles yielded an orange-brown spot near the origin plus two azopigments with R_f's identical with standard conjugated and unconjugated azopigments respectively. Only the unconjugated azopigment showed an increase in intensity in the light bile sample.

Chromatography of the ethyl anthranilate azo pigments from normal rat bile confirmed the pattern reported by Heirwegh and his coworkers (21, 22) (Fig. 8). By contrast, Gunn rat bile contained only traces of azopigment-δ

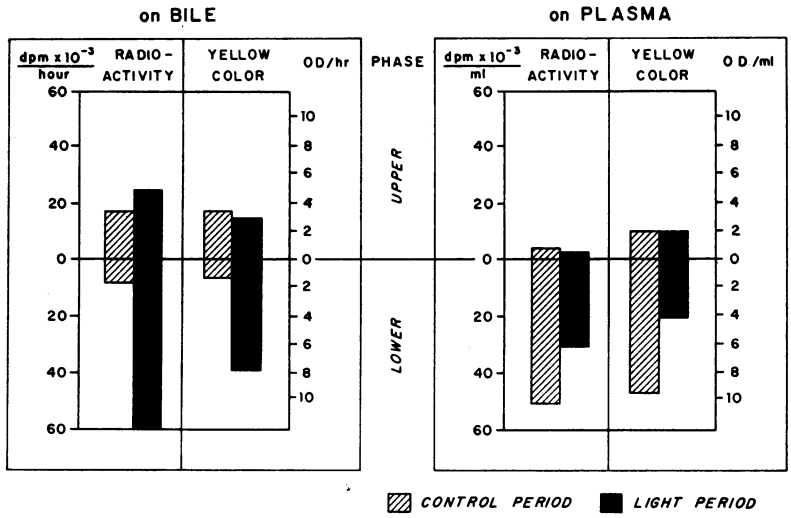

FIGURE 5 Folch solvent partitions of Gunn rat bile (left) and plasma (right) during control (shaded bars) and light (solid bars) periods. Values for bile represent excretion rates per hour, for plasma concentrations per milliliter. Yellow color was assessed as OD at absorption maximum, which was 450 mμ for all lower phases, and for upper phases was 410 mμ for control and 425 mμ for light periods. For each bar, which is the mean of four animals, total height represents total excretion in bile or total concentration in plasma, and heights above or below the zero line represent portions partitioned with the upper and lower phases respectively.

which is predominately azobilirubin glucuronide (22). The unconjugated α-azopigment and the orange-brown β-azopigment accounted for almost all the diazo reactivity in control Gunn bile, and both intensified strikingly during phototherapy. Radioassay (Fig. 9) confirmed these findings and also revealed colorless, nondiazotized material which did not extract into pentanone. This fraction, plus the traces of undiazotized bilirubin from spot

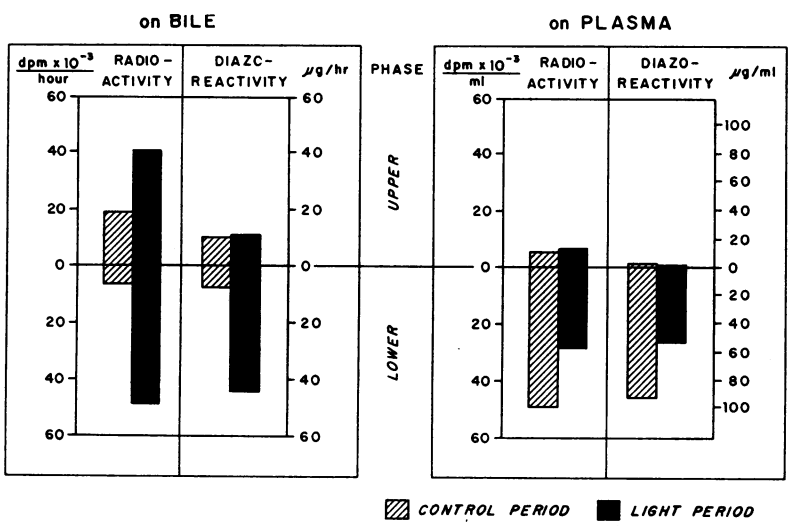

FIGURE 6 Weber–Schalm solvent partitions of azopigments from Gunn rat bile (left) and plasma (right) during control and light periods. Mean values from animals A through D were plotted as in Fig. 5, except that diazo reactivity is expressed in terms of bilirubin equivalents in micrograms, using the absorption coefficient for azobilirubin as the conversion factor for all azo pigments measured.

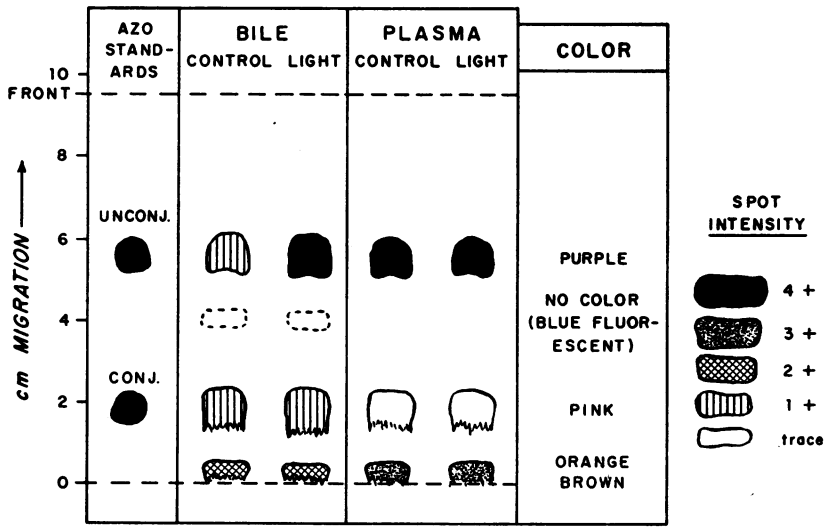

FIGURE 7 Ascending thin-layer chromatogram of sulfanilic acid azopigments from Gunn rat bile and plasma during control and light periods. Azopigment standards, prepared from normal rat bile, are shown at the left. Intensity of shading represents color intensity of each spot. Chromatography performed on silica gel with butan-2-one, propionic acid, water (4:1:1) as the developing solvent (20) (system F, Table I).

F at the solvent front, constituted total nondiazotized material and accounted for two-thirds of the label in control bile and one-half of the label in light bile. Total biliary excretion of these labeled nondiazotized derivatives doubled after exposure to light.

Character of pigments and azo pigments in plasma. More than 90% of the radioactivity and most of the yellow color and diazo reactivity in Gunn plasma extracted into the lower phases of the Folch and Weber-Schalm solvent partitions (Figs. 5 and 6). Following

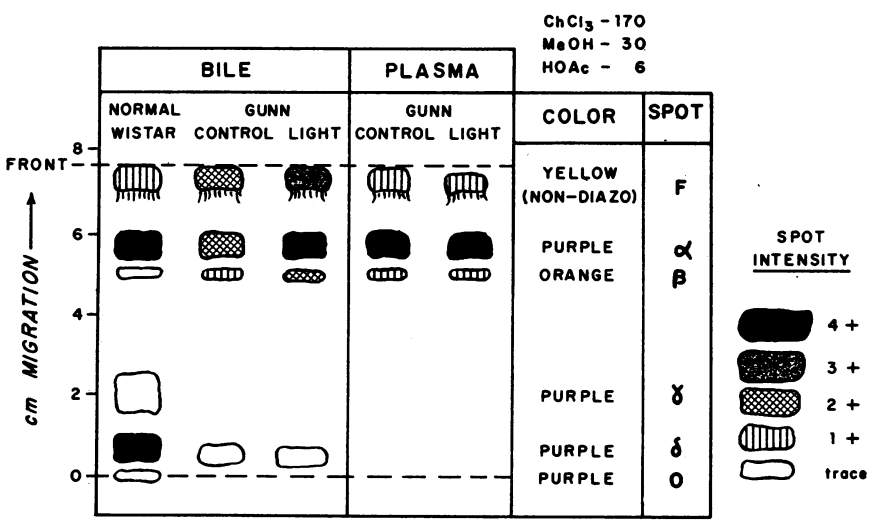

FIGURE 8 Ascending thin-layer chromatogram of total ethyl anthranilate azopigments from Gunn rat bile and plasma during control and light periods. For comparison, azopigments from normal Wistar rat bile are shown at the left. Chromatogram on silica gel with chloroform-methanol-acetic acid (85:15:3, v/v) as developing solvent (system E, Table I). Azo pigment spots designated as proposed by Heirwegh, Van Hees, Leroy, Van Roy, and Jansen (22); α is unconjugated azobilirubin and δ is azobilirubin glucuronide. Spot F at the solvent front includes the small fraction of unconjugated bilirubin which was not diazotized.

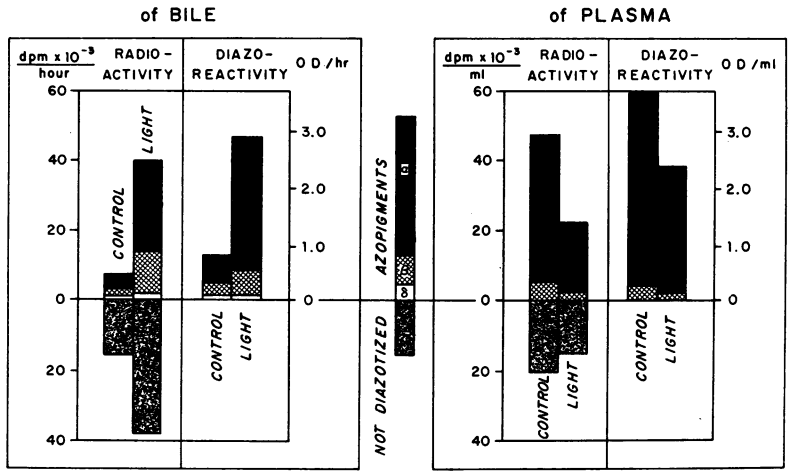

FIGURE 9 Radioactivity and diazo reactivity in ethyl anthranilate azo pigments δ, β, and α, eluted from chromatogram in Fig. 8, represented by the heights of respective bars above baseline. Radioactivity in undiazotized material, principally labeled colorless compounds not extracted into pentanone-butyl acetate, is represented as heights of bars below baseline. Mean values from four animals were plotted. with bile data expressed as excretion rates per hr and plasma data as concentrations per milliliter. Diazoreactivity was measured at 530 mμ. For each pair of bars, control period is shown at the left and light period on the right.

light treatment, almost all the decrease in label, color, and diazo reactivity in plasma occurred in these lower phases (Figs. 5 and 6), although there was also some decrease in the polar pigments in the upper phases. Chromatography of the Folch lower phases in system C revealed over 90% of the label in the bilirubin spot at the origin.

Chromatography of the sulfanilic acid (Fig. 7) and ethyl anthranilate (Fig. 8) azopigments from Gunn rat plasma yielded the same patterns as Gunn rat bile, with little conjugated azo pigment evident. Both before and during light therapy, over 90% of the extracted diazo reactivity and azo pigment radioactivity appeared in unconjugated α-azopigment, and the remainder migrated with the β-spot. These two azo pigment spots decreased proportionately during light exposure (Fig. 9). Thus, during both control and light periods in all four animals, the plasma diazo activity and radioactivity was principally in the form of bilirubin rather than its derivatives.

DISCUSSION

The results during the control period confirm Schmid and Hammaker's pioneer studies of bilirubin metabolism in the Gunn rat (9) although recoveries of radioactivity in the bile were somewhat lower in the present investigation (Table II). Differences in age, weight and strain (23), and in diet (24) may account in part for this discrepancy. The data obtained during the light period constitute the first reported study of the effects of light on metabolism of ^{14}C-bilirubin in vivo (1, 2), and indicate that phototherapy both accelerates the alternate pathways of bilirubin catabolism which exist in the Gunn rat under normal lighting conditions (9), and dramatically augments biliary excretion of unconjugated bilirubin. Callahan, Thaler, Karon, Bauer, and Schmid (25) have confirmed the increased biliary excretion of water-soluble bilirubin dervatives in comparable studies of phototherapy in children with a similar hereditary deficiency of bilirubin conjugation (Crigler-Najjar syndrome).

In control bile, unconjugated bilirubin accounted for less than 5% of the radioactivity and less than half the diazo reactivity, whereas in light bile it accounted for approximately half the radioactivity and most of the diazo reactivity, and was responsible for the difference between the absorption spectra of light and control biles. This enhanced biliary excretion of unconjugated bilirubin attained rates similar to the basal excretion of conjugated bilirubin in normal rats (26) but only about 2% of the reported T_m for unconjugated bilirubin in unilluminated normal (26) or Gunn (27) rats. Identification of this pigment as unconjugated bilirubin or some closely related analogue depended upon the following characteristics: (*a*) extraction into the lower (chloroform) phase of the Folch solvent partition; (*b*) positive diazo and Gmelin reactions; (*c*) absorption spectra similar to pure unconjugated bilirubin in Folch lower phase, petroleum ether, and Gunn rat bile; (*d*) chro-

matographic behavior identical with unconjugated bilirubin; (*e*) formation of characteristic unconjugated azo pigments with both sulfanilic acid (Weber-Schalm partition and Tenhunen chromatography) and ethyl anthranilate diazo reagents, and (*f*) Cocrystallization with added pure unconjugated bilirubin of most of the radioactivity in the Folch lower phase.

There thus seems to be little doubt that the pigments excreted during phototherapy consisted in large part of unconjugated bilirubin, but the mechanism of this process requires elucidation. Certain mechanisms which are known to enhance biliary excretion of unconjugated bilirubin cannot be invoked here since there was no accompanying increase in body temperature (28, 29) or bile volume (30, 31) and no appreciable increase in excretion of conjugated bilirubin (32). The rapidity of the effect suggests that enzyme induction is not involved, and the return of bilirubin excretion to control values during the recovery period indicates that the process is reversible. The action of phototherapy must differ also from the effect of phenobarbital on unconjugated hyperbilirubinemia (33, 34), since this drug does not diminish jaundice (35), nor accelerate bilirubin excretion (31, 35) in the homozygous jaundiced Gunn rat.

Goresky has shown that "the membrane transport system for biliary excretion of bilirubin diglucuronide has no apparent affinity for the unconjugated form" (36), and it seems doubtful that phototherapy would alter this condition. However, unconjugated bilirubin is normally present in bile, both in nonjaundiced (26) and Gunn (9, 27, and present study) rats, which indicates that the canalicular membrane has limited physiological permeability to this lipid-soluble bile pigment. It is tentatively suggested that phototherapy alters the state of the pigment and/or the hepatocyte membrane so that the liver becomes freely permeable to passive diffusion of bilirubin from blood to bile. This hypothesis is supported by the finding that, during phototherapy, the concentration of bilirubin in bile rose to a maximum value just below the minimum concentration attained in the plasma (Table II). This limiting condition may explain the long noted inability of phototherapy to completely eradicate the hyperbilirubinemia (37, 38).

Phototherapy also stimulated the catabolism of bilirubin to water-soluble derivatives which were excreted primarily in the bile and showed chromatographic and spectral properties identical with the pigments in control Gunn rat bile, but different from the derivatives formed during photodecomposition of bilirubin in vitro (6). Thus, the in vitro and in vivo photoprocesses are not the same, and reported studies performed with the in vitro products (6, 7) may have little relevance to the mechanism of excretion and possible neurotoxicity of the derivatives formed in vivo. The radiochromatograms in Fig. 4 showed also that the major bilirubin catabolites were the less polar compounds which migrated toward the solvent front, rather than the intensely yellow-fluorescent spot ($R_f = 0.35$ in Fig. 4) which had been featured by Garay et al. (16). Nonetheless, this spot was the only water-soluble catabolite which was diazo reactive (6) and it therefore may be the precursor of the azo pigment found in the upper phase of the Weber-Schalm partition, of the "conjugated" band on the chromatogram of sulfanilic acid azopigments (Fig. 7), and of the ethyl anthranilate β-azopigment (Fig. 8). In agreement with this possibility, both the yellow-fluorescent spot (16) and the β-azopigment (22) were present in small quantities in the bile of normal rats (16, 22) and of control Gunn rats (16, and Figs. 4 and 8), and both showed comparably increased excretion during phototherapy (Figs. 4 and 9). The chemical nature of this pigment is not known, but it is probably not a sulfate conjugate since these have not been found in Gunn rat bile (39).

During phototherapy, the pentdyopent reaction increased slightly in bile but more strongly in urine, confirming observations of Porto (40). However, the urine never contained more than a small fraction of the total radioactivity excreted, and the colorless water-soluble pigments in bile, which would include the propentdyopents (41), constituted a very small proportion of the biliary radioactivity. These data obtained in rats thus differ from findings of Porto that pentdyopent-positive dipyrroles may be the major products of the photocatabolism of bilirubin in human infants.

Data from the plasma confirmed Schmid and Hammaker's finding (9), that, under control lighting conditions, almost all of the circulating radioactivity and diazo reactivity was accounted for by labeled unconjugated bilirubin, validating the approximation that total plasma radioactivity divided by total plasma diazo reactivity equaled bilirubin specific activity during this period (9). During exposure to light, after an initial period of fluctuation, all four rats attained a new first-order decay of plasma-specific activity, but the responses fell otherwise into two groups (Fig. 2). Animals A and B exhibited a marked decline in plasma radioactivity and diazo reactivity due almost entirely to a decrease in the concentration of unconjugated bilirubin. This resulted in an increased proportion of labeled undiazotized compounds and an initial rise in the apparent specific activity of plasma (Fig. 2, left). Consequently, in these two rats during the light period, the apparent plasma specific activity was a less accurate gauge of bilirubin specific activity, and the absence of a steady state precluded calculations of bilirubin losses and pool sizes.

In rats C and D, by contrast (Fig. 2, right), despite a similar decline in plasma radioactivity and increase in excretion of labeled compounds, plasma bilirubin concentration decreased only slightly to a new stable level and

plasma specific activity declined semilogarithmically at an accelerated rate. This suggests that a new steady state was attained during the light period in these two animals. If this assumption is valid, bilirubin turnover and pool sizes during phototherapy can be calculated for rat C (14) who survived the full 16 hr of light exposure. These values, given in Table II, indicate that phototherapy increased pigment catabolism almost 2-fold and that all the radioactivity lost from the pool was recovered in the excreta, with 69% in bile, 13% in urine, and 25% in the stool. This, plus the decrease in the concentration of polar derivatives in plasma, indicated that the bilirubin catabolites formed during phototherapy, whose toxic potential is unknown, are not retained in the body but are rapidly and quantitatively excreted. However, the assumption of a new steady state during the light period must be made with caution, since Callahan et al. (25) were unable to achieve equilibration of infused ^{14}C-bilirubin during phototherapy of children with the Crigler-Najjar syndrome.

The accelerated decline in plasma specific activity during phototherapy of rats C and D may be due to: (*a*) preferential catabolism of the labeled bilirubin in the freely-exchangeable pool; (*b*) mobilization into this pool of poorly labeled pigment from slowly exchanged extravascular sites (9, 42), or (*c*) accelerated endogenous formation of unlabeled bilirubin. The first two possibilities cannot be excluded, but existence of a large, poorly, equilibrated pigment pool seems unlikely because the first-order decay of plasma specific activity in the Gunn rat remains constant for 4–6 days (9), even when large intercompartmental shifts of bilirubin are produced by intravenous injection of salicylate or albumin (43). This points to accelerated bilirubin production during phototherapy, yet there was no evidence of significant hemolysis in any of the animals as hematocrits declined almost equally and to a degree consistent with the frequent blood sampling. Therefore, if increased bilirubin production occurred, it may have been derived from the early labeled fraction (44). Increased formation of the hepatic fraction of early labeled bilirubin has been demonstrated during treatment of a jaundiced child with phenobarbital (45), and studies are underway to determine if phototherapy similarly stimulates the turnover of hepatic hemes from which this fraction is derived (46). Differences in this response in the four animals may relate to strain differences, since the two rats with little change in plasma bilirubin were the hooded variety, whereas the rats which responded well to phototherapy were albinos. Such variations in bilirubin production could account for the diversity of responses of plasma bilirubin observed during phototherapy of Gunn rats (37) and human infants (37, 38).

Water-soluble derivatives akin to those found in Gunn rat bile are present also in the bile of fetal monkeys (47), which suggests that similar "alternate pathways of bilirubin metabolism" may exist also in human neonates and be similarly augmented by phototherapy. Moreover, illumination of the human infant causes the stools to turn green, compatible with accelerated biliary excretion of bilirubin and its oxidation to biliverdin in the intestine. Nonetheless, caution seems advisable in application of the present animal studies to justify the safety of phototherapy in the human infant (3). The rats were exposed to illumination 3–6 times as intense as that customarily applied to icteric infants (3). Elderly Gunn rats were used which have a selective deficiency of bilirubin-UDPGA-glucuronyl transferase (8) and not the more generalized impairment of hepatic excretory function seen in the neonate (48) which might impede biliary excretion of photoderivatives as well as bilirubin. Moreover, bilirubin and its photoderivatives might undergo significant enterohepatic recirculation (49), which would diminish their net excretion in intact infants but not in the Gunn rat with an external bile fistula. Finally, the structure and toxicity of the photoderivatives remain unknown, and it is becoming evident that phototherapy may engender subtle ill effects, such as retarded growth (50) and delayed sexual maturation (51). Therefore, it would seem wise not to apply phototherapy routinely for prophylaxis of neonatal jaundice until the above questions have been answered by careful studies in the human infant.

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