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Bilirubin Dynamics in the Gunn Rat during Phototherapy

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Abstract □ Bilirubin dynamics were studied in homozygous Gunn rats under normal room lighting conditions and under conditions simulating phototherapy. A kinetic model was developed for the formation, distribution, and elimination of bilirubin. The decrease in plasma bilirubin concentration during illumination with low intensity [300 footcandles (fc)] and high intensity (1000–1100 fc) light was studied. The plasma bilirubin concentration in the rats decreased under phototherapy until a new steady-state concentration was reached, the decline being more rapid under high intensity light conditions. Gunn rats were also injected with a tracer dose of ^{14}C-bilirubin following a period during which the rats were illuminated with low or high intensity light. The distribution and elimination of the labeled bilirubin were followed under continuous illumination. The chosen kinetic model, when adapted to the set of data under investigation, fit all of the data concerning bilirubin kinetics in Gunn rats under continuous illumination.

Keyphrases □ Bilirubin—kinetics of formation, distribution, and elimination in rats, effect of phototherapy □ Kinetics—bilirubin formation, distribution, and elimination in rats, effect of phototherapy □ Phototherapy—effect on kinetics of bilirubin formation, distribution, and elimination, rats

Phototherapy was first described by Cremer *et al.* (1) and has since become a popular procedure for the treatment of neonatal hyperbilirubinemia. The efficacy of phototherapy is well established (2–7), but reservations have been expressed regarding the exposure of infants to blue or visible light intensities of 300–500 footcandles (fc), often continuously for as long as 3–5 days (8–14). It has been suggested that the generalized acceptance of this mode of therapy for neonatal hyperbilirubinemia has occurred without standardization of technique or individualization of application (15). Considerations of effects of variables such as light intensity and duration of exposure have been based primarily on clinical response following empirical selection of such parameters.

The present study was designed to investigate bilirubin kinetics in Gunn rats under normal room lighting conditions and under light exposure simulating phototherapy, with the objective of developing a kinetic model to predict the relative effectiveness of various phototherapy procedures. Gunn rats lack glucuronyltransferase activity for bilirubin conjugation and thus cannot excrete the pigment normally (16). Since Gunn rats show a markedly increased plasma bilirubin level, they are frequently used as a model in bilirubin studies.

Bilirubin kinetics previously were studied in humans (17–23) and in Gunn rats (17, 24–26). The effect of light on the catabolism of labeled bilirubin was studied in Gunn rats (27) and in infants with severe unconjugated hyperbilirubinemia (28), but in no report to date has a kinetic model been described that explains the change in the plasma bilirubin concentration due to phototherapy.

The approach used in the current study was based on the determination of the plasma level–time course of an intravenously injected tracer dose of ^{14}C-bilirubin in Gunn rats maintained under conditions of normal room lighting and under various conditions of illumination simulating phototherapy and on the determination of the rate at which the bilirubin plasma pool approaches a new steady-state level when Gunn rats are exposed to varying conditions of illumination.

EXPERIMENTAL

Reagents—Bilirubin[1] and riboflavin 5′-phosphate monosodium salt[2] were used as received from the supplier. All other solvents and reagents were analytical reagent grade.

Preparation of ^{14}C-Bilirubin Injections—This preparation was carried out in subdued light, as were all assays. Crystalline ^{14}C-bilirubin was prepared by a modification of the biosynthetic method of Barrett *et al.* (29), using Sprague–Dawley rats instead of dogs, and purified by the method of Ostrow *et al.* (30). After recrystallization (twice) from methanol, an aliquot was examined for radiochemical purity by TLC, using chloroform–acetic acid (99:1) on silica gel sheets[3], and by paper chromatography[4] with pyridine–ethyl acetate–water (1:2:1). The chromatograms were cut into 1-cm strips, and the amount of radioactivity on each strip was measured.

Ninety-five percent of the total activity was located in the bilirubin

[1] Lot 9324, Nutritional Biochemicals Corp., Cleveland, OH 44128.
[2] Mann Research, Orangeburg, NY 10962.
[3] ITLC type SG, Gelman Instrument Co., Ann Arbor, Mich.
[4] Whatman Grade 1, Scientific Products, Columbus, OH 43207.

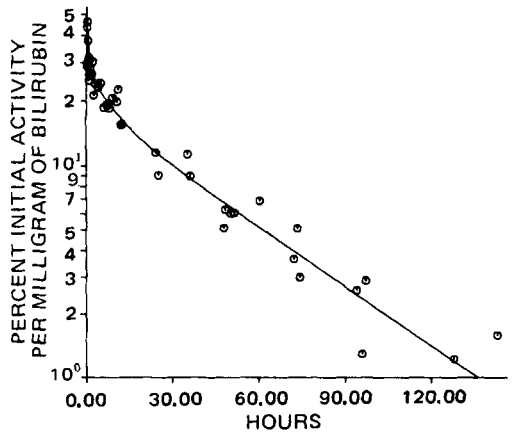


Figure 1—*Composite plasma ^{14}C-bilirubin data from six Gunn rats under normal room lighting conditions. The solid line represents a computer fit of data to a three-compartment model (Model 2).*

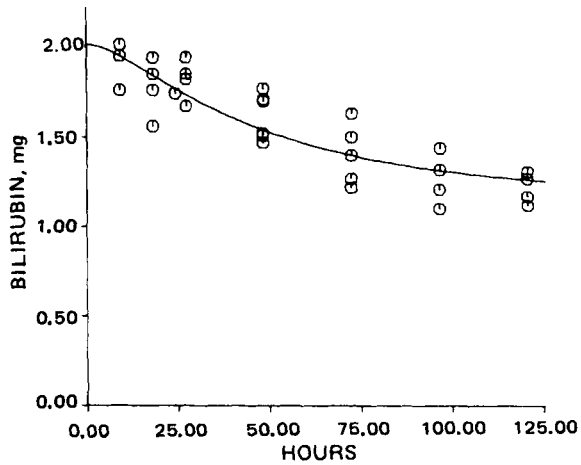


Figure 2—*Plasma bilirubin decline in six Gunn rats during continuous low intensity (300 fc) illumination. The solid line represents a computer fit of data to a three-compartment model (Model 11).*

region marked by the yellow-orange color and corresponding to the R_f value of an authentic bilirubin sample. The UV spectra of the crystalline ^{14}C-bilirubin in chloroform and in ethyl acetate showed no characteristics other than those of the authentic sample.

To prepare the injections, crystalline ^{14}C-bilirubin was further purified by TLC as described and eluted with ethyl acetate. The dried crystals were dissolved in a known volume of chloroform. A volume containing the required radioactivity was removed, and the solvent was evaporated under nitrogen. Crystalline ^{14}C-bilirubin was dissolved in 0.2 ml of 1% sodium carbonate, and 0.8 ml of freshly isolated rat plasma was added. An aliquot of the injection was assayed for activity and bilirubin content by the method described for the determination of plasma bilirubin specific activity. Each rat received an intravenous ^{14}C-bilirubin dose of 200,000–660,000 dpm.

Gunn Rat Studies—Adult homozygous male Gunn rats, 300–400 g, were shaved just prior to use. Six control rats were kept under normal room lighting after receiving intravenous injections of ^{14}C-bilirubin. Blood samples were taken periodically from the tail artery of the ether-anesthetized rats using 0.37-ml micro-blood-collecting tubes[5] fitted with disposable 20-gauge hypodermic needles.

Blood samples were centrifuged for 20 min at 5000×*g* and stored in the dark at −20° prior to ^{14}C-bilirubin specific activity determination. Rats used for phototherapy studies were placed in metabolism cages under a light canopy equipped with four daylight fluorescent tubes[6].

The cage to canopy distance was adjusted to allow the desired light intensity to reach the backs of the rats. Six rats were exposed to continuous low intensity (300 fc)[7] illumination[8], three rats received intravenous riboflavin 5′-phosphate injections of 75 mg/kg every 9 hr while being exposed continuously to low intensity illumination, and four rats were illuminated continuously with high intensity (1000–1100 fc)[7] light[8]. The plasma bilirubin concentration of all rats was followed for several days. The same sampling procedure as for the control rats was used.

In the third series of experiments, rats were illuminated for many hours before being injected with an intravenous tracer dose of ^{14}C-bilirubin. In the period following the injection, illumination was continued; blood samples were taken periodically and treated as in the control rat studies. Five rats were exposed to continuous low intensity illumination for 48 hr prior to injection, four rats received intravenous riboflavin 5′-phosphate injections (75 mg/kg every 9 hr) during the entire experiment while being illuminated continuously with low intensity light from 20 hr prior to the ^{14}C-bilirubin injection onwards, and four rats were exposed to continuous high intensity light for 12 hr prior to injection.

Each rat was used in only one experiment, since it was considered that the stress accompanying the prolonged light exposure studies was incompatible with a crossover design. All animals were produced by the same breeding colony.

Determination of Specific Activity of Plasma Bilirubin—Plasma bilirubin was isolated and purified by a two-stage process. The bilirubin was extracted from the plasma using a literature method (31) shown to separate bilirubin from its more polar degradation products. The lower phase, containing the bilirubin, was then further purified by TLC. A 0.1-ml plasma sample was added to a test tube containing 0.6 ml of ethyl acetate–lactic acid–water (62:36:2), and the mixture was shaken for 5 min. After the addition of 0.2 ml of chloroform, the tube was shaken for 5 min and centrifuged for 20 min.

The lower layer was transferred to a 5 × 20-cm thin-layer plate[3], which was then developed with chloroform–acetic acid (99:1). The yellow bilirubin band (R_f 0.7) was transferred to a screw-capped tube containing 1.5 ml of ethyl acetate, shaken for 1 min, and centrifuged for 20 min. After washing with water, the absorbance of the ethyl acetate layer was measured[9] at 445 nm, and the bilirubin concentration was determined using a standard curve. The UV spectrum of the isolated bilirubin was identical to that of an authentic sample, indicating that little or no degradation occurred.

The amount of radioactivity in an aliquot of the ethyl acetate solution was counted using a liquid scintillation counter[10] (about 50–1000 dpm/vial). Each vial was counted until 10,000 counts were made. The efficiency of counting was determined by automatic external standardization and was 75–85%. The activity of the bilirubin could then be expressed as disintegrations per minute per microgram.

Data were normalized to express each data point as a percent of dose per milligram of bilirubin for a 300-g rat with an initial plasma bilirubin level of 6 mg/100 ml, as described by Shipley and Clark (32):

$$\%\ \text{dose/mg of bilirubin at time } t = 10^5 \frac{(\text{dpm}/\mu\text{g})_t \times \text{rat weight (g)} \times \text{initial bilirubin concentration (mg/100 ml)}}{\text{dose (dpm)} \times 300 \times 6} \qquad \text{(Eq. 1)}$$

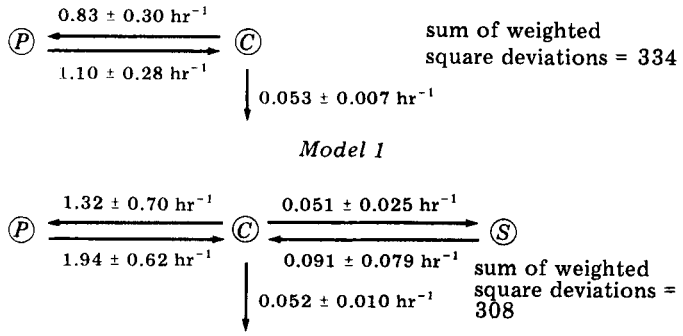
Determination of Plasma Bilirubin Concentration—In all studies not involving injections of tracer doses of ^{14}C-bilirubin, plasma bilirubin levels were determined by coupling bilirubin with diazotized sulfanilic acid, using caffeine and sodium benzoate as accelerators, as described by Jendrassik and Grof (33) and modified by Nosslin (34). The absorbance was measured[9] at 600 nm.

Data Analysis—The kinetic models used to describe the disappearance of ^{14}C-bilirubin from the plasma and the changes in the plasma bilirubin concentration were each defined by a series of differential equations or as the sum of exponentials. Least-squares estimates of the model parameters were obtained using a NONLIN program (35) and a digital computer[11]. All data points were given relative weight equal to the number of determinations per data point.

RESULTS

Control Data Studies—Individual rat data showed that the specific

[5] Caraway, Fisher Scientific Co., Louisville, KY 40299.
[6] F 40D, General Electric Co., Cleveland, OH 44117.
[7] One footcandle = 10.76 lux.
[8] Under the experimental condition, 300-fc illumination resulted in a radiant flux of 3.8 μw/cm^2/nm and 1100-fc illumination resulted in a radiant flux of 15 μw/cm^2/nm, as determined in the wavelength range of 425–475 nm with an Olympic Mark II Bili-meter, Olympic Medical Corp., Seattle, WA 98108.
[9] Cary 15 spectrophotometer, Cary Instruments, Monrovia, CA 91016.
[10] Packard Tri-Carb model 3375, Packard Instrument Co., Downers Grove, Ill.
[11] IBM 370 computer at the University of Kentucky.



activity declined exponentially after a distribution phase of a few hours, suggesting that bilirubin confers the characteristics of a multicompartment model on the Gunn rat. This result also was reported by other investigators (17, 26). Between 6 and 12 hr, the plasma bilirubin activity–time curves exhibited small secondary peaks, characteristic of a recycling process and similar to that noted for certain drugs (36). When averaging all of the data, this apparent recycling phenomenon is masked by rather large interanimal variations; this complexity was, therefore, ignored in subsequent data-fitting computations.

Plasma ^{14}C-bilirubin disappearance data from rats kept under normal lighting conditions were fitted to a two-compartment model (Model 1) and to a three-compartment model (Model 2). The data points are shown in Fig. 1.

Models 1 and 2 are characterized by a central compartment (C), which contains the plasma and the other fluids and tissues in rapid equilibrium with plasma, and a (rapidly) miscible peripheral compartment (P). The three-compartment model also includes a slowly miscible compartment (S). The elimination rate constants and all intercompartmental rate constants are of first order. The analysis of the sums of squares by the F ratio test (37) indicates that there is no significant improvement at the 5% significance level when fitting the data to a three-compartment model compared with the two-compartment model fitting. However, it will be shown that the decrease in the plasma bilirubin concentration due to the illumination with low intensity light can best be described by a three-compartment model. From the initial value of percent dose per milligram of bilirubin at zero time, the amount of bilirubin in the central compartment under normal steady-state conditions was calculated to be 2.01 mg. The biological half-life, $t_{1/2}$, in the postdistributive phase was 23 hr, and the bilirubin production rate was 0.105 mg/hr.

Estimates of $t_{1/2}$ and the bilirubin production rate were also obtained by describing the disappearance of ^{14}C-bilirubin by:

$$Y = Ae^{-\alpha t} + Be^{-\beta t} \qquad \text{(Eq. 2)}$$

where Y = percent dose per milligram at time t, and A, B, α, and β are parameters for which least-squares estimates were calculated (Table I).

Decrease of Plasma Bilirubin Concentration during Illumination—During the continuous illumination with low or high intensity light,

Table I—Data Obtained following Intravenous Injection of ^{14}C-Bilirubin to Gunn Rats under Varying Conditions of Illumination; $Y = Ae^{-\alpha t} + Be^{-\beta t}$ (Y = % dose/mg of Bilirubin at Time t)

Illumination	A, % dose/mg of Bilirubin	α, hr^{-1}	B, % dose/mg of Bilirubin	β, hr^{-1}	$t_{1/2}$, hr (0.693/β)	Bilirubin Production Rate[a], mg/hr
Controls (normal room light)	20.2 (4.4)[b]	1.94 (0.63)	25.4 (1.1)	0.0297 (0.0037)	23.3	0.116
300 fc	12.1 (3.4)	1.24 (0.65)	40.1 (1.8)	0.0371 (0.0039)	18.7	0.092
1100 fc	14.0 (2.3)	1.18 (0.36)	37.6 (1.2)	0.0522 (0.0034)	13.3	0.137

[a] Calculated as $100/(A/\alpha + B/\beta)$. [b] Standard deviations of the parameters are shown in parentheses.

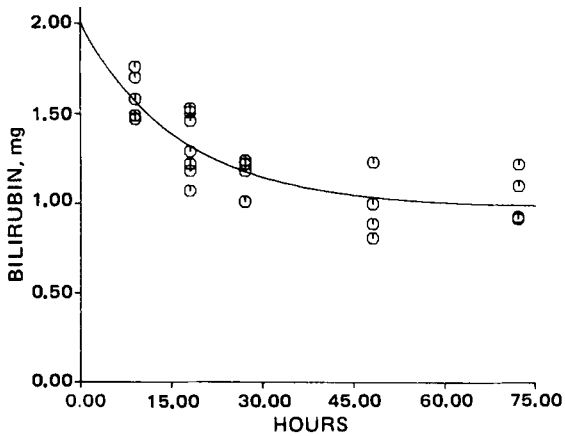


Figure 3—*Plasma bilirubin decline in seven Gunn rats during continuous severe phototherapy. The light intensity was either 1000–1100 fc or 300 fc with riboflavin as an adjunct. The solid line represents a computer fit of data to a two-compartment model (Model 3).*

the amount of bilirubin in the plasma decreased until a new steady-state level was reached (Figs. 2 and 3). No difference was detectable in the rates of decline of the plasma bilirubin concentration of rats illuminated with low intensity light while simultaneously receiving riboflavin 5′-monophosphate injections and of rats illuminated with high intensity light. Consequently, the data of these rats were pooled for analysis (Fig. 3).

On visual inspection of the graphs of Figs. 2 and 3, it appears that the new steady-state level of the amount of bilirubin in the plasma was approximately 60% of the initial value for rats illuminated with low intensity light and 50% for rats exposed to high intensity light. Any model used to describe the data must be compatible with these observations. The data were fitted to Models 3–12 (Fig. 4). Due to the restricted number of data points, it was not possible to obtain a computer solution if all of the parameters in the model under investigation were to be estimated in one computer run. Therefore, only the bilirubin production rate and the rate constant for the elimination process due to the phototherapy were introduced as adjustable parameters in Models 3–12. All other rate constants were given the values as obtained in the control rat studies.

The resulting values for the parameters, sums of weighted square de-

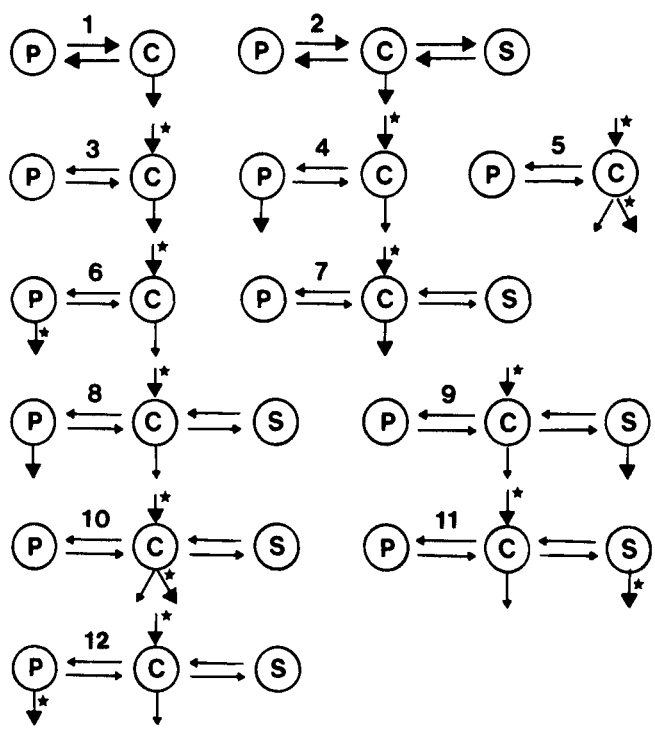


Figure 4—*Survey of kinetic models used in the data analysis. Zero-order rate constants are denoted by ★. Thick-lined arrows represent rate constants used as adjustable parameters in the computer fittings.*

Table II—Values of Characteristic Properties of the Models Used in the Computer Fit of the Data on the Decrease of the Plasma Bilirubin Level of Gunn Rats Due to Illumination

Model	Illumination Intensity, fc	*SWSD*[a]	Bilirubin Production Rate, mg/hr ± *SD*	Bilirubin[b] Excretion Rate ± *SD*	New Steady-State Level, % Initial Value
3	300	0.0472	0.00515 ± 0.00034	0.0105 ± 0.0012 hr^{-1}	<25
	1100	0.00487	0.102 ± 0.005	0.104 ± 0.006 hr^{-1}	48.3
4	300	0.121	0.0765 ± 0.0035	$0.157 \times 10^{-4} \pm 0.21 \times 10^{-5}$ hr^{-1}	65.0
	1100	0.00484	0.118 ± 0.003	0.0808 ± 0.0028 hr^{-1}	45.7
5	300	0.121	0.114 ± 0.005	0.0376 ± 0.0039 mg/hr	66.2
	1100	0.103	0.187 ± 0.030	0.149 ± 0.026 mg/hr	32.9
6	300	0.0545	0.115 ± 0.005	0.0475 ± 0.0039 mg/hr	58.4
	1100	6.11	0.12 ± 0.60	29.9 ± 133 mg/hr	<15
7	300	0.0150	0.0134 ± 0.0049	0.0182 ± 0.0050 hr^{-1}	<36
	1100	0.00851	0.113 ± 0.006	0.120 ± 0.004 hr^{-1}	47.3
8	300	0.0736	0.0663 ± 0.0029	$0.442 \times 10^{-4} \pm 0.56 \times 10^{-5}$ hr^{-1}	63.2
	1100	0.00762	0.116 ± 0.004	0.108 ± 0.006 hr^{-1}	47.3
9	300	0.0717	0.0728 ± 0.0028	0.00851 ± 0.00086 hr^{-1}	64.2
	1100	0.00506	0.0983 ± 0.0026	3.23 ± 0.27 hr^{-1}	47.5
10	300	0.0731	0.109 ± 0.006	0.0433 ± 0.0036 mg/hr	63.7
	1100	0.104	0.229 ± 0.058	0.273 ± 0.056 mg/hr	24.9
11	300	0.0248	0.105 ± 0.002	0.0431 ± 0.0019 mg/hr	59.2
	1100	0.00488	0.101 ± 0.002	1.27 ± 0.09 mg/hr or	48.3
				44.0 ± 2.8 mg/hr	48.3
12	300	0.0688	0.123 ± 0.006	0.0568 ± 0.0057 mg/hr	65.0
	1100	0.118	0.138 ± 0.030	0.114 ± 0.020 mg/hr	<35

[a] Sum of weighted square deviations. [b] Due to illumination.

viations, and the new steady-state plasma bilirubin levels as predicted by the models are shown in Table II.

^{14}C-Bilirubin Disappearance from Plasma under Phototherapy Conditions—Bilirubin kinetics were studied in rats that had been illuminated for a considerable period prior to the injection of labeled bilirubin. Ideally, the disappearance of the radioactive compound would be studied after the new steady state has been reached. This approach would require an illumination period of about 150 hr with low intensity light or 90 hr with high intensity light before injection of the labeled bilirubin. The illumination must be continued for a few more days after injection. When exposed to such prolonged periods of illumination, many of the Gunn rats died before the end of the experiment. Therefore, the rats were exposed for a shorter period to low or high intensity light before the injection, and the data were normalized as follows:

$$(\%\ \text{dose/mg of bilirubin})_{\text{time } t} = (\%\ \text{dose/mg of bilirubin})_{\text{observed}} \times \frac{(\text{amount plasma bilirubin})_{\text{time } t}}{(\text{amount plasma bilirubin})_{\text{new steady state}}} \quad \text{(Eq. 3)}$$

The amounts of bilirubin in plasma at time t were read from the graphs in Figs. 2 and 3. The new steady-state bilirubin levels for the low and high intensity light data were those predicted by Models 11 and 3, respectively (Table II). As before, the data from the rats illuminated with low intensity light while receiving riboflavin 5′-monophosphate injections were pooled with the high intensity light data, since there was no detectable difference between these two groups of data.

The decrease of the amount of radioactivity in the plasma of rats exposed to 300- (40 data points) and 1000–1100- (40 data points) fc light is shown in Figs. 5 and 6, respectively. The normalized plasma bilirubin specific activities (Eq. 1) could again be expressed as a simple two-exponential equation (Eq. 2). Values for A, B, α, and β, as well as for $t_{1/2}$ and the bilirubin production rate, are presented in Table I.

DISCUSSION

Since the products produced during the photocatabolism of bilirubin in the Gunn rat are not known (38), it was decided that bilirubin should not only be separated from its more polar degradation products by the much used extraction method of Weber and Schalm (31) but should subsequently be separated by TLC from unknown and possibly interfering products that might be present after the extraction in the organic layer.

In a previous report (39), it was established that riboflavin 5′-monophosphate administered intravenously to Gunn rats kept under low intensity light (300 fc) caused the plasma bilirubin level to decline to a new steady-state level at a faster rate than was achieved with low intensity light alone. These observations were confirmed in this study. Furthermore, no difference in the rate with which bilirubin was removed from the plasma was observed between the high intensity light data and the low intensity light data with simultaneous riboflavin 5′-monophosphate injections.

As is apparent from the numbers in Table II, none of the two-compartment models in Fig. 4 describes both the low and high intensity light data entirely satisfactorily, with the possible exception of Model 4. However, a significantly better fit (at the 5% significance level) was obtained with Model 11 when fitting the 300-fc data.

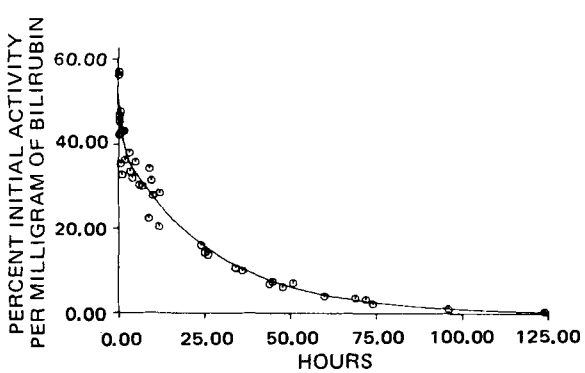


Figure 5—*Composite plasma ^{14}C-bilirubin data from five Gunn rats exposed to continuous low intensity (300 fc) illumination prior to and following the injection of the tracer dose. The solid line represents a computer fit of data for Eq. 2.*

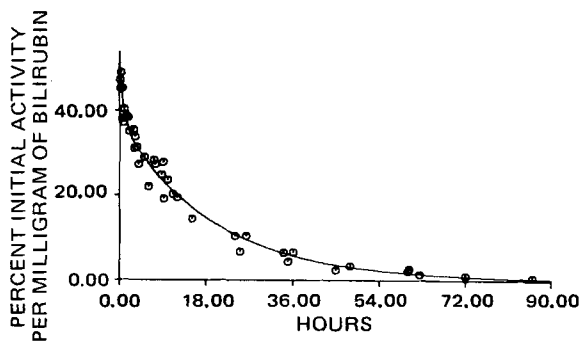


Figure 6—*Composite ^{14}C-plasma bilirubin data from eight Gunn rats exposed to continuous severe conditions of illumination (1000–1100 fc or 300 fc with riboflavin 5′-monophosphate as an adjunct) prior to and following the injection of the tracer dose. The solid line represents a computer fit of data to Eq. 2.*

Although Model 11 showed, for the low intensity light data, no significant improvement over Model 9 at the 5% significance level, Model 11 must still be preferred to Model 9 for several reasons. The bilirubin production rate as generated by the use of Model 11 is in better agreement with the value from the control rat studies than is the value obtained with Model 9; the coefficients of variation of the adjustable parameters are smaller for Model 11 than for Model 9, and the predicted new steady-state plasma bilirubin level is closer to the observed value (60%) when using Model 11. Consequently, Model 11 was considered the best possible model (of those in Fig. 4) to describe both the low and high intensity light data.

The high intensity light data could be fitted almost equally well to Models 3, 4, and 7–9. Depending on the initial value of the zero-order rate constant fed to the computer, very different estimates of that parameter were obtained for the high intensity light data; yet the bilirubin production rate, sum of weighted square deviations, and new steady-state values remained essentially unchanged. Once the zero-order elimination rate constant has exceeded a certain value under the influence of severe illumination conditions, the depletion of the slowly miscible compartment (S) occurs so rapidly that the model can be considered to collapse into the two-compartment Model 3 (Fig. 4).

It must be realized that, when searching for the best possible model as described here, a number of assumptions were made:

1. Only first-order and zero-order rate constants were considered.
2. The bilirubin production rate, as well as the intercompartmental rate constants, remained constant during phototherapy. The values in Table I suggest that, for the duration of the experiments, the bilirubin production rate remains essentially unaffected by illumination. The plasma levels in some rats tended to increase after prolonged periods of illumination (80–100 hr). These data points were not included in the analyses.
3. The rate of excretion of bilirubin from the central compartment, under control conditions, remained unaffected by subsequent illumination. When fitting the high intensity light data to Model 3, the obtained first-order elimination rate constant (0.104 hr^{-1}) was in excellent agreement with the expected value of 0.103 hr^{-1}, this value being the sum of the elimination rate constant (0.052 hr^{-1}) and the intercompartmental rate constant from C to S (0.051 hr^{-1}) from the control rat studies (Model 1), thus supporting this assumption.
4. In the jaundiced rat, bilirubin and its degradation products were reported to be partly excreted in the bile (17, 27).

There is no evidence that the liver in the Gunn rat is a separate compartment kinetically distinguishable from the central compartment, as has been suggested in reports concerning bilirubin kinetics in humans (20, 23). It was assumed that bilirubin was excreted solely from the central compartment under normal conditions. Obviously, this may be an oversimplification. On the basis of the present data, however, it was impossible to decide whether elimination under normal conditions occurs from one or more than one compartment; if elimination occurs from more than one compartment, it would be impossible to calculate the model constants independently (40).

5. Similarly, the degradation and elimination of bilirubin under phototherapy conditions might occur in more than one compartment.

Despite these and other possible complications, the chosen three-compartment model with zero-order elimination from the slowly miscible peripheral compartment has been shown to be excellently suited for the description of the changes in the plasma bilirubin concentration of the Gunn rat under continuous illumination with different light intensities.

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