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Research Article

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METABOLISM AND DISPOSITION OF C^{14}-BILIRUBIN IN CONGENITAL NONHEMOLYTIC JAUNDICE *

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In mammals, unconjugated bilirubin is rapidly cleared from the circulation and almost quantitatively excreted as a conjugate in the bile (2–4). The efficiency and rapidity of this process indicate that conjugation and subsequent biliary excretion represent the principal pathway of bilirubin disposition. If alternate pathways of bilirubin metabolism exist under physiologic conditions, their level of functional efficiency must be so low as to render detection difficult. When hepatic dysfunction causes interference with the efficiency of conjugation and excretion of bilirubin, however, resulting in hyperbilirubinemia, such alternate metabolic pathways may be expected to play a more important role in disposing of the accumulated pigment load (5). Moreover, when formation and hence biliary excretion of conjugated pigment is virtually abolished owing to an enzymatic defect in the hepatic conjugating apparatus, alternate pathways of bilirubin metabolism must assume the principal role in disposing of the bile pigment formed from the continuous breakdown of hemoglobin (6). Such metabolic anomalies associated with severe unconjugated hyperbilirubinemia exist as a rare congenital syndrome in man (Crigler-Najjar syndrome) (7, 8) and as a recessively inherited enzymatic defect in a mutant strain of Wistar rats (Gunn rats) (8, 9). In both instances, the level of unconjugated hyperbilirubinemia remains remarkably constant over months and years (8), indicating that a "steady state" has been established between pigment production and disposition.

In the present investigation, a patient with congenital unconjugated hyperbilirubinemia and a group of hyperbilirubinemic Gunn rats were injected with C^{14}-bilirubin to determine the turnover of the pigment and the metabolic disposition of the label. Separate and quantitative collection of bile, urine, and feces devoid of bile was achieved in the animal experiments by external biliary drainage. Equilibration of the injected labeled pigment with the total miscible pool of bilirubin in the body permitted estimation of the extravascular bilirubin pool, and, in the rats, of the tissue distribution of the pigment.

METHODS

Human studies

The patient was a 4½-year-old boy whose icterus was first detected when he was 3 days old and who has since remained severely jaundiced (8). He experienced minor incidental illnesses, including measles, otitis, and several upper respiratory infections, but developed normally and failed to exhibit somatic or mental abnormalities referable to his icterus. At the time of this study, he weighed 20 kg and measured 41½ inches. Physical examination revealed a deeply jaundiced boy without detectable abnormalities. Liver, spleen, and kidneys were not palpable, secondary stigmata of hepatic disease were lacking, and repeated neurological examinations failed to elicit positive findings. The hemoglobin concentration was 12.1 g per 100 ml, hematocrit, 39%, reticulocyte count, 0.8% and leukocyte count, 9,000 per mm^3 with a normal differential. Routine urine and stool examinations were negative. Conventional liver-function tests, including Bromsulphalein clearance, alkaline phosphatase activity, thymol turbidity, and cephalin flocculation were within normal limits. Serum albumin concentration was 4.6 g per 100 ml (10).

The patient has been followed by the senior author since shortly after birth; during this period, the following special investigations were carried out. Serum bilirubin concentrations (11) were determined on over 70 individual blood samples. Values for direct-reacting bilirubin ranged from 0.3 to 1.0 mg per 100 ml and for total bilirubin, fluctuated between 20 and 30 mg per 100 ml except during acute febrile illnesses, when bilirubin levels tended to be slightly higher, with occasional values exceeding 30 mg per 100 ml. On paper chromatography (12), serum bilirubin yielded exclusively the azo deriva-

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TABLE I

Urinary excretion of free N-acetyl-p-aminophenol (NAPA), NAPA glucuronide, and total conjugated p-aminophenol after intravenous injection of NAPA

Subject	Age	Serum bilirubin	Percentage of injected dose excreted in urine in 6 hours		
			Free NAPA	NAPA glucuronide	Total conjugated *p*-aminophenol
		mg/100 ml			
Patient	8 mos	24.4	5.5	8.9	49.9
Control boy	8 mos	0.3	4.0	28.0	78.5
Control boy	8 mos	0.1	3.3	28.2	58.0
Patient's father	32 yrs	0.5	2.7	33.8	53.8
Patient's mother	28 yrs	0.3	2.6	46.2	69.6
Normal men*		0.6	2.4	33.3	59.4

* Values are mean of 15 subjects.

tive of unconjugated bilirubin. Urine did not contain demonstrable bilirubin (13), and at the age of 6 months, fecal urobilinogen (14) was 0.23 mg per day. Bile aspirated through a biluminal tube from the duodenum before and 20 minutes after intraduodenal infusion of 15 ml 50% $MgSO_4$ contained 1.54 and 0.69 mg bilirubin per 100 ml (11), respectively; on paper chromatography (12), azo derivatives of conjugated bilirubin could not be detected. An oral cholecystogram revealed normal visualization of the gall bladder.

At the age of 8 months, the patient was given an iv injection of 10 mg per kg body weight of *N*-acetyl-*p*-aminophenol (NAPA) (15). The amounts of NAPA, NAPA glucuronide, and total conjugated *p*-aminophenol excreted in the urine during the subsequent 6-hour period were estimated (16), and the results expressed as percentage of the administered dose. Similar studies were carried out in two healthy boys of the same age and in the patient's nonicteric parents. The results are given in Table I.

On the day before the present investigation,[1] the patient's plasma volume was determined with T-1824 (17). He was then given an iv injection of 8.26 mg twice-recrystallized C^{14}-bilirubin (18), containing 1,134,000 disintegrations per minute (dpm) per mg, dissolved in 14 ml of 5% human albumin. During the subsequent 9 days, 4-ml samples of blood were drawn at frequent intervals, first every 15 minutes to 1 hour, then daily, and finally, every second day. In the serum obtained from each sample, bilirubin concentration and radioactivity (19) were determined and the specific activity of the pigment was calculated. Pooled samples of the sera collected from days 2 through 9 were used for separation of the protein fractions by continuous-flow electrophoresis (19) and for crystallization and radioassay of the extracted pigment (18, 20, 21).

All stools passed for 7 days after administration of C^{14}-bilirubin were collected and kept frozen until analysis. The stool samples were homogenized with 2 vol distilled water, and samples were used for the following determinations: *1*) Total radioactivity (21). *2*) Crystallization of fecal bilirubin. Samples of the fecal homogenate were weakly acidified with acetic acid and repeatedly extracted with a 2:1 mixture of methanol and chloroform. Unlabeled carrier bilirubin was added, the combined pigment crystallized, and the specific activity of the crystals determined (18). *3*) Fecal urobilinogen (14). *4*) Fecal mesobilifuscin. Mesobilifuscin methyl ester was prepared by a modification of Siedel and Möller's method (22). The purified dried pigment was used for elemental analysis and for assay of specific radioactivity (23).

All urine passed for 5 days after the administration of the C^{14}-bilirubin was collected, and the radioactivity determined (21). The remaining urine collected on days 1, 2, 3, and 5 was pooled, mixed with concentrated HCl to a final concentration of 1 N HCl, and then boiled for 1 hour. Mesobilifuscin methyl ester was prepared as described above for elemental analysis and for assay of radioactivity. Conjugated carrier bilirubin was added to the urine sample collected on day 4 for isolation, crystallization, and radioassay of the pigment.

Studies in rats

Preparation of experimental animals and collection of samples. Three male and five female homozygous, jaundiced Gunn rats were used. Their weights ranged from 200 to 400 g, the initial serum bilirubin, from 5.6 to 10.9 mg per 100 ml, and their hematocrits, from 40 to 49%. Total blood volume was assumed to be 5% of body weight (24), and plasma volume was calculated on the basis of the hematocrit.

In the first group of five male and female animals, external biliary drainage was established and a polyethylene square was sutured around the anus (21). The rats were then placed in restraining cages, permitting separate and quantitative collection of bile, urine, and feces for uninterrupted periods ranging from 53 to 96 hours. Pellets of rat chow and drinking solution (0.2% NaCl, 0.02% KCl, and 5% glucose in water) were offered freely.

[1] We are grateful to Dr. Sydney Gellis and his staff for their permission to study this patient on the Pediatric Service of the Boston City Hospital and for their help and cooperation.

One rat was pretreated with oral neomycin (0.1 g per kg body wt per day) for 5 days immediately before the experiment. A second group of three Gunn rats was placed in restraining cages without cannulation of the bile duct, but with the necessary provisions for separate and quantitative collection of feces and urine for uninterrupted periods ranging from 96 to 148 hours.

At time zero, the rats were injected intravenously with 170 to 1,100 μg of twice-recrystallized C^{14}-bilirubin (SA, 800 to 2,420 dpm per μg) dissolved in 1.0 to 2.8 ml of normal rat serum. Volumes of 0.2 to 0.3 ml of blood were collected from the tip of the tail, first at intervals of less than an hour and later, of 1 to 6 hours; during the latter part of the experiments, 2 to 3 blood samples were obtained per day. All urine and feces were collected continuously throughout the experiments. At autopsy, stool contained in the small and large intestines was recovered and analyzed separately. Bile was drained into tubes kept at 4° C in the dark and changed at frequent intervals.

Analytical procedures. Serum from each blood collection was used for determination of bilirubin concentration and radioactivity, and the specific activity of serum bilirubin was calculated. In pooled serum, obtained by exsanguinating the animals at the end of the experiment, serum bilirubin concentration and radioactivity were measured, and bilirubin was extracted and crystallized for determination of specific activity. Total radioactivity of *urine* was determined by methods previously described (21). In four instances pooled urine samples were mixed with unconjugated or conjugated carrier bilirubin for extraction, crystallization, and radioassay of the pigment. All samples of *feces* were homogenized with 4 vol of water, and portions were used for assay of total radioactivity (21). After addition of unlabeled carrier bilirubin to either the fecal homogenates or to their methanol-chloroform extracts, the pigment from seven stool collections was crystallized and its specific activity determined. All organic solvents used contained small amounts of hydroquinone to protect the bilirubin against oxidative breakdown (25).

In all *bile* specimens, total radioactivity was determined, and apparent bilirubin concentration was estimated by the diazo reaction. Five samples of Gunn rat bile were mixed with conjugated or unconjugated carrier bilirubin, and the pigment was extracted and crystallized for radioassay. In three instances, 4.5 to 10 ml of Gunn rat bile containing known amounts of radioactivity was injected intravenously into large male Sprague-Dawley rats with an external biliary fistula. In the recipients, all bile was collected for periods ranging from 6 to 18 hours after iv injection. Bilirubin concentration and total radioactivity in these bile specimens were determined, and bilirubin was crystallized for radioassay. Conjugated C^{14}-bilirubin excreted in the bile of the normal recipients was expressed as percentage of the total radioactivity in the injected Gunn rat bile. For comparison, normal rat bile containing conjugated C^{14}-bilirubin was injected into two normal recipients with external fistula, and the bile collected over the subsequent 5 hours was similarly analyzed.

Attempts at chromatographic identification of the radioactive catabolites in Gunn rat bile were unsuccessful, but adequate separation from the labeled pigments in normal rat bile was achieved by stepwise, ascending, thin-layer chromatography (26). Equal volumes of labeled Gunn rat bile and unlabeled normal rat bile, or of unlabeled Gunn rat bile and labeled normal rat bile were mixed and lyophilized, and the residue extracted with a 2:1 mixture of methanol and chloroform. After centrifugation, 0.01-ml samples of the supernatant fluid were applied to a dry, 1-mm-thick layer of MN-cellulose 300 g/CM [2] pasted on a 20- × 20-cm glass plate. The three successively employed developing systems consisted of *1*) chloroform, *2*) a 2:1 mixture of butanol and heptane, and *3*), a 10:15:10 mixture of butanol, methanol, and water. The dried chromatogram was divided into consecutive 2-cm squares; from these, the cellulose layer was scraped and suspended in 1 ml of 1 M Hyamine 10X for radioassay. Radioactivity in each square was expressed as percentage of the total isotope on the chromatogram.

Samples of bile from a normal and a Gunn rat, previously injected with C^{14}-bilirubin, were extracted repeatedly with butanol or chloroform at pH 5.0. The respective extracts were pooled for determination of bilirubin content and total radioactivity. Bile from each of these two animals, buffered to pH 6.8, was incubated with 50 mg of bacterial β-glucuronidase [3] at 37° C for 4 hours. The pH was then adjusted to 5.0, and the incubated substance extracted repeatedly with chloroform for determination of bilirubin content and radioactivity. Control incubations were carried out with heat-inactivated enzyme.

Gmelin (27) and pentdyopent (28) reactions were carried out on native normal and Gunn rat bile and on their chloroform and methanol-chloroform extracts. For spectroscopic analysis, Gunn rat bile or normal rat bile was diluted with water to a final volume of 4.0 ml. Two ml of the diluted bile was mixed with 0.5 ml diazo reagent and 2.5 ml methanol. The color reaction was allowed to develop in the dark for 30 minutes, and then optical density in the visible range was determined in a Beckman DU spectrophotometer. Spectral analysis was also carried out on butanol extracts of native normal and Gunn rat bile at pH 5.0 and on chloroform extracts of comparable bile specimens previously hydrolyzed in the dark for 20 minutes with 1 N NaOH.

Tissue analysis for radioactivity was done as follows. All rats were exsanguinated between 53 and 148 hours after the iv injection of C^{14}-bilirubin, and the following organs and tissues were removed for radioactive assay: liver, spleen, kidneys, brain, abdominal skin, femoral muscle, retroperitoneal or epididymal adipose tissue, small and large bowel. The intestine was opened, and after all fecal material collected was either combined

[2] Brinkmann Instruments, Inc., Great Neck, N. Y.

[3] Sigma Chemical Corp., St. Louis, Mo.

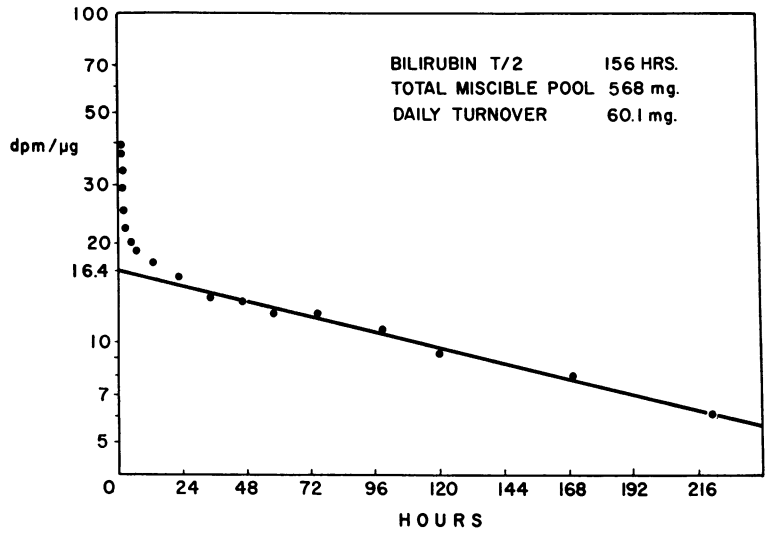

FIG. 1. SA OF SERUM BILIRUBIN IN THE 4½-YEAR-OLD PATIENT WITH CONGENITAL UNCONJUGATED HYPERBILIRUBINEMIA. At time zero, 4.3 μc C^{14}-bilirubin was injected intravenously.

or separated for small and large bowel, the tissue was washed in 0.9% saline. Tissue radioactivity was determined as described for feces, and isotope concentration was expressed in disintegrations per minute per gram wet weight. For calculation of bilirubin concentration, tissue radioactivity was divided by the specific activity of the serum bilirubin at death, and a correction factor was applied for labeled pigment bound to tissue albumin (29), on the assumption that the binding ratio was the same as that in the circulating plasma. In two rats, the pigment crystals of the renal papilla (30) were dissolved in chloroform for determination of total bilirubin content and radioactivity. In three instances, samples of liver were homogenized with 4 vol of 1 : 2 chloroform and methanol for crystallization and radioassay of the pigment.

Radioassay of all pigment, serum, excreta, and tissue extracts was performed in a Packard Tri-Carb liquid scintillation spectrometer. Radioactivity was expressed in disintegrations per minute with toluene-C^{14} as an internal standard (18). Counting time ranged from 2×10 to 2×30 minutes, depending on the activity present. Samples were considered to contain significant radioactivity when the number of counts in the counting vials exceeded by at least 3 SD that of appropriately prepared background vials (31).

TABLE II

Turnover of C^{14}-bilirubin and recovery of excreted C^{14}-activity in a 4½-year-old boy with congenital unconjugated hyperbilirubinemia

Days after administration of C^{14}-bilirubin	Serum bilirubin	Turnover of C^{14}-bilirubin	Feces				Urine	
			Wet wt	Uro-bilinogen	Total C^{14}-activity	C^{14}-activity recovered in crystalline bilirubin	Volume	C^{14}-activity recovered
	mg/100 ml	*dpm/24 hrs*	*g/24 hrs*	*mg/24 hrs*	*dpm/24 hrs*	*dpm/24 hrs*	*ml/24 hrs*	*dpm/24 hrs*
1	25.1–27.1 [10]*	880,000		No stool passed			610	65,580
2	25.1–26.7 [2]*	811,000		No stool passed			285	45,540
3	24.6	741,000	35.3	8.3	0	0	425	46,160
4	26.0	684,000	49.3	15.0	462,000	7,380	350	52,850
5	26.0	625,000	51.6	27.0	1,190,000	13,780	530	60,530
6	26.7	568,000	31.1	12.6	628,000	9,780	Not collected	
7	26.1–27.5 [2]*	512,000	57.7	21.0	816,000	25,150	Not collected	
Total (mean)	25.9	4,821,000	225.0	83.9	3,096,000	56,090	2,200	270,660

* Number of individual determinations.

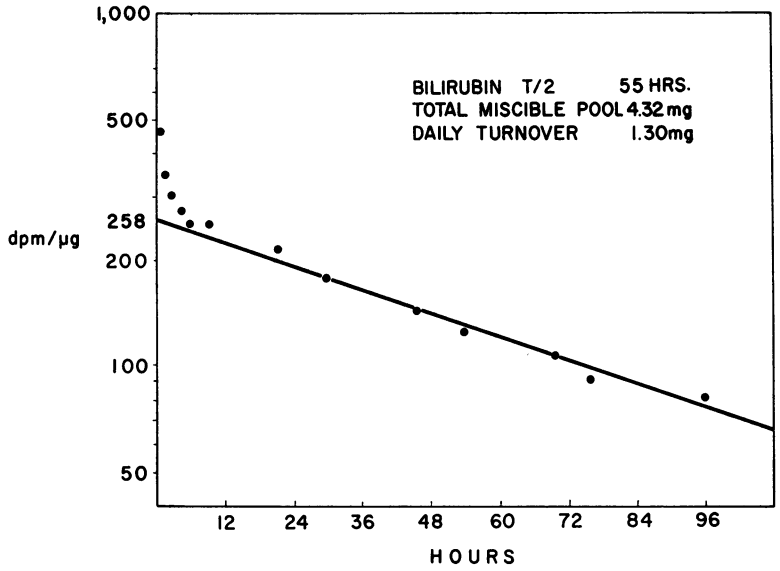

FIG. 2. SA OF SERUM BILIRUBIN IN A 400-G MALE GUNN RAT WITH EXTERNAL BILIARY DRAINAGE. At time zero, 0.64 μc C^{14}-bilirubin was injected intravenously.

RESULTS

Human studies

During this investigation, the patient's serum bilirubin concentration remained stable (Table II), indicating a "steady state" between bilirubin formation and disposition. After injection of C^{14}-bilirubin, mixing of the label with the rapidly exchanging bilirubin space resulted in a sharp initial fall of specific activity (Figure 1), while a second, slower phase of equilibration occurred during the subsequent 24 hours. Specific activity declined logarithmically from approximately 30 hours on (Figure 1), indicating that first-order kinetics had been reached that could be characterized by a single exponential rate of disappearance. In the pooled sera, collected from days 2 to 9, bilirubin SA was 11.8 dpm per μg when estimated on the basis of total serum radioactivity and diazo reaction, and 11.4 dpm per μg after extraction and crystallization of the pigment. All radioactivity migrated with the albumin fraction on electrophoretic separation of the serum proteins (19).

From these data and a measured plasma volume of 1,080 ml, the following values were calculated (32): circulating plasma pool of bilirubin, 280 mg; total miscible pool, 568 mg; bilirubin half life, 156 hours; and bilirubin turnover, 60.1 mg per 24 hours.

No stools were passed during the first 2 days of the study, but between days 3 and 7, 225 g of feces were collected, representing the total amount passed during this period (Table II). For the purpose of comparing fecal isotope excretion with the turnover of C^{14}-bilirubin in the body pool, an average delay of 2 days was allowed for intestinal transit time. Since the specimen passed on day 3 failed to exhibit significant radioactivity, the actual transit time during the early phase of this study may have been longer. For the present comparison, however, this appeared to be irrelevant because the biological half-time of the label as calculated from the fecal isotope excretion (Table II) was similar to that determined on the basis of pigment turnover in the serum (Figure 1). During the 5 days of stool collection, a total of 3,096,000 dpm was eliminated by the fecal route, corresponding to 82% of the calculated isotope turnover in the body pool during the comparable period. Only 56,090 dpm were recovered as crystallized bilirubin from these stool specimens (Table II). On the basis of the specific activity of serum bilirubin on the days corresponding to the fecal collections (Figure 1), the total amount of crystalline bilirubin recovered from the feces was calculated at less than 5 mg.

Cumulative fecal excretion of urobilinogen for the 5 days was 83.9 mg, representing 28% of

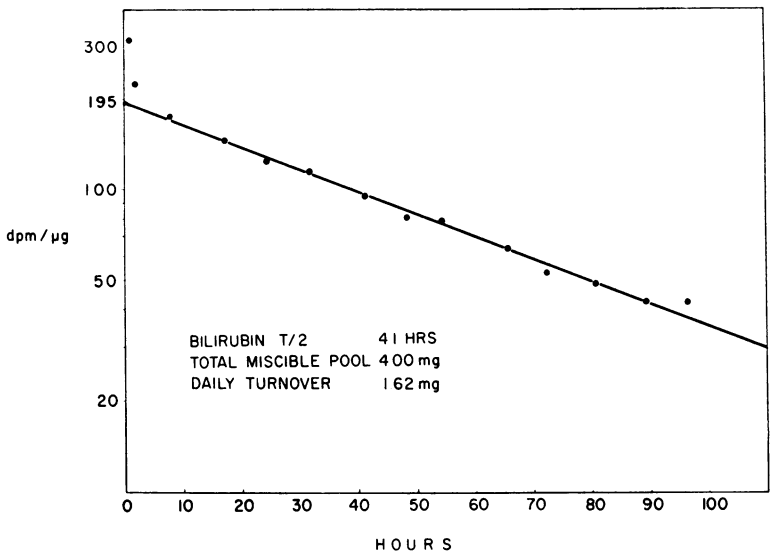

FIG. 3. SA OF SERUM BILIRUBIN IN A 355-G MALE GUNN RAT WITHOUT EXTERNAL BILIARY DRAINAGE. At time zero, 0.46 μc C^{14}-bilirubin was injected intravenously.

the corresponding total bilirubin turnover of 300 mg. Mesobilifuscin methyl ester was isolated from each fecal sample in amounts similar to those obtained from normal stool (22), but the pigment exhibited a specific activity of less than 10% of that of the serum bilirubin on corresponding days (Figure 1). Elemental analysis, performed on the pooled isolated mesobilifuscin methyl ester, revealed the following percentage composition: found—C, 58.6; H, 6.4; N, 7.2; and mol wt, 340; calculated—C, 64.2; H, 6.9; N, 8.8; and mol wt, 318.

The total of 270,660 dpm excreted in the urine (Table II) represents approximately 7% of the corresponding total isotope turnover. Bilirubin could not be demonstrated by the Gmelin reaction (27). When conjugated bilirubin as unlabeled carrier was added to a urine sample, only 680 dpm were recovered in the crystallized pigment, or less than 1.2% of the total urinary radioactivity. From 1,780 ml of pooled urine, 85 mg of purified dry mesobilifuscin methyl ester was obtained with the following elemental composition: found—C, 63.7; H, 6.0; and N, 6.9; calculated—C, 64.2; H, 6.9; and N, 8.8. The SA of the combined pigment sample was 0.14 dpm per μg, which is 4 to 10 times less than that of the mesobilifuscin isolated from the stool, and almost 100 times less than the mean specific activity of the serum bilirubin.

TABLE III

Pool size, biological half life, and turnover of bilirubin in Gunn rats

	Rats without external biliary drainage			Rats with external biliary drainage				
Weight, *g*	200	275	355	225	230	260	320	400
Initial serum bilirubin level, *mg per 100 ml*	6.0	9.2	8.0	8.8	10.9	6.0	6.8	5.6
Biological half life of C^{14}-bilirubin, *hours*	33	62	41	104	100	62	63	55
Bilirubin plasma pool, μg	360	763	845	585	750	475	650	672
Total miscible bilirubin pool, μg	3,520	5,470	4,000	3,270	3,480	2,980	3,760	4,320
Fractional turnover, *per 24 hours*	0.49	0.27	0.40	0.16	0.17	0.27	0.26	0.30

TABLE IV

*Bilirubin concentration in organs of eight Gunn rats injected with C^{14}-bilirubin**

Tissue	Bilirubin		
	Range	Mean	Corrected mean†
		μg/g or ml	
Serum	54–104	84	84
Liver	59–175	92	84
Kidney	27–100	44	37
Spleen	4–50	29	
Brain	1–14	6	<6
Intestine	20–61	43	37
Adipose	22–400	102	99
Skin	10–48	28	19
Muscle	3–29	12	10

* Pigment concentration per gram wet weight, calculated from tissue radioactivity divided by specific activity of serum bilirubin at death (see text).

† Correction applied for pigment bound to tissue albumin (29), assuming that the binding ratio was the same as in the plasma.

Studies in rats

Isotope turnover in vivo. In the eight Gunn rats injected with C^{14}-bilirubin, equilibration of the labeled pigment with the total miscible bilirubin pool required approximately 7 to 10 hours. Thereafter, disappearance of the isotope conformed to a first-order reaction (Figures 2 and 3), permitting determination of the biological half life of C^{14}-bilirubin (32) (Table III). In the pooled sera, obtained by exsanguinating the animals, bilirubin SA was 28 dpm per μg when estimated on the basis of total serum radioactivity and diazo reaction, and 32 dpm per μg when the pigment was extracted and crystallized. On the basis of plasma volume (24), serum concentration, and biological half life, the total miscible pool and the fractional turnover of bilirubin were calculated (32) for each rat. As shown in Table III, the total miscible pool was on the average 6 times larger than the amount of pigment in the circulation, and the fractional turnover per 24 hours ranged from 0.16 to 0.49.

Bilirubin content of tissues. Bilirubin concentration in the major organs varied considerably (Table IV). After correction for pigment presumed to be bound to the albumin present in these tissues (29), it was evident that significant portions of the extravascular bilirubin pool were contained in the liver, kidneys, intestine, and particularly in adipose tissue, whereas in brain, radioactivity was barely measurable. In the three instances in which C^{14}-bilirubin was crystallized from the liver, SA of the crystals was 26, 243, and 269 dpm per μg; SA of bilirubin in the comparable serum samples was 24, 256, and 201 dpm per μg. The bilirubin crystals recovered intact from the renal papilla exhibited less than half of the specific activity of the serum bilirubin obtained at death, indicating that this pigment fraction turned over more slowly than the rest of the miscible pool.

Recovery of isotope in excreta. Total turnover of C^{14}-isotope for the whole experimental period was calculated in each rat, and the values were compared with the combined recovery of radioactivity in the excreta. In the three rats without external bile drainage, combined recovery of radioactivity in urine and feces ranged from 70 to 100% (average, 83%) of the respective values calculated for isotope turnover. In all instances, feces passed during the first day after administration of the labeled pigment contained significant radioactivity. Values calculated for the biological half life of the label on the basis of fecal isotope excretion approximated those obtained from the rate of disappearance of radioactivity from the serum.

In the five animals with external biliary drainage, 32.5 to 114% (average, 68%) of the isotope calculated as lost from the body pool was recovered in the combined excreta. The lowest value was obtained in a rat which, for unknown reasons, exhibited a gradual decrease in bile flow and in fecal and urinary output. At sacrifice, this rat's hematocrit was below 30%, suggesting that the extracellular fluid compartment had expanded. In these rats, exclusion of the bile from the intestinal tract resulted in bulky stools, and fecal isotope excretion tended to reach a plateau rather than to fall commensurately with the progressive decrease in total isotope turnover (Figure 4). This was particularly evident in the rat pretreated with neomycin, which passed unusually voluminous stools containing a total of 76% of the isotope calculated to have been lost from the body pool. In the remaining four rats of this group, radioactivity recovered in the feces averaged 17% and in the urine, 3% of the calculated isotope turnover (Figure 4).

Characterization of the excreted isotope. In all instances, the *urine* exhibited a negative

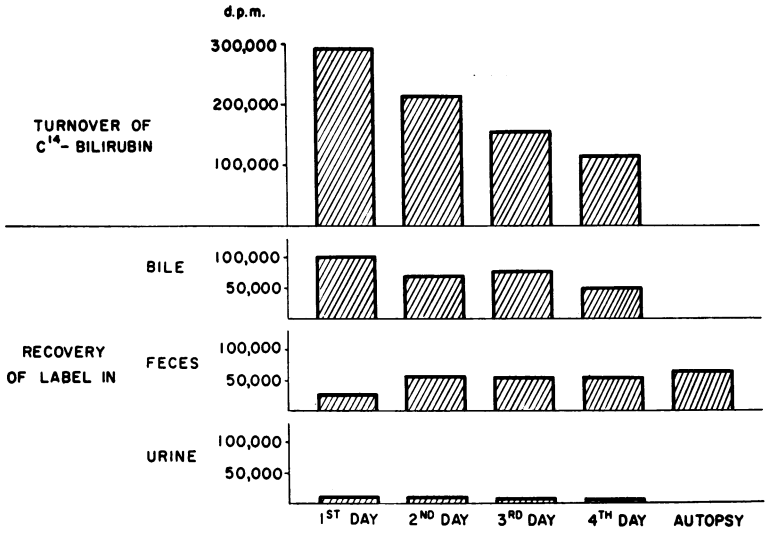

FIG. 4. TURNOVER OF C^{14}-BILIRUBIN AND EXCRETION OF RADIOACTIVE LABEL IN A GUNN RAT INJECTED WITH C^{14}-BILIRUBIN. Data for SA of serum bilirubin in this rat are given in Figure 2.

Gmelin reaction. When urine samples were mixed with unconjugated or conjugated carrier bilirubin for crystallization of the pigment, no significant label was detected in the crystals, and most of the radioactivity remained in the aqueous phase. In seven instances, *fecal* samples from rats with an external biliary fistula were mixed with unlabeled carrier bilirubin for extraction and crystallization of the pigment. With three samples, to which albumin-bound bilirubin had been added, the crystals contained 51, 49, and 26% of the total radioactivity present in the feces, whereas in the four instances in which the unlabeled bilirubin was added to the combined chloroform extracts, 32, 25, 11, and 8% of the isotope was present in the crystallized pigment. The last two values were obtained with feces collected at autopsy from the rat with the gradual decrease in fecal, biliary, and urinary excretion.

In the five Gunn rats with external bile drainage, the combined *bile* samples from each individual animal exhibited from 23 to 41% (average, 35%) of the radioactivity calculated to have been lost from the body pool (Figure 4). When samples of labeled Gunn rat bile were mixed with unlabeled normal rat bile for isolation and crystallization of the pigment, the crystals contained only 3 to 13% of the total radioactivity present, while the rest of the isotope remained in the aqueous supernatant fluid. On addition of carrier bilirubin in solution of albumin or chloroform, 3 to 5% of the total isotope was present in the crystallized pigment. After injection of radioactive Gunn rat bile into normal rats with external bile drainage, any labeled bilirubin present in the administered specimens would have been mixed and excreted with the recipients' endogenous bilirubin; this permitted direct crystallization of the pigment and eliminated the need for addition of carrier bilirubin *in vitro*. As shown in Table V, only 8.1% of the total radioactivity in the injected Gunn rat bile appeared in the recipients' bile in the form of labeled bilirubin. Calculated on the basis of excreted radioactivity, 15.1% of the isotope in the recipients' bile was recovered as C^{14}-bilirubin. By contrast, after injection of normal rat bile containing conjugated C^{14}-bilirubin, 66% of the injected or 80% of the excreted radioactivity was present in the bilirubin of the recipients' bile (Table V).

On thin-layer chromatography of extracts of lyophilized labeled Gunn rat bile, 88% of the isotope migrated in about equal proportions with the two butanol-containing systems, whereas only 12% advanced with the chloroform fraction (Figure 5). The latter contained most of the yellow pigment and exhibited a faintly positive Gmelin reaction, whereas the butanol-heptane and the butanol-methanol-water fractions were virtually colorless. In contrast, comparable extracts of

TABLE V

Recovery of isotope in bile of normal rats injected with labeled Gunn rat bile

Injected bile specimen		Collected bile specimen			
				Isotope in crystallized bilirubin	
Rat source	Isotope content	Duration of collection	Total radioactivity in bile	% of injected radioactivity	% of radioactivity excreted in bile
	dpm/specimen	*hours*	*dpm*		
Gunn	19,944	18	7,920	6.3	15.9
Gunn	20,928	18	12,875	8.7	14.1
Gunn	21,520	6*	13,150	9.3	15.2
Normal	68,840	5	59,232	69	80
Normal	34,480	5	28,002	64	80

* Bilirubin crystallized from the bile specimen collected from 6 to 18 hours did not exhibit significant radioactivity.

labeled normal rat bile revealed 79% of the radioactivity migrating in the chloroform fraction, whereas the butanol systems contained only minor fractions of the isotope.

After incubation of Gunn rat bile with β-glucuronidase, chloroform-extractable radioactivity did not differ significantly from that in comparable control experiments with inactivated enzyme (Table VI). In contrast, incubation of normal rat bile containing conjugated C^{14}-bilirubin with the enzyme resulted in a fourfold increment in chloroform-extractable isotope (Table VI).

Native Gunn rat bile and samples extracted with chloroform or with a 2:1 mixture of methanol and chloroform gave negative to faintly positive Gmelin (27) and pentdyopent (28) reactions, whereas with normal rat bile, these reactions were strongly positive. With the modified van den Bergh reaction, much less color developed with Gunn rat bile than with normal bile (Figure 6). Moreover, with the former, the small amount of azo derivatives obtained exhibited maximal absorption at 520 mμ, whereas with normal bile, the absorption peak lies in the 550 mμ band (33) (Figure 6). Since this indicated that Gunn rat bile contained constituents other than bilirubin that reacted with the diazo reagent, actual bilirubin concentrations in these specimens could not be estimated by this method. Additional evidence that the diazo-positive material was not identical with the excreted label was obtained on extraction with butanol. Whereas from normal bile, butanol extracted a significant amount of labeled yellow pigment exhibiting the characteristic spectral absorption of conjugated bilirubin (34) (Figure 7) and a positive diazo reaction (Table VI), comparable extracts of Gunn rat bile also contained radioactivity, but revealed a maximal absorption around 400 mμ (Figure 7) and only traces of diazo-positive material (Table VI). Similar spectroscopic differences were noted in chloroform extracts of normal and Gunn rat bile

TABLE VI

Radioactivity extracted from bile specimens after iv injection of C^{14}-bilirubin

Sample	Radioactivity extracted				
	Gunn rat bile		Normal rat bile		
	dpm	%	*dpm*	%	*SA of bilirubin, dpm/μg*
Initial bile specimen, *per ml*	3,974	100	34,420	100	222
Butanol extract, pH 5.0	1,270*	32*	15,150	44	168
Chloroform extract, pH 5.0	239*	6*	894	2.6	162
Incubated with inactivated β-glucuronidase, chloroform extract, pH 5.0	654*	16*	4,116	12	171
Incubated with active β-glucuronidase, chloroform extract, pH 5.0	701*	18*	15,935	46	163

* Extract contained only trace of diazo-reacting material.

previously subjected to alkaline hydrolysis (Figure 8).

DISCUSSION

Because of the large capacity of normal mammalian liver to conjugate and excrete bilirubin (2–4, 35), the pigment formed from breakdown

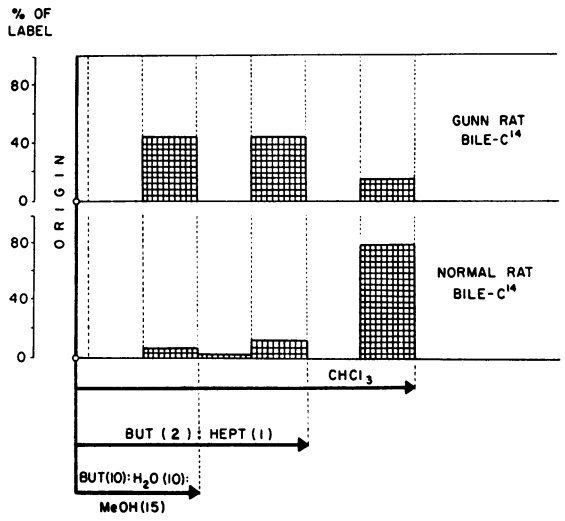

FIG. 5. STEPWISE ASCENDING THIN-LAYER CHROMATOGRAPHY OF EXTRACTS PREPARED FROM LABELED BILE OF NORMAL AND GUNN RATS. For details, see text.

of hemoglobin is rapidly cleared from the circulation and eliminated in the bile. Since this process results in a very short biological half life of bilirubin, an exogenous label injected in a single dose would not mix adequately with the endogenously produced pigment. On the other

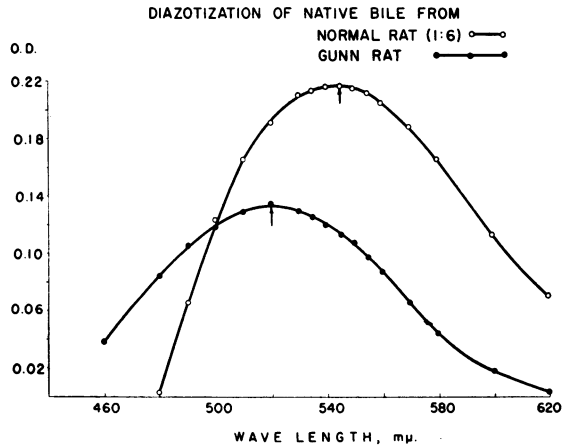

FIG. 6. SPECTRAL ABSORPTION OF DIAZO-TREATED BILE FROM NORMAL AND GUNN RATS.

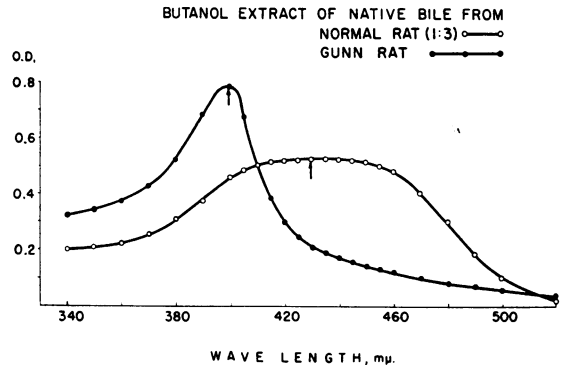

FIG. 7. SPECTRAL ABSORPTION OF A BUTANOL EXTRACT OF NATIVE BILE FROM NORMAL AND GUNN RATS.

hand, when C^{14}-bilirubin was administered intravenously to a patient and to animals with unconjugated hyperbilirubinemia, the disappearance rate of the label from the serum revealed kinetics (Figures 1–3) suggesting that virtually complete equilibration had been achieved. This was supported by the finding that in the patient and in the three rats without external bile drainage, the biological half life of the label as calculated from its rate of disappearance from the serum was

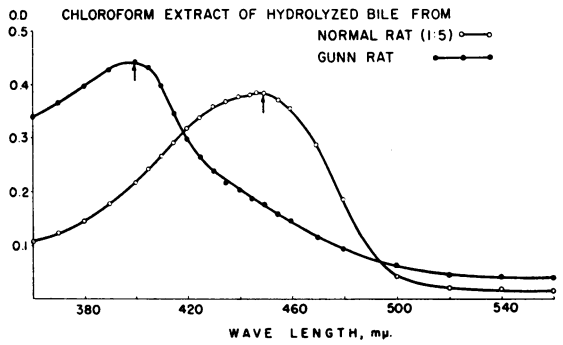

FIG. 8. SPECTRAL ABSORPTION OF A CHLOROFORM EXTRACT OF ALKALI-HYDROLYZED BILE FROM NORMAL AND GUNN RATS.

similar to that calculated from fecal excretion of the isotope. In the five rats with an external biliary fistula, variable weight loss associated with the operative trauma and the exclusion of bile from the intestinal tract created a more complex situation, making it difficult to ascertain that a true "steady state" was maintained throughout the experiment. This difficulty may explain why in this group of rats, bilirubin appeared to exhibit a slightly longer biological half life and the mis-

cible pool a smaller fractional turnover than in rats without surgery (Table III).

In the patient, daily bilirubin turnover amounted to 60.1 mg, closely approximating the anticipated value of 58.1 mg, calculated from the total red-cell volume, a mean erythrocyte life span of 120 days (36), and an estimated 12% increment for bile pigment formation from sources other than circulating hemoglobin (37). Similarly, in the three Gunn rats without external bile drainage, the magnitude of bilirubin turnover agreed in general with the values calculated from a mean erythrocyte life span of 60 days (38), allowing for additional pigment formation from random destruction of a minor red-cell fraction (39) and from nonhemoglobin sources (37). These findings indicate that in man and rats with this type of hyperbilirubinemia, bile pigment formation from endogenous sources is quantitatively comparable to that occurring under physiologic conditions.

On the other hand, the two species differed significantly in their bodily distribution of bilirubin. In the patient, the total pigment pool was divided about equally between the circulating plasma and the extravascular compartments. Since bilirubin is tightly bound to human albumin (19), it was not surprising that the pigment exhibited a total space of distribution similar to that reported for this protein (40, 41). The biological half life of bilirubin, however, was two to three times shorter (Figure 1) than that of albumin (40, 41), suggesting that in the tissues, part of the pigment became detached from its carrier protein and was destroyed at an accelerated rate (19, 42). In the rats, however, where the interaction between the pigment and albumin is weaker (19), five-sixths of the miscible bilirubin pool was present in the extravascular compartments (Table III). Since this space of distribution is far larger than that of extravascular albumin (29, 43), a significant fraction of the exchangeable bilirubin must have been bound to tissue proteins or lipids. As shown in Table IV, this binding occurred predominantly in the liver, kidney, intestine, and adipose tissue, but smaller amounts of pigment were present also in the spleen, skin, and muscle. Although all rats used in these experiments exhibited neurological damage resembling kernicterus (44), their brains contained only negligible radioactivity. This finding supports present concepts that after the neonatal period the blood-brain barrier becomes virtually impermeable to bilirubin (45), but it does not rule out the possibility of increased pigment concentrations occurring in selected local centers of the nervous system.

One of the major aims of this investigation was the detection and functional evaluation of the alternate pathways for bilirubin disposition that in the absence of effective conjugation, must be responsible for the maintenance of a "steady state" between pigment formation and elimination (6). A partial answer to this problem was obtained from the study of the patient and the three intact rats. In the patient, 300 mg of bilirubin were turned over during 5 days, but less than 30% of it could be accounted for as fecal urobilinogen and bilirubin (Table II). The exact source of this urobilinogen fraction could not be definitely established. Since with NAPA, glucuronide formation was found to be markedly reduced but not entirely absent (Table I), it is conceivable that small amounts of conjugated bilirubin could have been formed in the liver and excreted in the bile without being detected on duodenal aspiration. Although this possibility could not be definitely excluded, urobilinogen could also have been derived from unconjugated bilirubin reaching the intestinal lumen either with the bile (46), or across bowel wall, and subsequently being reduced by the fecal flora (47).

In the stool, two-thirds of the excreted isotope was present in unidentified catabolites of bilirubin that probably reached the intestinal tract with the bile. That most of this labeled material could not have been mesobilifuscin was demonstrated by the latter's low specific activity. This finding supports earlier observations (22) that most of the fecal mesobilifuscin is derived from metabolic sources other than circulating bilirubin or its intestinal breakdown products.

Urinary excretion played a minor role in the over-all pigment disposition, and the small amount of isotope appearing in urine was all present as water-soluble bilirubin catabolites. Similar observations were made in the three Gunn rats without external bile drainage, where 69 to 96% of the radioactivity calculated as lost from the body pool was recovered in the feces, whereas less than 5% of the label appeared in the urine.

Although in these studies most of the isotope

turned over appeared in the feces, little direct information was obtained about the form in which the labeled compounds reached the intestinal tract and by which routes. The following theoretical possibilities appeared to be worthy of consideration: *1*) that alternate conjugating mechanisms, such as sulfate formation (48), substitute for the defective glucuronidation (30); *2*) that unconjugated bilirubin is excreted in the bile (46) and perhaps across the intestinal mucosa (49); and *3*) that bilirubin is converted to more polar catabolites that can be excreted without the need for conjugation.

In the five rats with external biliary drainage, although no specific search was made for bilirubin sulfate (48), spectroscopy (Figures 6, 7), chromatography (Figure 5), and butanol extraction (Table VI) of the bile failed to reveal detectable amounts of conjugated bilirubin. Indeed, if these animals could excrete appreciable amounts of bilirubin by sulfate formation or by other conjugating mechanisms, significant retention of unconjugated pigment in the organism would not be expected. In all instances, the bile was found to contain small amounts of unconjugated bilirubin, but in terms of the over-all pigment disposition, the contribution of this excretory pathway appeared to be of minor significance. More important was the observation that in spite of complete diversion of the bile to the outside, a portion of the injected radioactivity was present in the feces. Moreover, a significant fraction of this label was contained in unconjugated bilirubin, suggesting that the latter had reached the bowel lumen across the intestinal mucosa. Since unconjugated bilirubin is absorbed from the gut (21) also, the fecal material appeared to contain a bilirubin pool exchanging with the miscible body pool. By oral administration of cholestyramine,[4] which tightly binds bilirubin, a portion of this fecal pigment was trapped in the intestine, causing a marked fall of the serum bilirubin level (49). These observations indicated that in Gunn rats, and perhaps in patients with unconjugated hyperbilirubinemia, direct transfer of pigment across the intestinal wall represents an ancillary pathway for bilirubin excretion. In the rats with external biliary drainage, however, the bulky consistency of the feces may have resulted in a larger than normal excretion of labeled pigment into the gut; this was particularly evident in the rat pretreated with neomycin.

Approximately one-third to two-fifths of the total isotope turnover appeared in the form of water-soluble catabolites of bilirubin excreted in the bile, and to a lesser degree, in the urine. In part, these pigment derivatives were yellow and soluble in butanol (Table VI), but they lacked the spectral properties of bilirubin (Figures 6–8) and failed to give a positive diazo reaction (Table VI). Moreover, after injection into normal rats, they were rapidly excreted in the bile without being converted to conjugated bilirubin (Table V). The exact structure of these pigment derivatives is unknown, but their solubility and spectroscopic properties appeared to be comparable to those of the breakdown products obtained on exposure of bilirubin to mild alkali (18) and to light (50) *in vitro*. The present findings suggest that in unconjugated hyperbilirubinemia, a similar breakdown of bilirubin may occur *in vivo*. To a limited extent, such pigment breakdown may occur also in animals with an intact conjugating mechanism, as we inferred from the finding that in the bile of normal rats injected with C^{14}-bilirubin, only 80% of the excreted isotope was accounted for in the isolated labeled pigment (Table V). Furthermore, in such bile specimens, the apparent specific activity of bilirubin was distinctly higher than that of the pigment fractions extracted with butanol or chloroform (Table VI). On the other hand, in Gunn rat bile, the diazo reaction gave spectrophotometric readings suggesting significantly higher bilirubin concentrations than were actually present. This result implies that in these specimens, substances other than bilirubin were responsible for the occurrence of an "atypical" diazo reaction (Figure 6). The nature of these compounds has not been determined, nor is it known whether similar diazo-positive excretory products are present in normal bile. Human T-tube bile, however, may contain small amounts of diazo-reacting material that is neither hydrolyzed by alkali, nor accounted for as sulfate conjugates of bilirubin (48).

These observations indicate that in unconjugated hyperbilirubinemia, several alternate pathways of pigment disposition can substitute for the deficient glucuronide formation (Figure 9).

[4] MK-135, Merck Sharp & Dohme Research Laboratories, West Point, Pa.

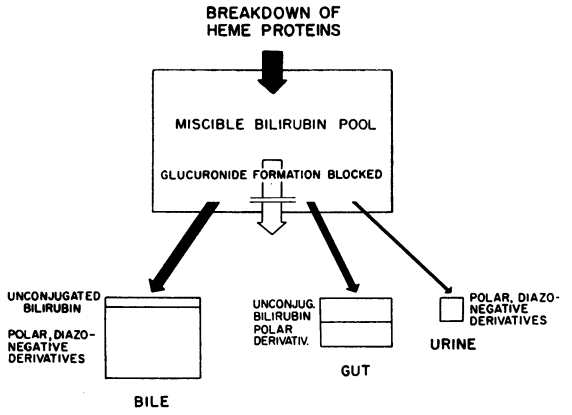

FIG. 9. SCHEMATIC REPRESENTATION OF ALTERNATE PATHS OF BILIRUBIN DISPOSITION IN HYPERBILIRUBINEMIC GUNN RATS WITH DEFECTIVE GLUCURONIDE FORMATION.

When their combined functional rate equals the rate of pigment formation, a "steady state" is achieved, resulting in a constant level of serum bilirubin. In patients with the Crigler-Najjar syndrome, such a balanced state is reached only at very high bilirubin concentrations (*7*, 8), which probably reflects the unusually strong affinity and protective action of human albumin for the pigment (19, 42). In Gunn rats, on the other hand, where bilirubin is less tightly bound to albumin (19) and hence escapes more easily into the extravascular compartments, the combined rates of pigment breakdown and of excretion through the intestinal wall equal that of pigment production at significantly lower bilirubin levels (30, 44). Since in human and rat sera, bilirubin catabolites were not detectable, it appears likely that they are very rapidly cleared from the circulation, or that pigment breakdown occurs at anatomical sites permitting irreversible egress of the resulting metabolites. It is possible that destruction of bilirubin in these tissue sites may be enhanced by intense illumination (51, 52), or by administration of certain drugs or chemicals (53, 54).

SUMMARY

1) In a patient with congenital unconjugated hyperbilirubinemia (Crigler-Najjar syndrome) and in eight rats with hereditary icterus (Gunn rats), tracer techniques were used to estimate the biological half life, total miscible pool, and fractional turnover of bilirubin. The metabolic disposition of the C^{14}-bilirubin was determined in the feces and urine, and in five of the rats, in the bile.

2) In the patient, the total miscible bilirubin pool was distributed about equally between the circulating plasma and the extravascular space, which was attributed to the very strong interaction of the pigment with human albumin. In the rats, however, where bilirubin is less tightly bound to albumin, five-sixths of the exchangeable pigment pool was present in the extravascular compartments.

3) In the absence of a functioning conjugating apparatus, elimination of bilirubin is accomplished through alternate metabolic pathways. These include breakdown of the pigment to more polar, diazo-negative derivatives, transfer of pigment across the intestinal mucosa into the gut, and excretion of unconjugated bilirubin in the bile. When the combined functional rate of these pathways equals that of pigment formation, a "steady state" is reached, leading to constant levels of serum bilirubin.

4) In hyperbilirubinemia of this type, the interaction of the pigment with serum albumin appears to be the major determinant regulating the serum concentration and the mode of disposition of bilirubin.

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