Journal of Clinical Investigation
Vol. 41, No. 8, 1962

THE FORMATION OF BILIRUBIN FROM HEMOGLOBIN *IN VIVO* *

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(Submitted for publication March 7, 1962; accepted March 30, 1962)

About 80 to 85 per cent of the fecal bile pigments are believed to be derived from the hemoglobin of senescent erythrocytes that are destroyed in the reticuloendothelial system (1). It is not known, however, whether the hemoglobin of sequestered red cells is converted quantitatively to bilirubin and eventually to urobilinogen, or is in part metabolized to other products (2, 3).

Earlier studies of this problem in man have revealed that the rate of fecal urobilinogen excretion falls short of the expected output as calculated from total red cell volume and mean erythrocyte life span (4–6). This has been attributed to incomplete intestinal conversion of bilirubin to urobilinogen (3, 4, 6, 7), destruction of urobilinogen in the large bowel (3, 4, 6), and loss of pigment through intestinal absorption (3, 4, 6, 8). Attempts to circumvent these difficulties by estimating bilirubin excretion through a T-tube in the common bile duct have been unsatisfactory (9, 10) because this technique does not permit complete bile collection. In animals with an external biliary fistula, this obstacle has been overcome, but the rate of endogenous bilirubin excretion was found to exhibit marked fluctuations (11, 12), which rendered difficult comparison of the observed values with the endogenous production of bilirubin calculated from hemoglobin turnover, or with excess bilirubin formation after intravenous injection of a hemoglobin load. In animals with chronic bile fistulae, these acute fluctuations in pigment output tend to balance out, but the necessity of giving oral bile supplements to replace drainage losses (11, 13) results in falsely high pigment excretion due to intestinal absorption and enterohepatic circulation of the bilirubin contained in the administered bile (8).

In the present investigation, these difficulties have been surmounted by injecting C^{14}-labeled, antibody-sensitized red cells into rats with an external bile fistula. This permitted both determination of the excretory rate of bilirubin-C^{14} in the bile and estimation of the efficiency of conversion of the injected hemoglobin to bilirubin, without regard for variations in endogenous bilirubin excretion. Since the injected red cells were also labeled with γ-emitting Fe^{59} or Cr^{51}, it was possible to follow their rate of removal from the circulation, and to determine the interval between sequestration of the cells and excretion of the resulting bilirubin-C^{14} in the bile.

Similar experiments were carried out with intravenously injected solutions of hemoglobin labeled with C^{14} and either Fe^{59} or Cr^{51}. Urinary loss of pigment was minimized or prevented by administering only small amounts of hemoglobin or, in some instances, by nephrectomizing the rats prior to the experiment.

MATERIALS AND METHODS

Preparation of red cells and hemoglobin labeled with C^{14} and Fe^{59}. Male Sprague-Dawley rats weighing approximately 300 g were given, at 48-hour intervals, three subcutaneous injections of 20 mg phenylhydrazine hydrochloride in neutralized 2 per cent aqueous solution. Beginning three days after the last phenylhydrazine injection, 0.2 mc glycine-2-C^{14} was administered subcutaneously twice daily to a total dose of 2.0 mc.[1] Over the same period, a total dose of 100 μc transferrin-bound $Fe^{59}Cl_3$ (14) was injected into a tail vein in three to five separate daily doses.[2] Two weeks after the last isotope administration, the rats were exsanguinated with heparinized syringes, and the red cells were separated and washed with cold isotonic saline.

* Supported in part by United States Public Health Service Grants A-1833 and RG-3507. This work was presented in part before the American Society for Clinical Investigation, Atlantic City, N. J., April 30, 1961.

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[1] Specific activity of glycine-2-C^{14}, 17 to 20 mc per mmole; New England Nuclear Corp., Boston, Mass.

[2] Specific activity of $Fe^{59}Cl_3$, 3 to 10 μc per μg; Abbott Laboratories, Chicago, Ill.

An aliquot of these red cells was sensitized *in vitro* by incubation with heated rabbit serum containing "incomplete" antibodies against rat erythrocytes (15). After these sensitized red cells were washed and resuspended in isotonic saline, they showed slight agglutination in saline, but strong agglutination in an appropriate Coombs serum (14) and in polyvinylpyrrolidone (16). On incubation for 2 hours at 37° C in a large volume of fresh rat serum, no hemolysis occurred.

Solutions of labeled hemoglobin were prepared by lysing an aliquot of donor red cells in cold distilled water. After restoring isotonicity with 5 per cent saline, the stroma was removed by centrifugation in the cold for 1 hour at 105,000 G. The clear supernatant was then adjusted to the desired hemoglobin concentration and to pH 7.8 by addition of appropriate volumes of isotonic saline and 0.15 M phosphate buffer, pH 8.1. The slightly alkaline pH was employed to obviate the slow, spontaneous precipitation that occurred with rat hemoglobin in aqueous solution.

Isolation and radioassay of labeled hemin. From a third aliquot of the C^{14}-, Fe^{59}-labeled red cells, hemin was isolated and crystallized (17). After recrystallization (18), the hemin was dissolved in pyridine and the concentration estimated spectrophotometrically, with a standard curve prepared by dissolving weighed amounts of hemin in similar fashion. The pyridine solutions were then diluted with 1 M Hyamine–methanol [3] to yield a final concentration of 10 μg labeled hemin per ml. For counting, 1 to 4 μg labeled hemin and 20 μg unlabeled carrier hemin, dissolved in a total of 1 ml of 1 M Hyamine–methanol, were mixed with 17 ml scintillator solution [4] in a 20 ml low potassium glass counting vial.[5] The vials were counted in a liquid scintillation spectrometer,[6] using toluene-C^{14} as the internal standard.[7] After 6 to 8 months, most of the Fe^{59} activity had decayed, and the specific activity of the hemin approached a constant value attributable solely to C^{14}. Absence of detectable gamma emission due to remaining Fe^{59} was further ascertained in a well-type scintillation counter. Allowing for the difference in molecular weights and for the loss of a labeled methene-bridge carbon atom (19), the C^{14}-specific activity of the bilirubin derived from the injected hemoglobin-C^{14} was calculated as follows:

$$\text{sp. act. of hemin} \times \frac{651.9}{584.7} \times \frac{7}{8} = \text{sp. act. of bilirubin}$$

or

$$\text{hemin (dpm per } \mu\text{g)} \times 0.976 = \text{bilirubin (dpm per } \mu\text{g).}$$

Clearance of injected labeled red cells or hemoglobin from the circulation. In 16 male Sprague-Dawley rats, weighing 450 to 600 g, an external bile fistula was produced (20) and the animals were then placed in loose restraining cages. Bile flow was maintained in excess of 0.7 ml per hour by appropriate hydration (21). After collecting bile for 3 hours, the rats were briefly anesthetized with ether and 1.6 to 2.0 ml of a suspension of the labeled red cells, or of the hemoglobin solution, was injected into the tail vein. From the total amount of hemoglobin administered (Table I), the expected quantity of bilirubin was calculated from the conversion ratio 1 g hemoglobin = 34.5 mg bilirubin (22).

The rate of removal of the administered red cells or hemoglobin from the circulation was determined by following the gamma activity of serial blood samples collected by incision of the tail. One-tenth ml blood was pipetted into 1 ml water for determination of whole blood radioactivity, and into 1 ml saline for measurement of plasma radioactivity after removal of red cells by centrifugation (15). The initial concentration of the injected material in the blood was estimated on the basis of the predicted blood volume, which for male rats of this size was assumed to be 4.5 per cent of body weight (15).

The rats were killed by cervical dislocation 22 hours after the injection of the labeled heme pigment. The liver, spleen, kidneys, lungs, and right femur were removed, blotted with damp gauze, weighed, and the Fe^{59} activity determined on specimens not exceeding 2 g in weight (14). Total Fe^{59} in the marrow was roughly estimated by multiplying the femur radioactivity by 14.4 (23). Urine and bile Fe^{59} or Cr^{51} radioactivity were determined on the total 22-hour specimens.

Because of the rapid reutilization and redistribution of iron from the metabolized hemoglobin (24, 25), the organ content of Fe^{59} after 22 hours is not a valid measure of the initial tissue distribution of injected heme. Therefore six of the animals (Table I) were injected with sensitized red cells or hemoglobin solution labeled with Cr^{51}, which is released more slowly from the tissues (25, 26). C^{14}-labeled red cells, prepared as described above, were labeled *in vitro* with $Na_2Cr^{51}O_4$ (15) prior to incubation with antiserum.[8] Hemoglobin was prepared from these cells as described. Crystalline hemin-C^{14} derived from these cells contained no Cr^{51}, as determined in a well-type scintillation counter. Blood and tissue samples from these rats were processed as above.

Rats R and S (Table I) received injections of hemoglobin solutions labeled only with C^{14}, which were prepared as above except that the donor rats were not given Fe^{59}. Rats J and K were bilaterally nephrectomized immediately before insertion of the biliary catheter.

Determination of bilirubin-C^{14} excretion. All bile excreted during the 22 hours of the experiment was collected

[3] Hydroxide of Hyamine 10X, Packard Instrument Co., La Grange, Ill.

[4] 2,5-Diphenyloxazole, 4 g per L, and 1,4-*bis*-2(5-phenyloxazolyl)-benzene, 50 mg per L, dissolved in dry toluene. Prepared from Liquifluor, Pilot Chemical Co., Watertown, Mass.

[5] Catalog no. 6001015, Packard Instrument Co., La Grange, Ill.

[6] Tri-Carb model 314-X, Packard Instrument Co., La Grange, Ill.

[7] Toluene-C^{14}, lot no. 61-12, New England Nuclear Corp., Boston, Mass.

[8] Specific activity of $Na_2Cr^{51}O_4$, 300 to 500 μc per μg; Abbott Laboratories, N. Chicago, Ill.

over consecutive periods ranging from 30 to 120 minutes. The collection tubes were kept on ice and protected from light, and then stored at $-20°$ C until analyzed. Under these conditions, the bilirubin does not deteriorate over periods of several weeks (21). For analysis, the individual samples were thawed, the volume recorded, and the bilirubin content determined by the diazo method (27). From each sample, bilirubin was crystallized (21) and the C^{14} specific activity was determined (21). Absence of Fe^{59} or Cr^{51} activity in the crystalline pigment was ascertained in the well-type scintillation counter. For each collection period, total excretion of C^{14} activity in bilirubin was calculated from the concentration and specific activity of the excreted bilirubin. The quantity of bilirubin derived from the injected hemoglobin-C^{14} was obtained by dividing the total C^{14} activity of bilirubin in each sample by the calculated specific activity of the bilirubin-C^{14} derived from the injected heme-C^{14}. Excretion of unlabeled bilirubin derived from endogenous sources was calculated as the difference between the total bilirubin output and the excretion of bilirubin-C^{14}. In rats R, S, T, U, V, and W (Table I), these determinations were performed only on pooled bile samples collected over the entire 22-hour period.

Related experiments. The fluctuations of endogenous bilirubin excretion under the conditions of this experiment were determined in three rats with a bile fistula to which *no* exogenous pigment load was given. Bilirubin excretion was measured during individual collection periods ranging from 30 to 60 minutes, and the effect of anesthesia and surgery on pigment output was determined.

The rate and efficiency of excretion of an intravenous load of unconjugated bilirubin were studied by injecting a 500-g male rat with crystalline bilirubin-C^{14} (21). Bilirubin-C^{14}, 0.93 mg with a specific activity of 168 dpm per μg, was dissolved in 0.2 ml 0.1 N NaOH, diluted to 1.0 ml with 0.1 M phosphate buffer, pH 7.4, and slowly added to 2.5 ml pooled normal rat serum. Three ml of this solution, containing 0.80 mg bilirubin-C^{14}, was injected into the femoral vein of an anesthetized rat with an external bile fistula. Bile was collected in 4- to 6-minute fractions for the first half hour, and in 15- to 30-minute fractions thereafter. Each bile sample was diluted with water to a volume of 1.0 ml and then divided into two equal portions. One-half was used for determination of bilirubin content and total radioactivity. The other halves of the bile samples were pooled in such a way that each pool represented one-half of the bile excreted during each hourly collection period. From each pool, bilirubin was crystallized and its specific activity determined.

RESULTS

Rate of heme clearance and of bilirubin excretion. For each experimental animal, data concerning the nature and quantity of the administered hemoglobin load are given in Table I. In all instances, cumulative curves of removal of labeled red cells or hemoglobin from the blood, and of excretion of labeled bilirubin in the bile, revealed similar patterns (Figure 1). Clearance of administered heme pigment is expressed as per cent

TABLE I

Bilirubin-C^{14} excretion in rat bile after administration of C^{14}-labeled heme pigment

Material injected	Rat	Isotopic label	Dose injected (as hemoglobin)	Heme-t_{50}*	Bilirubin-t_{50}†	Δt_{50}‡	Bilirubin-C^{14} recovered§
			mg/kg	*min*	*min*	*min*	%
Sensitized red cells	A	Cr^{51}, C^{14}	245	54	237	183	72.1
	B	Cr^{51}, C^{14}	188	75	345	270	69.9
	C	Fe^{59}, C^{14}	57.1	43	240	197	66.4
	D	Fe^{59}, C^{14}	63.3	13	185	172	63.3
	T	Cr^{51}, C^{14}	3.8				79.6
	U	Cr^{51}, C^{14}	3.5				65.0
Hemoglobin solution	E	Fe^{59}, C^{14}	60.2	15	185	170	63.4
	F	Fe^{59}, C^{14}	51.3	21	172	151	55.4
	R	C^{14}	62.5				58.3
	P	Fe^{59}, C^{14}	11.7	16	170	154	98.6
	Q	Fe^{59}, C^{14}	12.7	7	125	118	98.7
	S	C^{14}	12.5				97.5
	V	Cr^{51}, C^{14}	4.2				98.6
	W	Cr^{51}, C^{14}	3.9				76.1
	J‖	Fe^{59}, C^{14}	55.3	45	260	215	69.7
	K‖	Fe^{59}, C^{14}	50.0	77	260	183	62.8

* Heme-t_{50} = time for half the injected Fe^{59} or Cr^{51} to disappear from the circulation.
† Bilirubin-t_{50} = time for half the total amount of bilirubin-C^{14} excreted to appear in the bile.
‡ Δt_{50} = difference between heme-t_{50} and bilirubin-t_{50}.
§ Recovery of bilirubin-C^{14} is expressed as the per cent of the amount of bilirubin-C^{14} expected in bile if *all* the administered heme pigment were converted to bilirubin.
‖ Rats J and K were nephrectomized prior to hemoglobin-C^{14} injection.

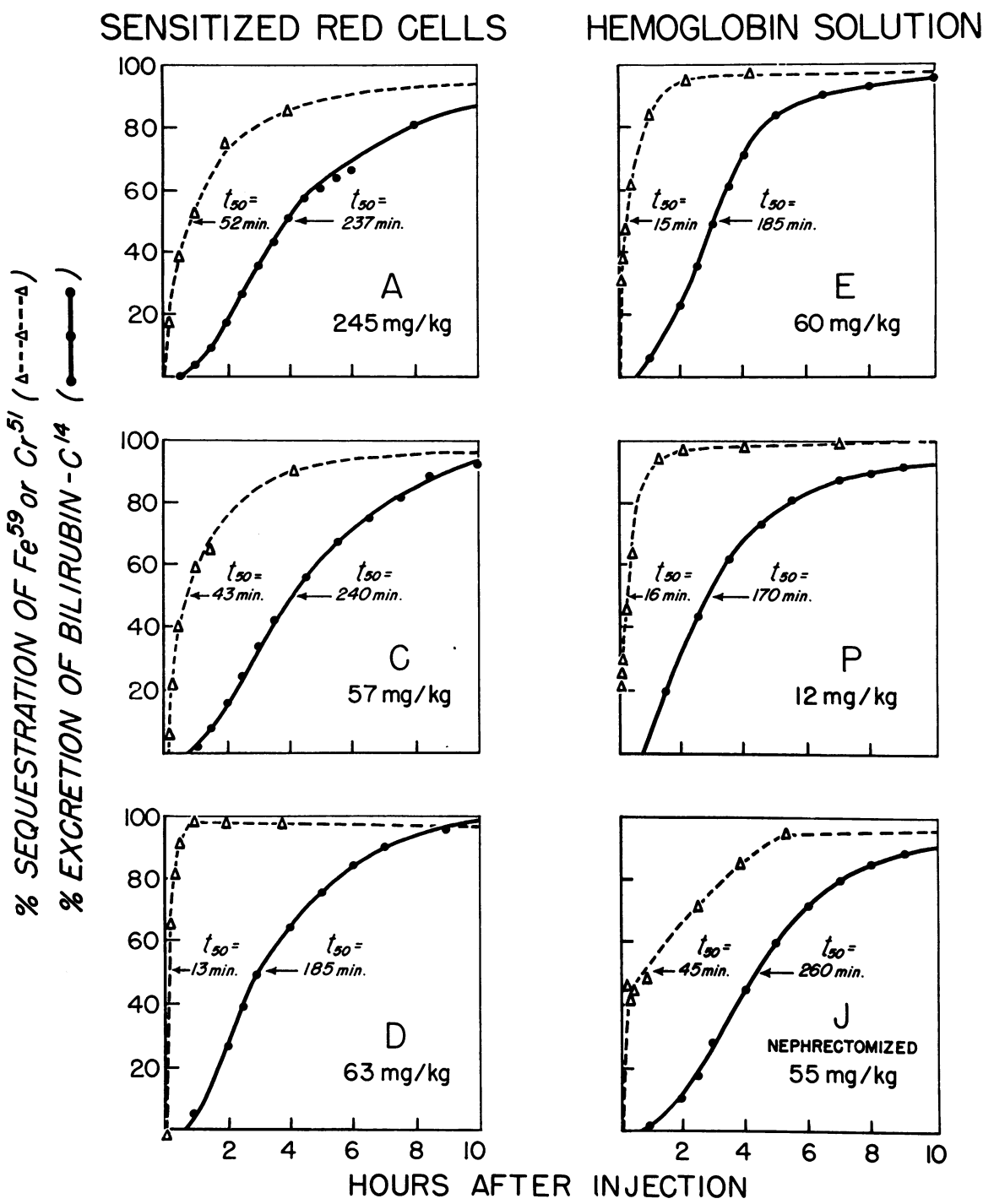

FIG. 1. CUMULATIVE CURVES OF RED CELL OR HEMOGLOBIN CLEARANCE AND OF BILIRUBIN-C^{14} EXCRETION IN SIX REPRESENTATIVE RATS. Sequestration of heme pigments is expressed in per cent of the injected dose of Cr^{51} or Fe^{59}. Excretion of bilirubin-C^{14} is expressed in per cent of the total quantity of labeled bilirubin that appeared in the bile during 22 hours.

of the *injected* dose of Fe^{59} or Cr^{51}, whereas bilirubin-C^{14} output is plotted as per cent of the total amount of isotopic bilirubin that was *excreted* in 22 hours. The time required after injection of heme pigment for half the labeled heme to be cleared from the circulation (heme-t_{50}) and for half the labeled bilirubin to be excreted in the bile (bilirubin-t_{50}) was determined from these curves (Table I). Since the curves are not exponential, these t_{50} values do not represent mathematically derived half-lives.

Sequestration of sensitized red cells began within a few minutes after their injection, with 50 per cent of the cells being removed in 15 to 75 minutes, and 70 to 80 per cent within 2 hours (Figure 1; A, C, and D). Excretion of bilirubin-C^{14} began within 30 to 50 minutes after the onset of sequestration, after 1 hour reached a nearly constant rate lasting 3 to 4 hours, and was almost completed within 7 to 8 hours. With all doses of red cells injected, the bilirubin excretion increasingly lagged behind erythrocyte sequestration (Figure 1). At the t_{50} level, the interval between the two curves (Δt_{50}) ranged from 172 to 270 minutes (Table I).

After injection of labeled hemoglobin in solution, the patterns of heme clearance and bilirubin-C^{14} excretion were similar to those obtained after administration of red cells (Figure 1; E and P). In general, hemoglobin was cleared more rapidly than sensitized red cells (heme-t_{50} = 7 to 21 minutes), resulting in an accelerated rate of bilirubin-C^{14} excretion (Δt_{50} = 118 to 170 minutes) (Table I). As with red cells, excretion of labeled bilirubin began within 30 to 50 minutes after the hemoglobin injection. In the nephrectomized rats (J and K), as in the intact animals, initial removal of hemoglobin was rapid, with approximately 40 per cent of the injected dose being cleared within 15 minutes (Figure 1). In the absence of the kidneys, however, the remaining hemoglobin was cleared more slowly, resulting in delayed excretion of labeled bilirubin (Table I).

With all doses both of sensitized red cells and of hemoglobin solution, irrespective of the presence or absence of the kidneys, the rate of bilirubin-C^{14} excretion appeared to depend upon the rate of heme-C^{14} sequestration. Moreover, the longer the time required for the clearance of the administered heme pigment (heme-t_{50}), the greater was the interval (Δt_{50}) between heme sequestration and excretion of labeled bilirubin ($r = 0.76$; $p < 0.01$) (28).

Figure 2 illustrates absolute rates of bilirubin-C^{14} excretion in four animals, two of which were injected with sensitized red cells (A and C) and two with hemoglobin solution (E and P). As expected, the rate of bilirubin-C^{14} excretion depended upon the quantity of heme pigment administered, the maximum being reached between 2 and 3 hours after heme injection. Since virtually all injected red cells or hemoglobin had been cleared from the circulation during the period of 22 hours, and since, in most instances, excretion of bilirubin-C^{14} was thereafter no longer detectable, it was possible to compare directly the total amount of heme pigment injected with the total amount of labeled bilirubin excreted.

Recovery of bilirubin-C^{14} from the injected heme-C^{14}. Total excretion of bilirubin-C^{14} during the 22 hour period, as per cent of the amount expected from the administered heme-C^{14}, is given in Table I and in Figure 3. In the six animals that received different doses of sensitized red cells, recovery of bilirubin-C^{14} ranged from 63 to 80 per cent. Comparable or slightly lower recovery of labeled bilirubin was obtained in rats E, F, and R

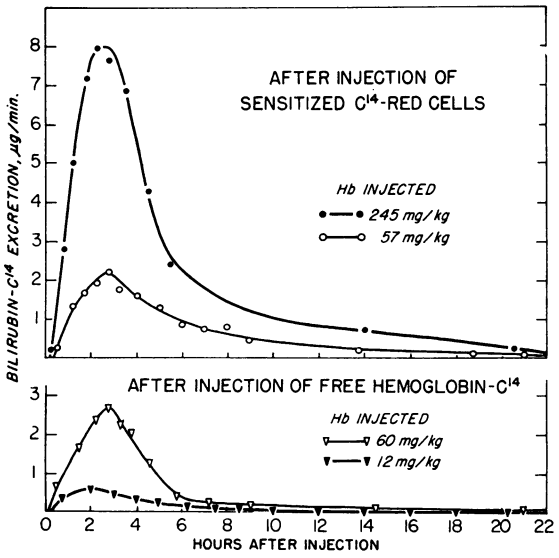

FIG. 2. ABSOLUTE RATE OF BILIRUBIN-C^{14} EXCRETION IN BILE AFTER ADMINISTRATION OF LABELED, SENSITIZED RED CELLS OR HEMOGLOBIN SOLUTION TO RATS.

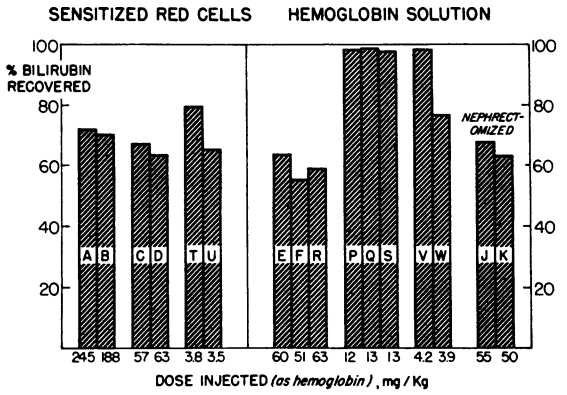

FIG. 3. RECOVERY OF BILIRUBIN-C^{14} IN RAT BILE AFTER ADMINISTRATION OF LABELED, SENSITIZED RED CELLS OR HEMOGLOBIN SOLUTION. Results are expressed in per cent of the amount of labeled bilirubin expected if all the administered heme had been converted to bilirubin. Each bar represents one animal, and the number below the bar indicates the dose of administered heme pigment expressed as milligrams of hemoglobin per kilogram of body weight.

(Figure 3), which were injected with an amount of hemoglobin in solution equivalent to that in the red cells given to rats C and D. Nephrectomy did not alter the recovery of bilirubin, as shown by animals J and K, which received the same dose of hemoglobin solution as rats E, F, and R (Figure 3). When smaller quantities of hemoglobin, calculated not to exceed the anticipated hemoglobin-binding capacity of rat plasma,[9] were administered, recovery of bilirubin-C^{14} in the bile of 4 out of 5 animals approached the values expected if all injected hemoglobin were quantitatively converted to bilirubin (rats P, Q, S, and V; Figure 3).

Organ uptake of labeled red cells and hemoglobin. Since recovery of Fe^{59} or Cr^{51} in the tissues of the recipient rats was determined only 22

[9] Haptoglobin levels in rat plasma were kindly determined by Dr. Eloise Giblett, Seattle, Wash. In two groups of normal rats, mean haptoglobin levels bound 34 and 45 mg hemoglobin per 100 ml plasma.

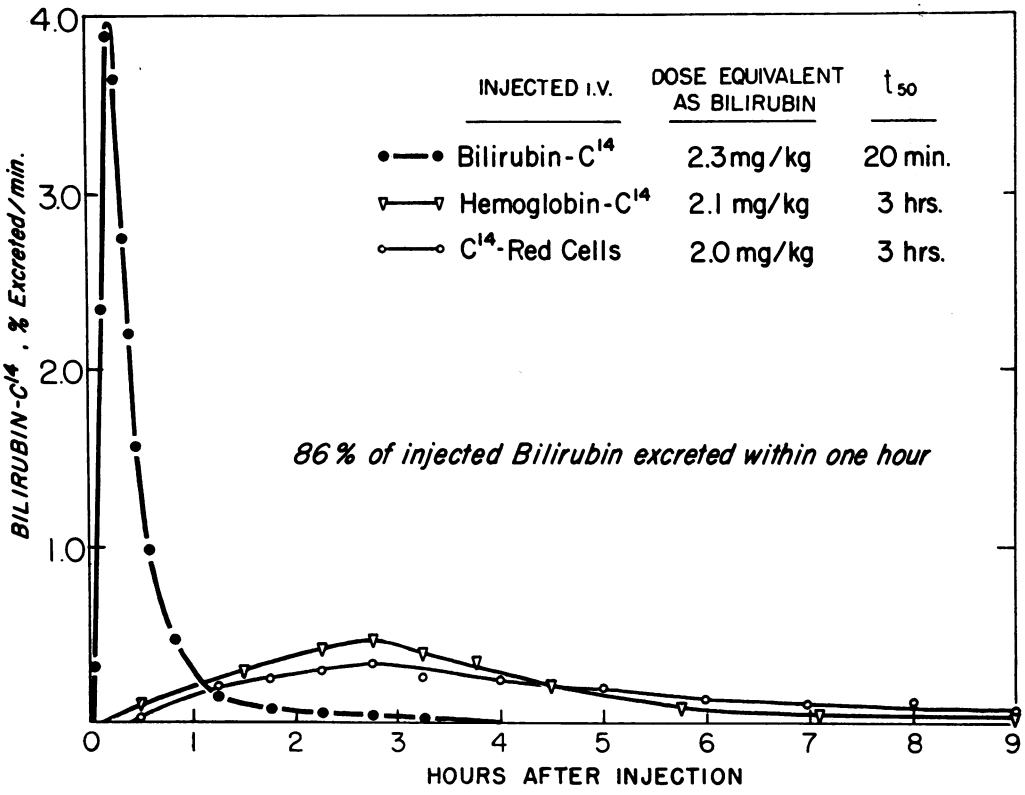

FIG. 4. EXCRETION OF LABELED BILIRUBIN IN RAT BILE AFTER INTRAVENOUS INJECTION OF EQUIVALENT DOSES OF UNCONJUGATED BILIRUBIN-C^{14}, HEMOGLOBIN-C^{14} IN SOLUTION, AND SENSITIZED C^{14}-LABELED RED CELLS. The excretory rate of bilirubin-C^{14} is expressed in per cent of the dose of pigment administered.

hours after injection of the labeled heme pigment, the data are of limited value and will not be given in detail. As expected (15, 29), virtually all sensitized red cells were sequestered in approximately equal proportions by spleen and liver, with no significant uptake of Cr^{51} or Fe^{59} by the kidneys nor significant excretion of radioactivity in the urine. After injection of comparable quantities of hemoglobin solution, clearance from the circulation also was virtually complete, but the tissue distribution of radioactivity was different (23, 30). Hepatic uptake greatly exceeded splenic uptake, which was never more than 3 per cent of the injected dose. Moreover, approximately half of the injected Fe^{59} or Cr^{51} was recovered in the kidneys and urine, suggesting that the total plasma hemoglobin-binding capacity may have been exceeded even with the smallest dose of hemoglobin administered (30, 31). However, estimation of urinary hemoglobin excretion, by a quantitative benzidine technique (32), revealed that less than 2 mg hemoglobin was lost in the urine, corresponding to less than 4 per cent of the largest dose of hemoglobin injected. Thus, most of the urinary Fe^{59} must have been excreted after splitting of the heme molecule, leaving the porphyrin moiety to be metabolized to bilirubin and excreted in the bile.

Related findings. After intravenous administration of protein-bound, unconjugated bilirubin-C^{14}, labeled pigment appeared in the bile within 3 minutes (Figure 4). Of the injected pigment, 50 per cent was excreted as conjugated bilirubin in 20 minutes, 86 per cent in 1 hour, and 96 per cent in 4 hours.

In the three rats that were not injected with exogenous hemoglobin or red cells (Figure 5), the rate of bilirubin excretion after bile duct catheterization first rose and then declined before leveling off at a constant rate after approximately 8 hours. Pentobarbital-ether anesthesia sharply reduced bile volume and pigment output (12, 33), followed by a rebound which lasted for 4 hours after cessation of ether administration. Injection of 20 mg pentobarbital per kg alone did not affect bile and pigment excretion.

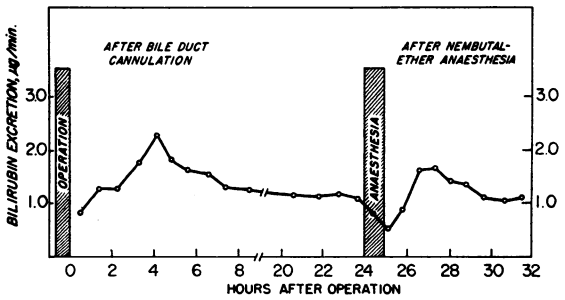

FIG. 5. RATES OF BILIRUBIN EXCRETION IN A 420 G MALE SPRAGUE-DAWLEY RAT AFTER BILE DUCT CATHETERIZATION UNDER PENTOBARBITAL-ETHER ANESTHESIA, AND DURING AND AFTER SUCH SUBSEQUENT ANESTHESIA.

In all rats intravenous injection of labeled red cells or hemoglobin solution was followed by a sharp rise and subsequent fall in the excretion of unlabeled endogenous bilirubin in rough proportion to the amount of administered heme. The magnitude of this effect exceeded the variations in bilirubin excretion which resulted from anesthesia alone. As outlined under Methods, this did not interfere with the calculations involved in the present experiments, since the C^{14}-label permitted identification of all bile pigment derived from the injected labeled heme.

DISCUSSION

In the present experiments rats with a bile fistula were given an intravenous injection of one of the following heme pigment preparations: *1*) suspensions of antibody-sensitized red cells which were primarily sequestered in the spleen and liver (15, 29); or *2*) various amounts of hemoglobin in solution, which were predominantly removed by the liver and the kidneys (23, 30, 31). In spite of these differences, the labeled heme pigment was cleared from the circulation and excreted as radioactive bilirubin in the bile at similar rates. This indicates that the antibody-sensitized erythrocytes were lysed almost immediately after their sequestration. It further suggests that the reticuloendothelial cells of the liver, the spleen, and perhaps the kidneys, are all capable of converting hemoglobin to bilirubin.

Although a small portion of the injected heme-C^{14} was converted to labeled bile pigment within 30 minutes (33, 34), a rapid, nearly constant rate of excretion of bilirubin-C^{14} was not achieved until approximately 50 minutes after the onset of sequestration of the administered hemoglobin or red cells (Figure 1). This delay in bile pigment formation is in agreement with previous observations in man, wherein serum levels of bilirubin did not

begin to rise until 1 hour after administration of sensitized red cells (29) or hemoglobin (35). For all doses of hemoglobin and red cells, the mean interval between removal of half the injected heme pigment from the circulation and the appearance of half the excreted bilirubin-C^{14} in the bile was approximately 3 hours (Table I). On the other hand, a comparable intravenous load of bilirubin-C^{14} was almost completely excreted within 1 hour, confirming prior reports (36–38). This indicates that the delay between heme sequestration and bilirubin excretion occurred in the reticuloendothelial system (39) after lysis of the red cells and before release of the bile pigment into the circulation.

This delay does not seem to be due to overload of the conversion mechanism, since widely different doses of heme pigment were converted to bilirubin at similar rates. Indeed, the mechanism for bilirubin formation did not appear to be saturated by a load of heme pigment equivalent to approximately 50 times the physiological turnover of rat red cells (25). Furthermore, the labeled red cells in any given injection were all generated within a 5-day period, and were metabolized to bile pigment at the same rate as a comparable dose of extracorpuscular hemoglobin. They thus behaved as a relatively uniform population of red cells, which was sequestered and metabolized in random fashion. Consequently, the observed lag may be considered consistent with the kinetics of a precursor-product relationship involving an intervening rate-limiting step (40), although the nature of these conversion processes is not yet clear (41).

Despite virtually complete removal from the circulation, 20 to 45 per cent of the administered heme-C^{14} was *not* recovered as bilirubin-C^{14} in the bile of those animals which received sensitized red cells or large doses of hemoglobin in solution (42, 43). Urinary loss of heme pigment never exceeded 4 per cent of the administered dose of dissolved hemoglobin, and was absent in nephrectomized animals and after injection of sensitized red cells. Moreover, less than 3 per cent of the administered Fe^{59} or Cr^{51} was excreted in the bile, and bilirubin was never detected in the urine. Therefore, urinary and biliary loss of heme pigment could not account for the significant discrepancy between the quantity of heme-C^{14} injected and the amount of bilirubin-C^{14} excreted.

It also appears unlikely that significant amounts of heme-C^{14}, or bilirubin derived therefrom, would be retained in the tissues of the recipient animal 22 hours after administration. However, this possibility could not be definitely excluded, since the C^{14} label was also present in the globin as well as in the heme of the injected red cells or hemoglobin. Thus, residual C^{14} activity in the tissues could not be assumed to represent retained heme or bile pigments. On the other hand, the virtual cessation of bilirubin-C^{14} excretion within 22 hours after injection of the labeled red cells or hemoglobin strongly suggests that the metabolism and elimination of the administered pigment had been completed within this period. Indeed, it seems improbable that labeled heme pigment or bilirubin should accumulate in the tissues at a time when excretion of unlabeled bilirubin, derived from endogenous red cells, continued unabated.

The failure to account for a significant fraction of the administered heme-C^{14} in these instances is most likely due to its conversion to excretory products other than bilirubin. Although earlier investigators have suggested that dipyrrolic compounds, such as mesobilifuscin (7, 44) and propentdyopent (44, 45), may be normally occurring excretory products of heme metabolism, recent findings have failed to confirm this concept (46). The present experiments constitute strong evidence for the existence of alternate pathways of heme catabolism, but they do not establish the identity of the compounds involved.

Intravenously injected bilirubin-C^{14} was almost completely recovered in the bile. This would indicate that the postulated alternate excretory products may only be derived from heme or perhaps from bilirubin within the reticuloendothelial system, but cannot be formed from bilirubin in the circulation. Moreover, the greatest percentage conversion to bilirubin was observed after the lowest doses of hemoglobin in solution. Under these conditions the pigment is believed to be removed from the circulation as a complex with haptoglobin (47). This suggests that the metabolic disposition of intracorpuscular hemoglobin and of unbound plasma hemoglobin may differ from that of the hemoglobin-haptoglobin complex.

After injection of labeled red cells, biliary excretion of *unlabeled* bile pigment was temporarily increased, possibly as a result of accelerated destruction of *endogenous* red cells by the recipient animal. Since this effect was also observed after administration of dissolved hemoglobin, it does not appear to be the result of the elution of antibody from the injected to the endogenous red cells, nor of indiscriminate trapping of host cells by an actively sequestering spleen. These observations complicate interpretation of earlier experiments in which increments in bile pigment excretion were assumed to reflect quantitatively catabolism of administered loads of heme pigment (42, 43, 48). The use of labeled hemoglobin obviates these difficulties, and permits direct comparison of the quantity of injected heme pigment with that of the excreted bile pigment.

SUMMARY

1. Rats with a bile fistula were injected with antibody-sensitized red cells labeled with C^{14} and with either Fe^{59} or Cr^{51}. Similar experiments were performed with equivalent doses of labeled hemoglobin in solution. In all instances the removal of the administered heme pigment from the circulation and the excretion of bilirubin-C^{14} in the bile were determined.

2. After clearance from the circulation, intra- and extracorpuscular hemoglobin were converted to bilirubin at similar rates. Labeled bilirubin appeared in the bile within 30 minutes after the onset of heme sequestration, and 50 per cent was excreted in approximately 3 hours.

3. A delay was observed between removal of heme pigment from the circulation and excretion of labeled bilirubin, which appeared to be due primarily to the time required for the conversion of heme to bilirubin in the reticuloendothelial system.

4. With sensitized red cells, only 63 to 80 per cent of the administered heme could be recovered as bilirubin-C^{14}. Similar bile pigment recovery was obtained after injection of relatively large doses of hemoglobin in solution. In contrast, after administration of small doses of hemoglobin, calculated not to exceed the binding capacity of plasma haptoglobin, conversion to bilirubin-C^{14} was nearly complete.

5. Possible explanations for this difference are discussed, and it is suggested that the heme not accounted for was converted to excretory products other than bilirubin.

ACKNOWLEDGMENT

The authors wish to acknowledge the continued interest and advice of Dr. William B. Castle and the expert technical assistance of Louise Engel, Lydia Hammaker, and Deanna Samuelson.

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