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Research Article

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Quantitative Studies of the Delivery of Hepatic-Synthesized Bilirubin to Plasma Utilizing δ-Aminolevulinic Acid-4-^{14}C and Bilirubin-^{3}H in Man

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ABSTRACT After the simultaneous intravenous administration of unconjugated bilirubin-^{3}H and δ-aminolevulinic acid-4-^{14}C, the plasma disappearance curves of unconjugated bilirubin-^{3}H and the plasma appearance curves of biosynthesized unconjugated bilirubin-^{14}C have been defined in seven patients, three of whom had acute intermittent porphyria (AIP). The incorporation of ^{14}C into plasma unconjugated bilirubin, derived by an analysis which involves deconvolution of the two plasma curves, varied between 13.1 and 23.5% (mean 19.3%) of the injected dose in the nonporphyric patients and between 5.4 and 13.6% (mean 8.3%) of the injected dose in the porphyric patients. In five of the patients, the stercobilin-^{14}C specific activity in a pooled specimen of feces was measured, enabling the following further values to be calculated: (*a*) the total ^{14}C radioactivity incorporated into bilirubin (21.0 and 25.3% [mean 23.2%] of the injected dose in two of the nonporphyric patients and between 8.5 and 25.3% [mean 14.2%] of the injected dose in the porphyric patients), and (*b*) the proportion of hepatic synthesized bilirubin delivered directly to plasma in the unconjugated form (between 0.520 and 0.904; mean for nonporphyric patients 0.712; mean for porphyric patients 0.614). The results demonstrate that a large proportion of bilirubin derived from hepatic hemes passes through the plasma in the unconjugated form before conjugation and secretion into bile.

INTRODUCTION

Unconjugated bilirubin, a product of heme catabolism, is extracted from plasma by the hepatic parenchymal cell, where it is conjugated and secreted into the bile. The presence of small but measurable quantities of conjugated bilirubin in normal human plasma is frequently cited as evidence that conjugated bilirubin may reflux from the liver to plasma. That unconjugated bilirubin may also pass from liver cell to plasma has been predicted from kinetic models of the metabolism of unconjugated bilirubin (1–3), and has been inferred from the analysis of multiple transhepatic indicator dilution studies (4).

The studies reported in this paper support the concept that passage of unconjugated bilirubin between liver and plasma is bidirectional. The studies demonstrate that a significant proportion of the bilirubin derived from the turnover of hepatic nonhemoglobin hemes appears in the plasma in the unconjugated form before conjugation and biliary excretion. The data also indicate differences in the incorporation of the label of δ-aminolevulinic acid-4-^{14}C (ALA-^{14}C)[1] into bilirubin

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[1] *Abbreviations used in this paper:* AIP, acute intermittent porphyria; ALA-^{14}C, δ-aminolevulinic acid-4-^{14}C; BRP, bilirubin production rate: DFP-^{3}H, diisopropylfluorophosphate-^{3}H; PBG, porphobilinogen; TRCV, total circulating red cell volume.

in porphyric, compared with nonporphyric, human subjects.

METHODS

Patients

All of the studies described were performed on patients admitted to the Metabolism Ward of the National Cancer Institute. Details of the seven patients studied are given in Table I. At the time of study, all were considered to be in a metabolically steady state, with particular reference to body weight, diet, and plasma unconjugated bilirubin concentration. The studies were conducted with the fully informed consent and cooperation of each patient.

Materials

Bilirubin-^3H. Bilirubin-^{3}H was prepared biosynthetically from δ-aminolevulinic acid-2,3-^{3}H in bile fistula dogs (5).

δ-Aminolevulinic acid-4-^{14}C. δ-Aminolevulinic acid-4-^{14}C, obtained from New England Nuclear Corp., Boston, Mass., was dispensed in ampoules containing 47 μCi (specific activity 37.4 mCi/mmole) in 5 ml N saline and stored at −20°C.

Design of studies

At zero time known volumes of bilirubin-^{3}H (15–30 μCi in 0.5 mg) dissolved in an alkaline albumin solution (2, 3) and δ-aminolevulinic acid-4-^{14}C (30–40 μCi) were injected simultaneously through the tubing of an intravenous infusion. Appropriate standard solutions for counting were made from portions of both of the injected solutions.

A base line blood specimen was taken immediately before the administration of the labeled ALA and bilirubin. Subsequent specimens were taken at intervals of approximately 10 min for the 1st hr, 15 min for the next 2 hr, and 20 min for the subsequent 2 hr. A further 4–8 specimens were taken at increasing intervals during the ensuing 36–72 hr.

In five of the seven patients, feces were collected in 24-hr lots for 6–10 days. None of the patients was constipated. The weight of each lot was measured and the specimen stored at −20°C.

In addition, the total red blood cell volume was measured in each subject using ^{51}Cr-labeled autologous erythrocytes and, in five of the seven patients, the survival $t\frac{1}{2}$ of ^{51}Cr-labeled autologous red cells was also determined (6, 7) (Table I).

Laboratory procedures

Daily urinary excretion of porphobilinogen (PBG) (milligrams/day). In the three patients with acute intermittent porphyria (AIP) daily urinary PBG excretion was measured by the method of Mauzerall and Granick (8) (Table I).

Plasma unconjugated bilirubin-^3H and bilirubin-^{14}C radioactivities (disintegrations per minute/milliliter plasma). Plasma specimens were processed by the methods previously described (2, 3). Portions of the lower layer of the Weber and Schalm (9) plasma separation were counted for both ^{3}H and ^{14}C radioactivity, and the derived figures for plasma unconjugated bilirubin-^{3}H and bilirubin-^{14}C radioactivities were expressed as disintegrations per minute/milliliter plasma.

Plasma unconjugated bilirubin concentration (milligrams/100 ml). At least 10 determinations of the plasma unconjugated bilirubin concentration were made during each study by the method of Weber and Schalm (9) and the mean plasma unconjugated bilirubin concentration determined. Standard errors averaged ±7.3% of the mean values (range 6.2–9.3%).

Fecal stercobilin-^{14}C specific activity (disintegrations per minute/milligram stercobilin). Each individual stool specimen was homogenized and 10% portions pooled. Stercobilin was crystalized from the pooled homogenate by the method of Watson et al. (10) and recrystallized until constant specific activity was obtained. Crystals obtained had optical rotations which varied between α_D −2800 and −4000. Portions of the crystals were weighed, dissolved in chloroform, bleached under ultraviolet light, dried under a nitrogen stream, dissolved directly into a liquid scintillation counting solution, and counted for ^{14}C.

Measurement of ^3H and ^{14}C radioactivity. All counting was carried out in a three channel liquid scintillation coun-

TABLE I

Patients Studied

Patient	Diagnosis	Age	Sex	Weight	Hematocrit	Reticulocytes	Total red cell volume	^{51}Cr red cell half-life	Urinary porphobilinogen
		yr		*kg*	%	%	*ml/kg*	*days*	*mg/day**
1	Obesity	19	M	82.9	46.5	0.6	28.5	31	—
2	Myotonic dystrophy	37	M	77.1	44	—	22.6	28	—
3	Red cell hypoplasia Diabetes mellitus	40	M	56.3	35‡	0.0	21.6‡	15	—
4	Red cell hypoplasia	24	M	67.6	35‡	0.5	15.8‡	19	—
5	AIP	42	M	87.8	46.9	1.6	20.8	27	16
6	AIP	23	F	58.1	47.7	0.3	19.2	—	93
7	AIP	27	M	59.6	37.3	1.2	28.2	—	116

* Mean of days 1–4 inclusive.

‡ After blood transfusion.

ter.[2] Channel A was peaked for ^{3}H and channel C for ^{14}C excluding ^{3}H. The counting fluid used was Turner's solution (toluene 700 ml, methanol 300 ml, 2,5-diphenyloxazole 1 g, *p-bis*[2-(5-phenyloxozolyl)]-benzene 0.1 g). Counting was continued until a minimum of 10,000 counts had been collected in appropriate channels. All samples were recounted after the addition of ^{14}C internal standard and, in the case of plasma samples, counted further after the addition of ^{3}H internal standard to enable appropriate corrections for quenching to be made.

Validity of methods

Because the measurement of plasma unconjugated bilirubin-^{14}C radioactivity was made in plasma samples, at least some of which could contain appreciable ^{14}C radioactivity in the form of ALA-4-^{14}C and/or other compounds besides unconjugated bilirubin, the following further experiments were conducted to test the validity of the method used.

(*a*) When PBG-^{14}C (obtained by applying the isolation procedure of Cookson and Rimington [11] to the urine of a patient with AIP who had received δ-ALA-^{14}C intravenously) or δ-ALA-^{14}C were added to plasma samples, which were then subjected to the Weber and Schalm (9) separation, the lower (unconjugated bilirubin) layer contained less than 0.01% of the ^{14}C added as ALA and less than 0.03% of the ^{14}C added as PBG. Similarly, the ^{14}C radioactivity found in the lower layer of a Weber and Schalm extraction of a plasma sample obtained 10 min after the intravenous administration of δ-ALA-^{14}C to a patient was indistinguishable from background.

(*b*) Hemin-^{14}C, of specific activity 8500 dpm/mg, was isolated from the blood of a rat 10 days after the intravenous administration of 100 μCi glycine-2-^{14}C by the method of Labbe and Nishida (12). Protoporphyrin-^{14}C was prepared and purified from this material by the method of Morell and Stewart (13). This protoporphyrin-^{14}C was added to plasma, which was then partitioned by the method of Weber and Schalm (9). An average of 98.4% of the protoporphyrin-^{14}C was recovered in the polar layer, and in no case was more than 1.9% recovered in the nonpolar layer which contains unconjugated bilirubin. Although the partitioning of copro- and uroporphyrin in the Weber-Schalm system was not tested directly, these substances are more polar and less soluble in chloroform than protoporphyrin as a consequence of the greater number of carboxyl groups in these molecules (14). Further, these two substances are known to be readily extracted into acidified ethyl acetate (14), which constitutes the polar layer of the Weber-Schalm system. Hence, it is likely that the recovery of copro- and uroporphyrin in the lower layer of the Weber-Schalm system would be even lower than that of protoporphyrin.

(*c*) When stercobilin-^{14}C, prepared by the method of Watson et al. (10), was added to plasma and the plasma partitioned by the Weber and Schalm method (9), 8.7% was recovered in the lower layer. Thus, contamination of the lower layer with labeled stercobilin is a potential source of error in the estimation of plasma unconjugated bilirubin-^{14}C radioactivity used in these studies. However, in subjects with normal liver function the plasma concentration of urobilin averaged 2.3±1.7 (SD) μg/100 ml (15), or less than 1% of the plasma bilirubin concentration observed in any of our patients. Furthermore, formation of stercobilin-^{14}C in the current studies would require the conversion of administered ALA-^{14}C to labeled heme, catabolism of this heme to bilirubin-^{14}C, secretion of the labeled bilirubin into the bile, its subsequent passage to the distal ileum and colon, and its reduction at these sites by bacterial action to stercobilin-^{14}C. This implies a considerable time lag between the injection of ALA-^{14}C and the availability of appreciable quantities of labeled stercobilin for intestinal absorption. There was virtually no input to plasma of ^{14}C radioactivity in a chemical form extractable into the lower layer of the Weber-Schalm system between 24 and 72 hr, the time period when maximal quantities of labeled stercobilin would be available within the gut (3). Hence, it is unlikely that any appreciable quantity of the ^{14}C radioactivity found in the lower layer of the Weber and Schalm (9) separation of plasma samples, taken during the initial 24 hr of a study, would be due to stercobilin-^{14}C.

(*d*) In three additional patients a total of seven plasma samples were taken between 1 and 3 hr after the intravenous injection of δ-ALA-4-^{14}C. The plasma unconjugated bilirubin-^{14}C specific activity of these samples was determined by both (i) the method of Barrett, Berk, Menken, and Berlin (2) as modified by Berk, Howe, Bloomer, and Berlin (3) used in these studies and (ii) from bilirubin crystallized from the lower layer of the Weber and Schalm (9) plasma separation with the addition of cold carrier bilirubin. The ratio of the specific activities obtained using method i to those using method ii averaged 0.96±0.03 (mean ±SEM). These ratios compare favorably with other comparable data in the literature (16–18).

(*e*) In patient 1 (an individual with normal hematological findings, hospitalized for management of obesity), hemin was crystallized by the method of Labbe and Nishida (12) from five blood samples obtained between 7 and 14 days after injection of ALA-^{14}C, plated at infinite thinness on aluminium planchets, and counted in a thin window low background counter.[3] In none of the samples were counts obtained which were significantly different from background. These data confirm earlier observations (17, 19–24) that when δ-aminolevulinic acid-4-^{14}C is used to label bilirubin biosynthetically, the incorporation of the label into red cell heme is exceedingly small. Thus it could be assumed that the label of ALA-^{14}C appearing in bilirubin is not derived from erythroid sources.

CALCULATIONS

The proportion of ^{14}C radioactivity, administered as ALA-4-^{14}C, incorporated into plasma unconjugated bilirubin. The experimental measurements of the plasma unconjugated bilirubin-^{3}H radioactivity-time curve (G(t) dpm/ml, see Fig. 1*A*) were fitted to three exponential functions by a technique which minimizes the root mean square error (25, 26). The relationship between G(t) and the plasma radioactivity time curve of unconjugated bilirubin-^{14}C, biosynthesized from ALA-^{14}C (X(t) dpm/ml, see Fig. 1*A*) is given by the following equation:

$$X(t) = \frac{1}{G(0)} \int_0^t M(t - T)G(T)dT, \qquad (1)$$

where M(t) = rate of entry to the plasma of unconjugated bilirubin-^{14}C, newly synthesized from hepatic hemes (disintegrations per minute/milliliter per minute).

[2] Nuclear-Chicago Corporation, Des Plaines, Ill.

[3] Sharp Laboratories, La Jolla, Calif.

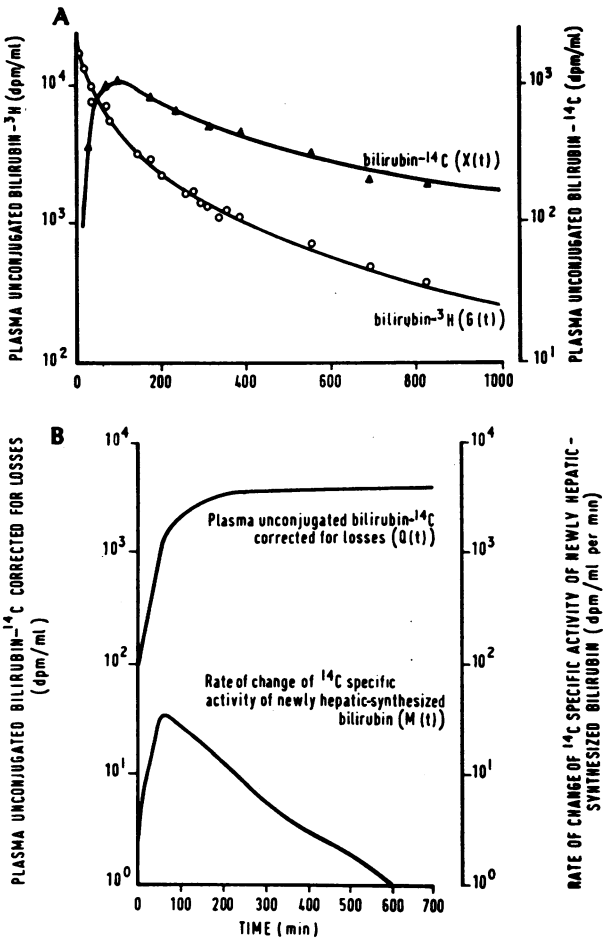

FIGURE 1 *A*. Plasma curves of unconjugated bilirubin-^{3}H (G(t)) and bilirubin-^{14}C (X(t)) radioactivity after the simultaneous injection of bilirubin-^{3}H and δ-ALA-4-^{14}C. *B*. The rate of input to the plasma of bilirubin-^{14}C, newly biosynthesized from hepatic hemes (M(t)), derived by deconvoluting the two curves in *A*, and the plasma unconjugated bilirubin-^{14}C radioactivity curve corrected for losses of unconjugated bilirubin-^{14}C from the plasma due to distribution and hepatic uptake (Q(t)), derived by integrating M(t) with respect to time.

After expressing equation 1 in discrete notation, the function M(t) was derived by deconvolution of individual experimental estimates of X(t), using a step size of 1 min, on an IBM 360/60 digital computer[4] (Fig. 1*B*). This procedure is analogous in principle to that described previously by Robinson and coworkers (*22*, *23*), in their analysis of plasma labeled bilirubin curves in Gunn rats and a patient with the Crigler-Najjar syndrome. An important difference between our calculations and those of Robinson and coworkers (*22*, *23*) arises from fundamental differences in bilirubin metabolism in the subjects studied. In the particular jaundiced animals and patient studied by Robinson's group, very slow excretion of labeled bilirubin led them to describe a weighting function, analogous to G(t), by a single exponential function. The seven patients described in this report had normal rates of disappearance of labeled bilirubin from the plasma and hence, to describe adequately G(t), it was necessary to fit experimental values of this function to three exponentials (3).

Integration of M(t) with respect to time, also employing an IBM 360/60 digital computer, gave a hypothetical plasma unconjugated bilirubin-^{14}C radioactivity-time curve (Q(t) dpm/ml) (Fig. 1*B*), which represents the pattern of accumulation of ^{14}C-labeled unconjugated bilirubin in the plasma that would have occurred in the absence of hepatic uptake and extrahepatic extravascular distribution of bilirubin. In all studies the function M(t) had declined to virtually zero by 1000 min and hence, values for Q(1000) could be taken as valid approximations for Q(∞). This derivation of the functions M(t) and Q(t) is independent of any kinetic model of unconjugated bilirubin metabolism. Let:

(*a*) ^3H_b = injected dose of unconjugated bilirubin-^{3}H (dpm),

so that

$\frac{^3H_b}{G(0)}$ = initial volume of distribution of unconjugated bilirubin (ml);

(*b*) $^{14}C_a$ = injected dose of ALA-4-^{14}C (dpm);

(*c*) $^{14}C_b$ = total ^{14}C radioactivity incorporated into plasma unconjugated bilirubin (dpm).

Then

$$^{14}C_b = Q(\infty) \times \frac{^3H_b}{G(0)}, \qquad (2a)$$

and

$$\frac{^{14}C_b}{^{14}C_a} = \frac{Q(\infty)}{^{14}C_a} \times \frac{^3H_b}{G(0)} = \text{proportion of the label of administered ALA-}^{14}\text{C incorporated into plasma unconjugated bilirubin.} \qquad (2b)$$

The proportion of bilirubin, synthesized from hepatic hemes, delivered directly to plasma in the unconjugated form. This proportion is given by the ratio of the total ^{14}C radioactivity incorporated into plasma unconjugated bilirubin (see equation 2a) to the total ^{14}C radioactivity incorporated into bilirubin ($R_b(t_o - t_\infty)$). One method of measuring the total radioactivity incorporated into bilirubin necessitates determining the specific activity of any one representative metabolic product of bilirubin. Stercobilin is a suitable substance in this context. Although the specific activity must be determined from samples collected for a sufficient period to ensure complete excretion of the labeled product, it is not necessary that the methods employed provide complete recovery of the product, or even accurately quantitate the amount of product excreted. Since measurements of total ^{14}C radioactivity indicated that recovery was virtually complete by the end of the period of stool collection, then

$$R_b(t_o - t_\infty) = N \times M_B \times \bar{s} \times \frac{594}{584.7}, \qquad (3)$$

[4] International Business Machines Corp., Armonk, N. Y.

where N = number of days of stool collection, M_B = total synthesis of bilirubin (milligrams per day), $\bar{s}$ = ^{14}C specific activity of stercobilin in the pooled stool specimen, 594 = mean molecular weight of stercobilin (27), 584.7 = molecular weight of bilirubin (27). Values for M_B were derived from the mean plasma unconjugated bilirubin concentration and the experimental estimates of G(t), employing the methods of curve fitting and calculation described by Berman and Weiss (28) and Berk et al. (3), respectively. These methods do not depend on any kinetic model of unconjugated bilirubin metabolism, but do incorporate the well established principles of the plasma curve integral method of Nosslin (29) for estimating the fractional turnover rate of substances in plasma.

It follows that

$$\frac{^{14}C_b}{R_b(t_0 - t_\infty)} = \frac{Q(\infty)}{N \times M_B \times \bar{s}} \times \frac{^{3}H_b}{G(0)} \times \frac{584.7}{594}. \quad (4)$$

RESULTS

The principal experimental results are summarized in Table II. The mean plasma concentration of unconjugated bilirubin was normal in each instance. The total bilirubin production rate (BRP) varied betwen 202 and 309 mg/day (3.14 — 3.73 mg/kg per day) in the nonporphyric subjects (numbers 1–4) and between 148 and 377 mg/day (2.55 — 4.29 mg/kg per day) in the three patients with acute intermittent porphyria (numbers 5–7). The total bilirubin production rate in each individual is within the range previously defined for normal volunteers (3). However bilirubin production rates were not always within the normal range when expressed in terms of total circulating red cell volume (BRP/TRCV). In 22 normal volunteers the ratio BRP/TRCV was 0.14±0.02 (mean±SD) mg bilirubin/ml RBC per day (30). In a previous study in patients with acute intermittent porphyria, alterations in total red cell volume and bilirubin production occurred in parallel, so that the ratio BRP/TRCV ranged from 0.12 to 0.16 (31). In the current study, the BRP/TRCV ratio was normal in two nonporphyric patients (numbers 1 and 2) and in two patients with porphyria (numbers 6 and 7). The ratio was elevated in patients 3–5, implying an increased rate of bilirubin production relative to the observed red cell mass. In patients 3 and 4, this could be attributed to hemolysis, as indicated by a reduction in the $t_{\frac{1}{2}}$ of ^{51}Cr-labeled erythrocytes.

In patient 5, with acute intermittent porphyria, the molar production of carbon monoxide, measured by a modification of the method of Coburn, Blakemore, and Forster (32)[5] was identical to that of bilirubin, confirming that this individual had an increased rate of heme turnover. Hemolysis was excluded by the repeated finding, by several techniques (^{51}Cr, DFP-^{3}H, glycine-2-^{14}C) of normal red cell survival. Consequently the possibility of increased hepatic production of bilirubin was suggested in this case and indeed hepatic bilirubin production in this patient has been estimated to be twice that for patients 6 and 7, as reported elsewhere (33). Of nine patients with acute intermittent porphyria in whom measurements of bilirubin production and red cell volume have been made, patient 5 is the only one in whom an increase in the BRP/TRCV ratio has been observed. This may reflect the chronic use by this patient of chloral hydrate, a known inducer of hepatic microsomal enzymes (34). A number of other unusual features in this patient have previously been reported (35).

The incorporation of ^{14}C from ALA-4-^{14}C into plasma unconjugated bilirubin varied from 13.1 to 23.5% (mean 19.3%) of the injected dose in the four nonporphyric patients. It was 5.4 and 5.8% of the dose in the two typical porphyrics (numbers 6 and 7) and 13.6% in patient 5.

The peak plasma bilirubin-^{14}C specific activity after the administration of ALA-^{14}C was 3 times as high in the four nonporphyric subjects (mean: 6.9 dpm/μg plasma unconjugated bilirubin per μCi ALA-^{14}C administered: range 3.5–10.0) as in the three porphyric patients (mean: 2.1 dpm/μg per μCi, range 1.0–3.1).

The total incorporation of ^{14}C into bilirubin was 21.0 and 25.3% (mean 23.2%) of the injected dose in the two nonporphyric patients in whom complete stool collection made this calculation possible. These values, which depend on the accuracy of our determinations of total bilirubin production, are in very satisfactory agreement with measurements of the incorporation of ALA-^{14}C into bile bilirubin in dogs (36) and rats (37) with external biliary fistulae and into fecal stercobilin in human subjects (38). In the two typical porphyric patients, total incorporation of ALA-^{14}C into bilirubin was markedly reduced (8.5 and 8.7%). In patient 5, the value was 25.3%.

In the first 24 hr 31–56% (mean 47%) of the label administered as ALA-^{14}C was recovered in the urine of the four nonporphyric patients, compared with 39–43% (mean 41%) in the urine of the three porphyric patients.

Of the total ^{14}C radioactivity incorporated into bilirubin after the administration of ALA-^{14}C, 52.0–90.4% appeared in the plasma as unconjugated bilirubin before its ultimate excretion in the bile. The mean value for this proportion was similar in the porphyric (mean 61.4%) and nonporphyric (mean 71.2%) patients.

DISCUSSION

A fundamental assumption inherent in the present studies is that both the administered unconjugated bilirubin-^{3}H and ALA-4-^{14}C behave in the body as true metabolic tracers. Whereas the mass of labeled unconjugated bilirubin administered was clearly small in relation to the

[5] Determined by Dr. F. Lee Rodkey, Naval Medical Research Institute, Bethesda, Md.

TABLE II

The Incorporation of ^{14}C, Administered as δ-Aminolevulinic Acid-4-^{14}C, into Bilirubin

Patient	Plasma unconjugated bilirubin concentration	Bilirubin synthetic rate		BRP/TRCV	Duration of stool collection	Fecal stercobilin specific activity	Incorporation of ^{14}C into bilirubin (proportion of injected dose)		Proportion of hepatic synthesized bilirubin appearing as plasma unconjugated bilirubin
							Plasma unconjugated bilirubin	Total	
	mg/100 ml	*mg/day*	*mg/kg per day*	*mg bilirubin/ml RBC per day*	*days*	*dpm/mg*	$^{14}C_b/^{14}C_a$	$R_b(t_0 - t_\infty)/^{14}C_a$	$^{14}C_b/R_b(t_0 - t_\infty)$
1	0.54	309	3.73	0.13	10	2355	0.131	0.253	0.520
2	0.64	242	3.14	0.14	7	10013	0.189	0.210	0.904
3	0.26	202	3.59	0.17	—	—	0.218	—	—
4	0.41	224	3.31	0.21	—	—	0.235	—	—
5	0.44	377	4.29	0.21	6	8416	0.136	0.253	0.535
6	0.25	148	2.55	0.13	10	4831	0.058	0.087	0.668
7	0.35	202	3.39	0.12	10	3250	0.054	0.085	0.639

BRP/TRCV, ratio of bilirubin synthetic rate to total red cell volume; $^{14}C_a$, injected dose of δ-ALA-4-^{14}C (dpm); $^{14}C_b$, total ^{14}C radioactivity incorporated into plasma unconjugated bilirubin (dpm); $R_b(t_0 - t_\infty)$, total ^{14}C radioactivity incorporated into bilirubin (dpm).

in vivo pool of unconjugated bilirubin, it is less clear that the mass of labeled ALA administered is also small in relation to the hepatic pool of ALA. No measurements of the hepatic pool of ALA in man are available. However, it has been estimated that the liver synthesizes about 1 μmole of ALA per min in normal subjects, and presumably a greater quantity in patients with acute intermittent porphyria, in whom levels of ALA-synthetase are demonstrably increased (39, 40). The total dose of ALA-^{14}C administered in each study was approximately 1 μmole. As a result of distribution and circulation, the injected material was presumably delivered to the liver over a finite time interval rather than as an instantaneous pulse. Furthermore a proportion of the dose would presumably be excreted in urine without prior passage through the liver. Thus, it would seem reasonable to conclude that the doses of ALA-4-^{14}C administered in this study were small enough to behave as true tracers. Consequently, it would not be expected that the doses of ALA injected would result in any detectable qualitative or quantitative changes in heme or bilirubin biosynthesis.

A second assumption of the current studies is that virtually all of the labeled bilirubin which is formed from injected ALA-^{14}C is of hepatic origin. While ALA is presumably an intermediate in the biosynthesis of heme in all cells, the membrane of the immature red blood cell is virtually impermeable to this precursor (17, 19–24). Thus, while approximately 25% of the label of ALA is incorporated into bilirubin, incorporation of this label into red cell heme varies from 0.00 to 0.24% of the administered dose in both nonporphyric and porphyric subjects (17, 20, 23, 41). Thus as an absolute maximum, less than 1% of the labeled bilirubin recovered after the administration of labeled ALA may be of red cell origin, and the studies cited (17, 20, 23, 41) suggest that an even smaller figure is usually the rule. Furthermore, although the administration of labeled ALA results in the labeling of nonhemoglobin heme proteins in tissues other than the liver (24, 42, 43), there is no evidence that these extrahepatic heme proteins given rise to bilirubin under physiologic conditions in vivo (37, 44). After injection of ALA-4-^{14}C into the bile fistula dog, liver hemes alone contained enough ^{14}C radioactivity to account for all of the early-labeled bilirubin ^{14}C subsequently recovered in the bile (42). Furthermore, after the administration of ALA-4-^{14}C the rate and magnitude of incorporation of ^{14}C into labeled bilirubin by the isolated, perfused rat liver is similar to that observed in whole animals (44). Thus, the available data support the assumption that the bilirubin-^{14}C produced in the current studies is of hepatic origin.

The principal finding of the current studies—that a large proportion of the bilirubin-^{14}C formed in the liver from ALA-^{14}C appears as unconjugated bilirubin in the plasma before its ultimate biliary excretion—indicates that the flux of unconjugated bilirubin between liver and plasma is bidirectional. Incorporation of ^{14}C, administered as ALA-^{14}C, into plasma bilirubin has been demonstrated previously by Yamamoto, Skanderbeg, Zipursky, and Israels (17) and Dowdle, Mustard, Spong, and Eales (41), but their reports did not specify whether the label was in unconjugated or conjugated bilirubin. The ^{14}C-labeling of plasma bilirubin after the administration of ALA-^{14}C to a patient with the Crigler-Najjar syndrome

(23) and to Gunn rats (22) indicates that in the absence of bilirubin conjugation, unconjugated bilirubin passes from the liver to plasma. The results of the present studies indicate that even when conjugation of bilirubin is normal, there is appreciable passage of unconjugated bilirubin from liver to plasma. This result had been predicted from previous analyses of kinetic models of unconjugated bilirubin metabolism in man (1–3), and from the analysis of multiple transhepatic indicator dilution studies in the dog (4). Bidirectional passage of other organic anions, such as sulfobromophthalein between liver and plasma has also been demonstrated (45).

Since hepatic bilirubin production may be increased by certain drugs (46) and in experimental liver injury (47), the current studies indicate that increased hepatic production of bilirubin may be a cause of, or contribute to, unconjugated hyperbilirubinemia. Whether this occurs in human disease is unknown. It is known, however, that the administration of many of the drugs, which appear to increase hepatic bilirubin production, is also associated with a reduction in the plasma bilirubin concentration due to the simultaneous acceleration of hepatic bilirubin clearance (48, 49).

Incidental observations in the present studies are that patients with typical acute intermittent porphyria incorporated only one-third as much of the label of ALA-^{14}C into bilirubin as do nonporphyric subjects (8.6 vs. 23%), and that the peak specific activity of plasma bilirubin-^{14}C was appreciably lower in porphyric than in nonprophyric patients in relation to the injected dose of ALA-^{14}C. There are several possible explanations for these findings. First, in view of the large excretion of ALA and PBG in the urine of porphyric patients, it is possible that a large proportion of the injected isotope is lost in the urine, resulting in a lower "effective dose" of label to the hepatic precursor pool. Although the data are inconclusive, neither our own observations nor those of Dowdle et al. (41), indicate any significant differences between porphyrics and nonporphyrics with respect to the amount of isotope excreted in the urine in the 24 hr after ALA-^{14}C administration. Second, it is possible that in porphyric patients there is greater incorporation of hepatic ALA and PBG into pathways which do not result in bilirubin formation. For example, in drug-induced experimental porphyria in the rat, after administration of labeled heme precursors relatively more of the label appears in early labeled carbon monoxide than in early labeled bilirubin (50). There are inadequate data to evaluate this possibility in human porphyria. Finally, it is possible that the observations reflect greater dilution of the administered label in an increased endogenous hepatic pool of ALA or PBG, as suggested by Dowdle et al. (41). In patients with acute intermittent porphyria, increased levels of ALA synthetase (39, 40) would be expected to result in increased hepatic pools of heme precursors. Under these circumstances, the decreased incorporation of the label of injected ALA-^{14}C into bilirubin may be a direct consequence of the reduced levels of PBG deaminase. This need not imply a reduction of hepatic bilirubin synthesis, since the bilirubin formed in porphyric livers, with their larger endogenous precursor pools, would be of lower specific activity. Indeed, previous studies of total bilirubin formation, red cell volume and red cell life-span (31), and of the formation of "early labeled" bilirubin (30) in patients with AIP lead to the conclusion that hepatic bilirubin production is not appreciably different in porphyrics than in normals. This being so, values for hepatic bilirubin production determined in patients with acute intermittent porphyria (33) may be similar to those for normal subjects. It may well be that the total incorporation of ^{14}C into bilirubin, after administration of ALA-4-^{14}C to patients with various conditions, including porphyria, may be inversely proportional to the size of the hepatic ALA and PBG pools and directly proportional to the levels of hepatic PBG deaminase.

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