Catabolism of Heme In Vivo: Comparison of the Simultaneous Production of Bilirubin and Carbon Monoxide

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ABSTRACT The quantitative relationship between the catabolism of heme and the formation of bilirubin and carbon monoxide (CO) was studied in untreated rats and in animals treated with phenobarbital or the porphyrogenic drug, allylisopropylacetamide (AIA). A novel metabolic chamber permitting continuous collection of the bile and breath was utilized for quantitation of bilirubin-^{14}C and ^{14}CO after the administration of hematin-^{14}C or glycine-^{14}C.

After intravenous infusion of hematin-^{14}C, control and phenobarbital-treated rats produced equimolar amounts of labeled bilirubin and CO; a minor fraction of the infused radioactivity appeared in the bile in other metabolites. The equimolar relationship in the formation of bilirubin and CO was also observed after pulse-labeling with glycine-2-^{14}C; in phenobarbital-treated rats both metabolites were formed at an increased rate as compared to controls. By contrast, AIA treatment reduced the fractional conversion of hematin-^{14}C to bilirubin and CO; a major fraction of the infused radioactivity appeared in the bile in metabolites other than bilirubin. In addition, in AIA-treated animals the molar CO/bilirubin recovery ratio was consistently greater than 1.0. Comparable results were obtained in AIA-treated rats after pulse-labeling with glycine-2-^{14}C.

These findings suggest that (*a*) in control and phenobarbital-treated rats infused hematin and heme formed in the liver are converted predominantly to bilirubin and CO, appearing in equimolar amounts; only a minor fraction of the hematin is degraded to other metabolites; (*b*) treatment with phenobarbital results in a proportional increase in the formation of both bilirubin and CO, reflecting increased heme synthesis and degradation in the liver; and (*c*) treatment with the porphyrogenic drug AIA shifts the CO/bilirubin ratio in favor of the gas, and enhances the formation of nonbilirubin metabolites.

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INTRODUCTION

Recent evidence indicates that catabolism of hemoglobin heme in vivo is associated with the formation of carbon monoxide (CO) (2–4). It appears most likely that in mammals this gas originates solely from the oxidized α-methene bridge carbon atom of heme (5), since this is the position at which the ferriprotoporphyrin ring is cleaved by the microsomal heme oxygenase (6). This implies that for each mole of heme degraded, 1 mole of CO and 1 mole of bilirubin are formed. Supportive evidence for such a quantitative conversion was deduced from studies in man (7, 8) and in animals (9, 10). It is pertinent, however, that in a number of recent experimental studies using isotopic techniques, the yield of bilirubin and of CO appears to fall short of the amount expected on the basis of stoichiometric calculations (11–13). These and other findings (14, 15) suggested the possibility that part of the heme degraded in vivo may be converted to metabolites other than bilirubin and CO. The most direct experimental approach to this problem would be a search for these suspected alternate metabolites of heme. Unfortunately, this approach is not feasible at present because of lack of methods for the isolation and exact identification of the various pyrrolic compounds that may be formed in such degradative pathways (16). Therefore, the following more indirect approaches were chosen: first, in experimental animals given a measured dose of isotopically labeled hematin intravenously, the rate of formation of radioactive bilirubin and CO was quantitated and the total amount of these labeled products was compared with the total recovery of isotopic tracer in the bile. This permitted determination of the metabolic fate of the administered hematin. Second, endogenous heme com-

pounds were pulse-labeled with glycine-2-^{14}C. Subsequent metabolism of this tagged heme resulted in production of labeled bilirubin (17) and CO (18). Isotopic bile pigment formed during the initial few hours after such pulse-labeling largely reflects turnover of heme in the liver (19). It is thus possible to compare hepatic heme metabolism in normal animals with that in conditions in which heme synthesis in the liver is enhanced, as occurs, for example, in animals treated with phenobarbital or the porphyrogenic drug, allylisopropylacetamide (AIA) (20, 21).

EXPERIMENTAL PLAN

The experiments to be described were carried out in rats with an external bile fistula, housed in a novel metabolic cage (Fig. 1) which permitted continuous collection of the exhaled ^{14}CO and $^{14}CO_2$. The animals were injected with a single dose of glycine-2-^{14}C, hematin-^{14}C, or bilirubin-^{14}C, the latter two being labeled in the methene bridge carbon atoms (22). Formation of ^{14}CO was also assayed in an experimental animal given glycine-1-^{14}C, which is not a specific precursor of the α-methene bridge carbon atom of ferriprotoporphyrin (22).

Fig. 2 graphically outlines the experimental protocol. The day of injection of the labeled material (hematin, glycine, or bilirubin) was arbitrarily designated as day zero. Beginning on day −4 and throughout the experiment, the rats were fasted, but had free access to water. From day −3 to day 2 inclusively, the animals were given daily subcutaneous injections of 300 mg/kg of AIA,[1] or 80 mg/kg

[1] Kindly supplied by Hoffman-La Roche, Inc., Nutley, N. J.

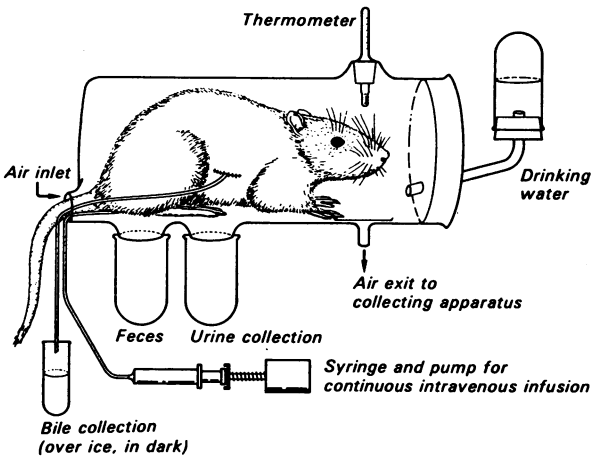

FIGURE 1 Diagram of metabolism chamber and collecting apparatus for continuous intravenous infusion and quantitative collection of urine, feces, bile, and expired air in individual rats.

of phenobarbital; these substances were dissolved in isotonic saline with a total injected volume of 2 ml/100 g body weight. A third group of rats (control group) received only a daily injection of saline of the same volume.

On day −1, the animals were anesthetized with ether and the bile duct and a femoral vein were cannulated. For the remainder of the experiment, hydration was maintained by continuous intravenous infusion of a solution containing 0.9% NaCl and 0.09% KCl given at the rate of 1 ml/hr. The rats were placed in a restraining cage (Aerospace Industries, Inc., Garnerville, N. Y.) permitting uninterrupted collection of bile. On day zero, immediately before the administration

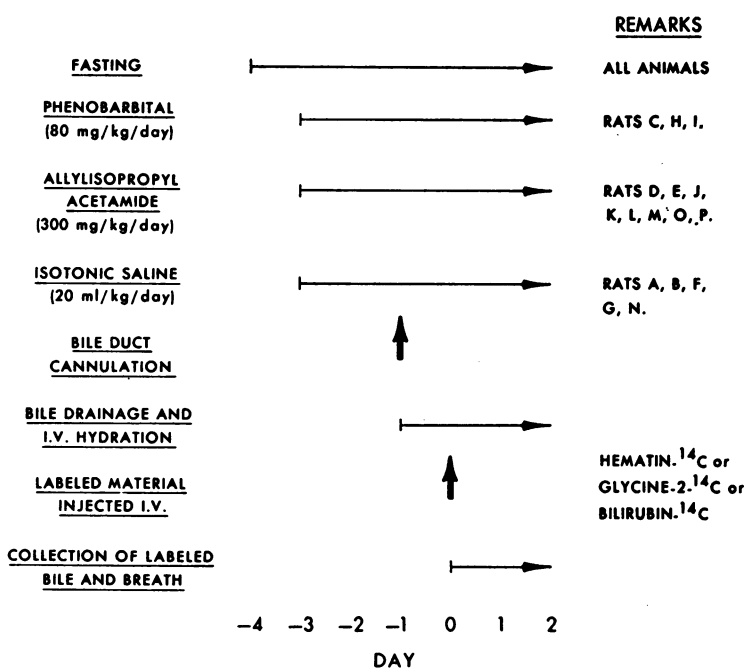

FIGURE 2 Experimental protocol (see text for details).

TABLE I

Details of Experimental Procedure

Rat	Initial wt	Treatment day −3 to day 2	Labeled material injected		Expression of results
			Nature	Dose	
	g			*dpm*	
A	240	None	Hematin-^{14}C	1.89×10^5	Cumulative radioactivity (dpm) in bile, bilirubin and CO, as per cent of injected dose
B	340	None	"	1.57×10^5	
C	420	Phenobarbital	"	1.89×10^5	
D	290	AIA	"	1.90×10^5	
E	250	AIA	"	1.90×10^5	
F	300	None	Glycine-2-^{14}C	1.11×10^8	Cumulative radioactivity (dpm) in bilirubin and CO
G	320	None	"	"	
H	370	Phenobarbital	"	"	
I	310	Phenobarbital	"	"	
J	330	AIA	"	"	
K	300	AIA	"	"	
L	260	AIA	"	"	
M	350	AIA	"	"	
N	310	None	Bilirubin-^{14}C	5.69×10^5	Cumulative radioactivity in total bile and CO as per cent of injected dose
O	300	AIA	"	"	
P	380	AIA	Glycine-1-^{14}C	1.11×10^8	Radioactivity in bilirubin and CO

of the isotopic material to be studied, the animal in its restraining cage was put inside a matching metabolic chamber (Aerospace Industries, Inc.) with an air flow of 300–400 ml/min. Accumulation of moisture in the chamber was prevented by a small packet of $CaSO_4$. The temperature within the metabolic chamber was continuously monitored and maintained slightly above room temperature (25°C) by a variable heating element.

Bile and breath were collected continuously for 49 hr, a period arbitrarily selected for convenience. Both collecting tubes (for bilirubin and for CO) were changed simultaneously according to the following schedule: during the first 8 hr at intervals of 1–2 hr; during the subsequent 24 hr at intervals of 3–5 hr; and for the remainder of the experiment from 6–12 hr. In those experiments in which only bile was collected, sample collections were terminated after 24 hr.

METHODS

Male Sprague-Dawley rats with an initial weight of 240–420 g were studied (Table I). Labeled hematin was prepared from rats treated with glycine-2-^{14}C (23) or from δ-aminolevulinic acid-4-^{14}C (ALA-4-^{14}C), and exhibited a specific activity of 3.9×10^5 dpm/mg, and 6.6×10^5 dpm/mg, respectively. Hematin prepared from glycine-2-^{14}C was administered in a total dose of 400–500 μg dissolved in 0.1 ml of 0.05 N NaOH and 1 ml of rat serum, and was used for studies of simultaneous ^{14}CO and bilirubin-^{14}C production. Hematin prepared from ALA-4-^{14}C is not labeled in any of the methene bridge carbon atoms, and thus is not a metabolic precursor of ^{14}CO (22); it was given in a total dose of 100 μg/animal. Glycine-2-^{14}C or glycine-1-^{14}C with a specific activity of 20–28 μCi/μmole was dissolved in isotonic saline. 50 μCi were injected through the femoral catheter in a single pulse. Labeled bilirubin, with a specific activity ranging from 1.05×10^6 to 6.8×10^6 dpm/mg, was dissolved and injected as described previously (24).

The bile was collected in tubes in the dark at 4°C. The bilirubin was quantitated by the diazo method, carrier pigment was added, and the specific activity of the radioactive bilirubin was determined after three crystallizations by methods described previously (17, 24). Radioactive bilirubin produced in this manner has a constant specific activity (17) and on mass spectrometry is free of contaminating nonbilirubin tetrapyrroles.[2] Total radioactivity in the bile samples was analyzed in a liquid scintillation spectrometer.

Air exiting from the metabolic chamber was dried by anhydrous $CaSO_4$, passed through an ionization chamber, and the signal generated, representing ^{14}C activity in the expired air, was continuously recorded (25). Carbon dioxide (CO_2) was then measured separately by an infrared detector. These instruments were calibrated immediately before each experiment with suitable standard gases (26). Subsequently all $^{14}CO_2$ was removed from the air stream by a soda lime absorber (26), and the air passed through a Hopcalite cannister (Mine Safety Appliances Co., Pittsburg, Pa.) at room temperature, which served to oxidize the ^{14}CO to $^{14}CO_2$. The labeled $^{14}CO_2$ thus generally was trapped in ethanolamine (26), aliquots of which were counted in a liquid scintillation spectrometer.

After the last collection, 1 hr was allowed for removal of the animal from its chamber and cage before blood was obtained by cardiac puncture. The hematocrit was determined, and the saline-washed red blood cells were used for

[2] Tenhunen, R., H. S. Marver, N. Pimstone, R. Schmid, D. Y. Cooper, and W. Trager. Unpublished observations.

crystallization of hemin (23) and for determination of its specific activity (12). In rats given AIA, a qualitative test for urinary porphobilinogen was carried out (27) on day zero or earlier.

Results of bilirubin-^{14}C and ^{14}CO production were expressed in terms of cumulative radioactivity (disintegrations per minute) appearing in these two products. Glycine-2-^{14}C can label eight carbon atoms in heme, seven in bilirubin, and one in CO (22). Therefore, to permit comparison of bilirubin and CO production on a molar basis, the radioactivity in bilirubin was divided by 7 (12).[3] In animals injected with hematin-^{14}C or bilirubin-^{14}C, cumulative excretion of carbon-14 in whole bile is expressed as per cent of the administered radioactivity.

The mathematical formulations for analysis of the kinetics of $^{14}CO_2$ production have been presented elsewhere (28) and include appropriate corrections for washout times inherent in the metabolism and ionization chambers. Results of $^{14}CO_2$ kinetics are expressed in terms of (*a*) the time required for $^{14}CO_2$ to reach maximal specific activity; (*b*) the percentage of injected radioactivity appearing in $^{14}CO_2$ over a specified time interval; and (*c*) total CO_2 formation in millimoles per hour per 100 g body weight.

RESULTS

Simultaneous production of ^{14}CO and bilirubin-^{14}C was studied in 16 rats, of which 5 were injected with hematin-^{14}C (prepared from glycine-2-^{14}C), 8 with glycine-2-^{14}C, 2 with bilirubin-^{14}C, and 1 with glycine-1-^{14}C (Table I). Five animals received no specific treatment, three were given phenobarbital, and eight allylisopropylacetamide (AIA). The total weight loss from the beginning of the fasting until sacrifice averaged 21% of the initial weight and there were no consistent differences between treatment groups. The hematocrit at the completion of the experiments ranged from 47 to 66%, with a mean of 54%.

[3] 100% recovery of hematin as bilirubin = $\frac{7}{8}$ of injected activity and 100% recovery of hematin as CO = $\frac{1}{8}$ of injected activity.

TABLE II

Cumulative Appearance of Radioactivity in Bile, Bilirubin, and CO in Rats Injected with Hematin-^{14}C

Rat	Treatment	Cumulative radioactivity recovered in 49 hr			Molar recovery ratio CO: bilirubin
		In bile	In bilirubin	In CO	
		% injected dose	*molar recovery, %*	*molar recovery, %*	
A	None	*	60.6‡	59.0‡	0.98
B	None	84.1	49.1	48.4	0.98
C	Phenobarbital	86.0	66.8	67.7	1.01
D	AIA	*	23.1	30.2	1.31
E	AIA	85.4	37.1	44.5	1.20

* Not measured.

‡ See Methods section for details of calculations.

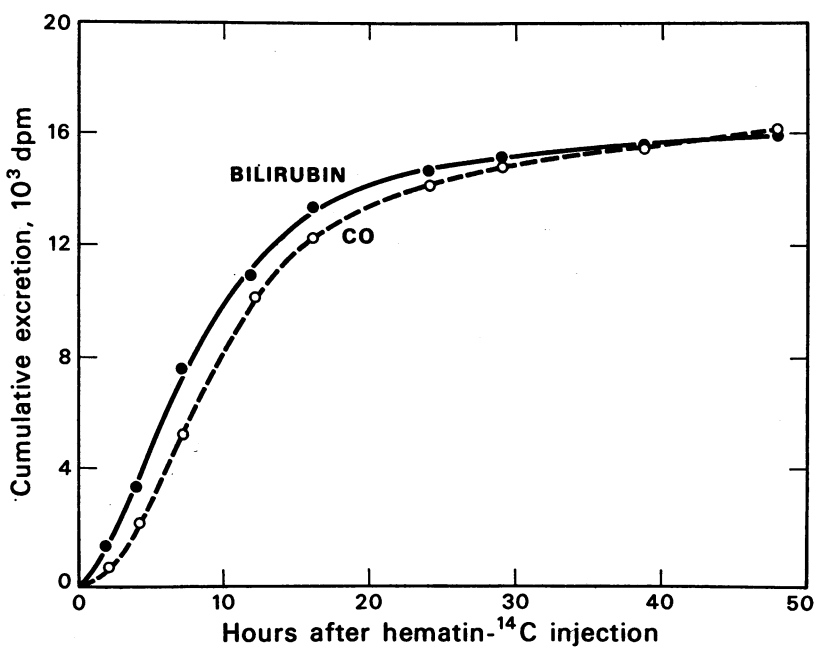

FIGURE 3 Cumulative production of bilirubin-^{14}C and ^{14}CO in a phenobarbital-treated rat injected with hematin-^{14}C in a single pulse at time zero. In this and the following figures, bilirubin production is indicated by closed symbols and solid lines, while CO production is indicated by open symbols and dashed lines. In this and in all subsequent figures, the radioactivity of bilirubin was divided by 7 to permit comparison with CO formation.

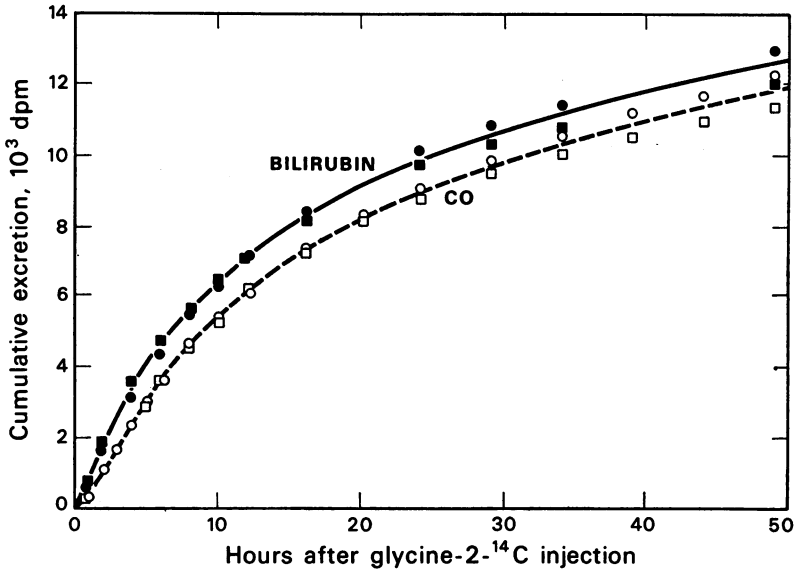

FIGURE 4 Cumulative production of bilirubin-^{14}C (closed symbols) and ^{14}CO (open symbols) in two control rats injected with glycine-2-^{14}C in a single pulse at time zero (*rat F:* squares, *rat G:* circles).

In an additional 13 rats, cumulative excretion of radioactivity in whole bile and in bilirubin-^{14}C was studied without concomitant measurement of ^{14}CO. Eight of these animals were injected with hematin-^{14}C (prepared from ALA-4-^{14}C) and five with bilirubin-^{14}C. Five of these additional animals received no specific treatment, three were given phenobarbital, and five were treated with AIA.

Formation of bilirubin and CO from hematin-^{14}C in control and phenobarbital-treated rats. In control rats A and B injected with hematin-^{14}C, total recovery of labeled bilirubin was 61 and 49% (Table II) which is in agreement with findings published previously (11). The corresponding values for recovery of ^{14}CO were 59 and 48%, with a resulting ratio of CO to bilirubin close to unity (Table II).

In rat C, treated with phenobarbital, recovery of labeled bilirubin and CO was 67 and 68%, respectively, with the CO/bilirubin ratio again close to unity (Fig. 3, Table II).

Formation of bilirubin and CO from glycine-2-^{14}C in control and phenobarbital-treated rats. In the two control animals, F and G, given glycine-2-^{14}C, the rate of appearance of the label in bilirubin and CO was very similar (Fig. 4). In confirmation of earlier studies, the maximal rate of formation of bilirubin-^{14}C (29) and ^{14}CO (26) occurred during the initial 4 hr after glycine-2-^{14}C administration. The ratio of cumulative radioactivity in bilirubin and CO formed during the entire 49 hr period was close to unity (Table III).

In rats H and I treated with phenobarbital, fractional incorporation of glycine-2-^{14}C into bilirubin was greater than in control animals (Fig. 5, Table III), confirming earlier findings (30). A proportional increase of labeling also occurred in CO so that the ratio of the total amount of isotope in the two products was 1.01 and 1.02 (Table III). The maximal rate of formation of bilirubin-^{14}C and ^{14}CO occurred during the initial 4 hr after glycine-2-^{14}C administration.

Formation of bilirubin and CO in rats treated with AIA. In all eight rats treated with AIA, the qualitative test for urinary porphobilinogen turned positive on day

TABLE III

Cumulative Appearance of Radioactivity in Bilirubin and CO in Rats Injected with Glycine-2-^{14}C

Rat	Treatment	Cumulative 49 hr recovery		Molar recovery ratio CO: bilirubin
		In bilirubin	In CO	
		dpm/7	*dpm*	
F	None	12,040*	11,390	0.95*
G	None	13,100	12,350	0.94
H	Phenobarbital	21,140	21,350	1.01
I	Phenobarbital	22,210	22,700	1.02
J	AIA	6,360	15,980	2.51
K	AIA	12,500	25,500	2.04
L	AIA	16,350	29,040	1.78
M	AIA	10,300	22,920	2.22

* See Methods section for details of calculations.

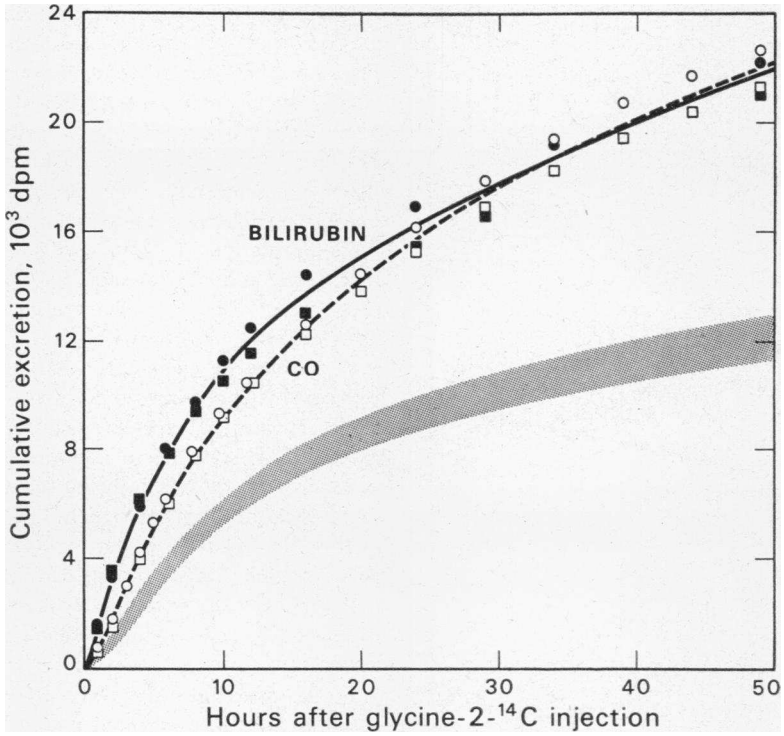

FIGURE 5 Cumulative production of bilirubin-^{14}C and ^{14}CO in two phenobarbital-treated rats injected with glycine-2-^{14}C in a single pulse at time zero. The range of values in the two control rats (Fig. 4) is shown as the hatched area. Open and closed symbols are as described in Fig. 3 legend (*rat H:* squares, *rat I:* circles).

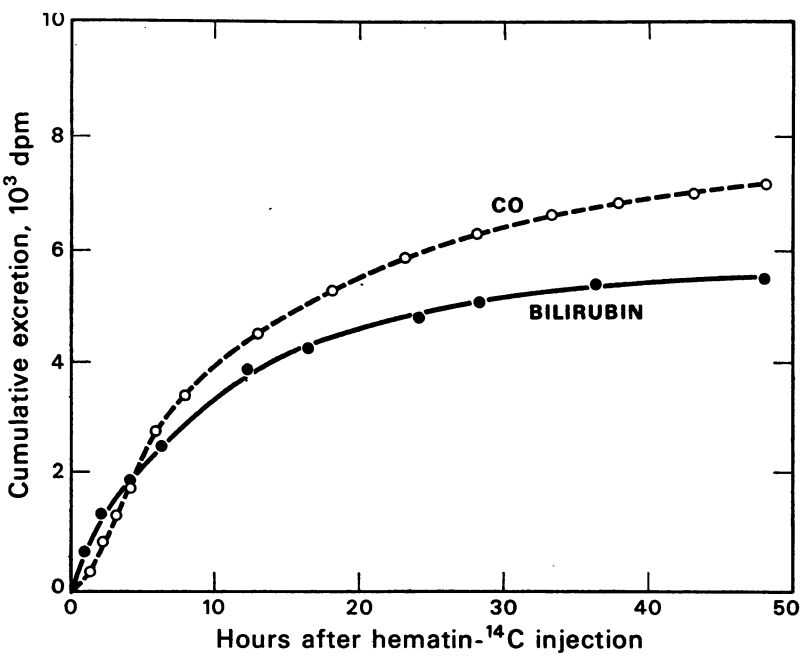

FIGURE 6 Cumulative production of bilirubin-^{14}C and ^{14}CO in an AIA-treated rat injected with hematin-^{14}C at time zero (*rat D*).

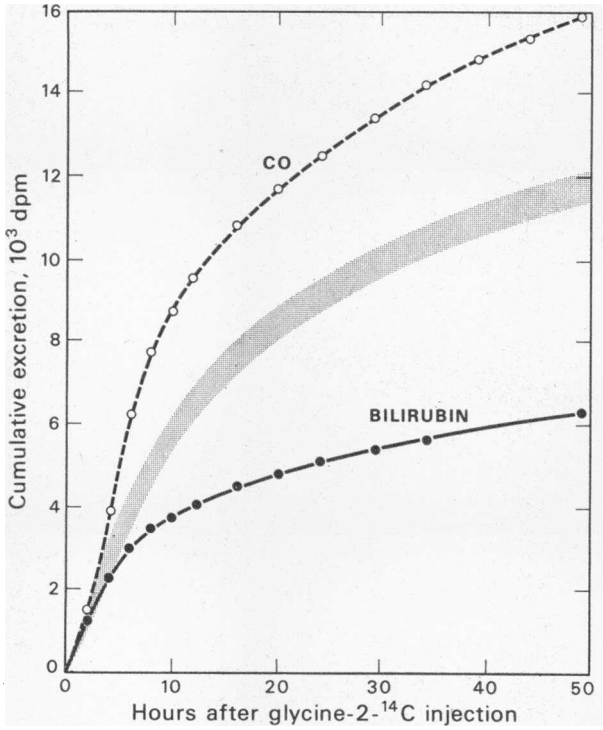

FIGURE 7 Cumulative production of bilirubin-^{14}C and ^{14}CO in an AIA-treated rat injected with glycine-2-^{14}C in a single pulse at time zero (*rat J*). The range of values in the two control rats (Fig. 4) is shown as the hatched area.

zero or earlier. When the AIA-treated rats D and E were injected with hematin-^{14}C (Fig. 6, Table II), the CO/bilirubin isotope ratio was 1.31 and 1.20. In addition, recovery of labeled CO and bilirubin (Table II) was less than in control animals, although the total radioactivity in the bile was comparable to that in untreated or phenobarbital-treated rats. In rats J, K, L, and M given glycine-2-^{14}C, cumulative incorporation of the labeled glycine into bilirubin in 49 hr averaged 11,380 dpm (Table III), which is comparable to the average value in untreated (control) animals (12,570 dpm). On the other hand, appearance of isotope in the exhaled CO was greatly increased, and approached or exceeded that seen in the phenobarbital-treated rats (Table III). This resulted in a marked increase in the ratio between isotope in CO and in bilirubin (Fig. 7), which ranged from 1.78 to 2.51 (Table III).

The previously described greenish discoloration of the bile of AIA-treated rats was noted (31). These pigments did not interfere with the diazo reaction of bilirubin added to the bile samples. No attempt was made to identify or isolate these pigments nor was a search made for mesobilifuscin or other potential breakdown products of heme.

Ancillary investigations. In order to rule out the formation of CO from sources other than the α-methene bridge carbon atom of heme, rat P treated with AIA was given 50 μCi of glycine-1-^{14}C. As previously mentioned, the carboxyl (No. 1) carbon atom of glycine is not used for any of the carbon atoms of the protoporphyrin ring (22). Neither the exhaled CO nor the excreted bilirubin contained detectable radioactivity, while formation of $^{14}CO_2$ was comparable in magnitude to that in animals injected with glycine labeled in the methylene (No. 2) carbon atom. Moreover, in rat P, no significant radioactivity was present in the hemoglobin heme of circulating red blood cells obtained at the conclusion of the experiment. By contrast, in rats J. K, L, and M treated with AIA and injected with glycine-2-^{14}C, the heme contained 211–428 dpm/mg.

Control rat N was injected with bilirubin-^{14}C containing 5.69×10^5 dpm, of which 4.81×10^5 were recovered in the bile (84.5% recovery). Rat O, treated with AIA, was given the same amount of bilirubin-^{14}C and cumulative recovery of radioactivity in the bile was 82%. No radioactivity was demonstrable in the breath CO of either animal.

In the additional five rats injected with bilirubin-^{14}C in which bile was collected for 24 hr, recovery of radioactivity in whole bile was 90 and 106% in the two untreated animals, and 90, 90, and 100% in the three AIA-treated rats. Bilirubin-^{14}C crystallized from these bile samples represented 86 and 88% of total radioactivity present in the bile of the untreated animals, and 80, 85, and 103% in the AIA-treated rats.

In the eight rats injected with hematin-^{14}C in which the 24 hr cumulative excretion of bilirubin-^{14}C and total bile radioactivity was determined, the results were as follows: in the three untreated rats total biliary excretion of radioactivity averaged 76% of the injected dose;

TABLE IV

Kinetics of CO_2 Formation in Experimental Rats Injected with Glycine-2-^{14}C

Rat	Cumulative radioactivity in $^{14}CO_2$		Time of maximum $^{14}CO_2$ specific activity	Total CO_2 production
	Initial 2 hr	Initial 12 hr		
	% of injected dose		*min*	*mmoles/hr per 100 g body wt*
F	12.4	30.8	23	2.56
G	15.7	38.5	24	3.49
H	11.0	26.2	27	2.69
I	11.6	25.4	23	3.23
J	11.7	24.0	25	2.73
K	12.3	27.4	27	2.75
L	15.1	31.3	24	3.59
M	17.3	30.8	22	3.00

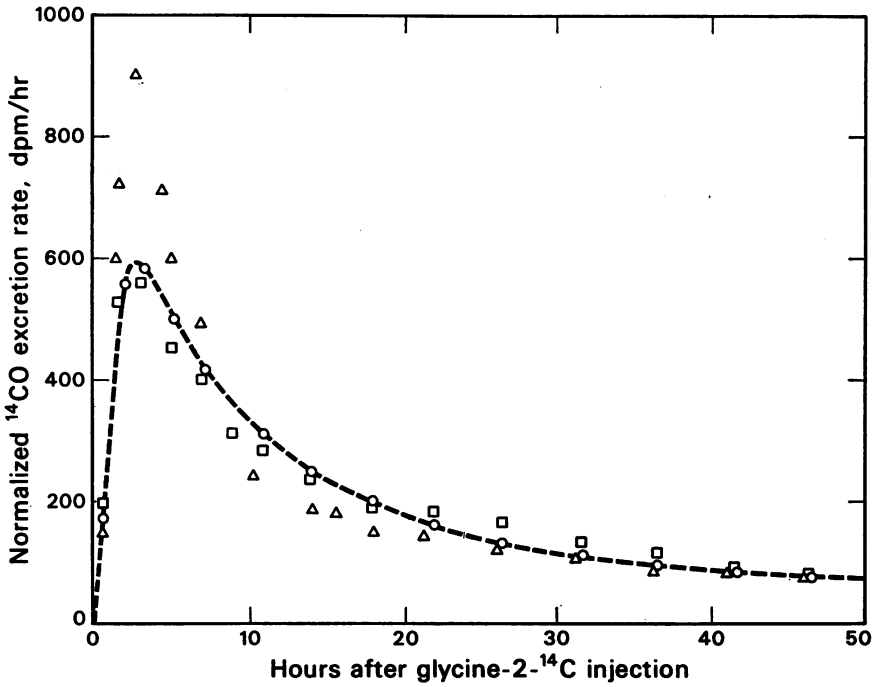

FIGURE 8 Rate of ^{14}CO production from glycine-2-^{14}C after normalization of the curves to constant area (10,000 dpm in 49 hr). The dashed line and open circles represent normalized average ^{14}CO excretion rates in the two control rats. Squares represent normalized average ^{14}CO excretion rates in the two phenobarbital-treated rats, while the triangles indicate the average values in four AIA-treated rats.

bilirubin excretion represented 63% (range: 62–65%) of the total injected dose. In the three phenobarbital-treated rats, the respective values averaged 72 and 55%. In the two AIA-treated rats, total excretion of radioactivity in bile averaged 67%, with bilirubin representing only 38% in both instances.

In all eight rats injected with glycine-2-^{14}C (rats F–M), the kinetics of $^{14}CO_2$ formation exhibited similar patterns (Table IV). No statistically significant difference (Student's t test [32]) was found in cumulative formation of $^{14}CO_2$ either during the initial 2 hr of the experiments or during the first 12 hr. Moreover, the total amount of CO_2 produced per hour per 100 g body weight and the time required for $^{14}CO_2$ to reach maximum specific activity were similar in all animals (Table IV).

For the rats injected with glycine-2-^{14}C, the curves of the average rate of ^{14}CO production were compared by normalization to a constant area, corresponding to 10,000 dpm exhaled cumulatively during 49 hr (Fig. 8). In the control and phenobarbital-treated animals, the shape of the curves was similar, with peak ^{14}CO formation occurring 2–4 hr after the isotope administration. In the four rats treated with AIA, the peak at 2–4 hr was higher and the downslope steeper.

DISCUSSION

The present findings indicate that when heme is catabolized in vivo, equimolar amounts of bilirubin and CO are formed. Thus, in rats A and B (Table II) infused with hematin-^{14}C, the quantities of labeled bile pigment and labeled CO produced were virtually equivalent. Pretreatment with phenobarbital, which enhances hepatic heme turnover (21), did not interfere with this equimolar relationship (Fig. 3, Table II). It was evident, however, that the conversion of the infused hematin to these two catabolic products was incomplete; a significant fraction of the isotope in the administered hematin was not recovered in labeled bilirubin or CO (Table II). This finding is consistent with previously reported observations that after infusion of hematin-^{14}C or hemoglobin-^{14}C into rats with an external bile fistula, the recovery of labeled bilirubin fell short of the expected quantities (12).

A probable explanation for this discrepancy is provided by the finding that in all instances the bile contained more radioactivity than could be accounted for by the total isotope appearing in bilirubin or CO (Table II). In view of the relatively small amounts of hematin administered, it is improbable that this excess radioactivity in the bile could have been unaltered

hematin that had escaped metabolic conversion (11). A more likely explanation is that part of the hematin was converted in the liver to labeled metabolites that differed from bilirubin and CO which subsequently were excreted in the bile. The nature of these alternate metabolites is unknown; deductive reasoning suggests that they may be di- or monopyrroles (14, 17).

The existence of pathways of heme catabolism that lead to metabolites other than bilirubin is not a novel concept (14). It repeatedly has been postulated that heme compounds may be degraded to pyrrolic derivatives that are smaller and more water soluble than bilirubin (12, 33), but the exact mechanisms, the chemical structure of the end products, and the functional significance of these alternate degradative routes have not been established with certainty. The present findings strongly support these postulates and indicate that *in these alternate pathways* bilirubin is not an intermediate. This suggests that the metabolic routes by which heme is degraded may be determined by the nature of the initial attack on the ferriprotoporphyrin ring; this could result either from chemical modification of the heme molecule or from alterations in metabolic conditions. While opening of the tetrapyrrole ring at the α-methene bridge catalyzed by microsomal heme oxygenase (6) leads to formation of equimolar amounts of bilirubin and CO, other pyrrolic derivatives and no CO may be produced when the ring opening occurs by different mechanisms. This concept is supported by the previously recorded observation (34) that chemical modification of the hematin or hemoglobin molecule may profoundly alter the metabolic fate of the heme moiety in the organism. Thus, Heinz bodies consisting of oxidatively denatured and precipitated hemoglobin (35) are broken down to, and excreted as, diazo-negative and water soluble metabolites (34); bilirubin is neither an intermediate nor an end product in this process. It is possible that in these instances, the opening of the ferriprotoporphyrin ring occurs by nonenzymatic mechanisms similar to the coupled oxidation proposed by Lemberg (36).

In normal and phenobarbital-treated rats, most of the injected hematin was converted to bilirubin and CO (Table II), while the alternate pathways of heme degradation played only a secondary role. By contrast, in five rats treated with the porphyrogenic drug allylisopropylacetamide (AIA), more than half of the injected hematin was converted to metabolites other than bilirubin, indicating that the functional activity of the alternate routes of heme catabolism is enhanced by this compound. Moreover, in rats D and E, less bilirubin-^{14}C was formed than ^{14}CO (Table II) suggesting an additional catabolic anomaly; this will be considered in more detail subsequently.

In the present studies of the catabolism of heme, methodologic difficulties required the use of labeled, albumin-bound hematin rather than labeled hemoglobin or hemoglobin-containing red cells. However, the following considerations suggest that the catabolism of hematin may serve as a valid model for the catabolism of hemoglobin and other hemoproteins. First, hematin and hemoglobin are taken up preferentially by the liver (11, 37) whereas sequestration of senescent erythrocytes normally occurs primarily in the spleen (38). This anatomic difference may be of little biologic significance, however, as both organs contain microsomal heme oxygenase which converts the heme moiety to bile pigment (6). Hematin, methemalbumin, isolated α- and β-chains of hemoglobin and methemoglobin all serve as substrate for microsomal heme oxygenase (39). Second, in the intact organism the kinetics of conversion to bilirubin are similar for hematin (11), hemoglobin, and rapidly sequestered erythrocytes (12). Finally, Green and Kench (40) and Bunn and Jandl (41) recently demonstrated that in methemoglobin, the ferriprotoporphyrin ring is readily dissociated from the globin and is bound by other proteins, including albumin. Together these observations suggest that the separation of the heme moiety from globin may be the initial step in the catabolism of hemoglobin and that hematin or a closely related ferriprotoporphyrin may be the first intermediate in this degradative pathway.

Recent studies have demonstrated that turnover of heme compounds in the liver (19) is a significant source of bile pigment. Among these the cytochromes of the endoplasmic reticulum appear to play a major role because of their relatively high concentration (30). In addition, liver may contain one or several metabolically "unassigned" heme pools (30, 42) that turn over very rapidly and may serve as precursors in the formation of hepatic hemoproteins. These heme fractions and hemoproteins appear to account for most of the "early-labeled" bile pigment formed during the initial hours after pulse-labeling with an isotopic precursor (19).

In normal rats given glycine-2-^{14}C as the labeled heme precursor, bilirubin-^{14}C was formed at rates similar to those obtained in previous experiments (29). In addition, as in the animals injected with exogenous hematin-^{14}C, 1 mole of ^{14}CO was produced for each mole of bilirubin excreted (Fig. 4, Tables II and III). These findings suggest that heme formed in the liver and hematin administered parenterally are degraded by qualitatively similar mechanisms: in both instances cleavage of the ferriprotoporphyrin ring at the α-methene bridge results in formation of equimolar amounts of bilirubin and CO. It should be noted, however, that in the experiments with glycine-2-^{14}C, the recovered bilirubin-^{14}C and ^{14}CO do not permit an estimate of total hepatic

heme turnover because the specific activity and magnitude of the precursor pool(s) are unknown. Moreover, in analogy with the findings after infusion of hematin-^{14}C, it is likely that a fraction of the heme synthesized in the liver is converted not to bilirubin and CO but to metabolites that at present cannot be identified or quantitated. Thus, the "early-labeled" bilirubin and CO, which are largely of hepatic origin, may actually underestimate the total heme turnover in the liver.

Despite these methodologic limitations, the observations in phenobarbital-treated rats indicate a proportionality between heme turnover in the liver and bilirubin-^{14}C and ^{14}CO formation. Phenobarbital induces δ-aminolevulinic acid (ALA) synthetase (20), magnifies hepatic heme synthesis (21), and increases the level of microsomal cytochromes (43); concomitantly the "early-labeled" bile pigment fraction is significantly enhanced (30). In rats treated with phenobarbital (Fig. 5, Table III) this increased formation of "early-labeled" bilirubin was associated mole for mole with a correspondingly enhanced ^{14}CO production. It is improbable that this increase in labeling was due to phenobarbital-induced alterations in the dilution and disposition of the administered tracer glycine because the conversion of the latter to $^{14}CO_2$ was quantitatively similar in treated and control animals (Table IV). Thus, phenobarbital appears to produce an increase in the rate of hepatic bilirubin and CO formation that is proportional to the drug-induced enhancement of heme synthesis in the liver. This concept implicitly assumes a comparable drug-induced activation of the pathways of heme degradation that involve cleavage of the ferriprotoporphyrin ring to metabolites other than bilirubin and CO. It should be noted that this effect of phenobarbital on bile pigment metabolism may be demonstrated only by the use of glycine-2-^{14}C as the metabolic tracer. With ALA-4-^{14}C as the isotopic precursor, no enhancement of bilirubin labeling would be expected because the tracer ALA is diluted by larger amounts of endogenous ALA formed in the liver in response to the phenobarbital treatment. Experiments in our laboratory have borne out this prediction.[4]

The demonstration that phenobarbital increases the production of hepatic heme and its subsequent conversion to equimolar amounts of bilirubin and CO suggests that other drugs and endogenous compounds which share with phenobarbital the property of inducing ALA-synthetase (20) and of enhancing hepatic heme synthesis (21) may have a similar effect. That this may be an erroneous conclusion was demonstrated by the findings obtained in the animals treated with allylisopropylacetamide (AIA) (Table III). AIA is a water-soluble congener of Sedormid which induces hepatic porphyria in fasted laboratory animals (44, 45). In this condition, hepatic ALA synthetase and the rate of heme synthesis in the liver (46) are increased to levels in excess of those achieved by treatment with phenobarbital. On the other hand, in contrast to phenobarbital, AIA does not produce significant changes in the concentration of microsomal cytochromes nor is there an increase in the total hepatic heme concentration (46). Preliminary data suggest that AIA either accelerates the turnover rate of a hepatic precursor heme pool or enhances the breakdown of microsomal cytochromes, or both (47); either mechanism would be expected to result in an augmentation of the "early-labeled" bile pigment and CO fractions. However, the present findings in rats J, K, L, and M (Fig. 7, Table III) and the single similar observation previously reported (29) indicate that despite the documented increase in hepatic heme turnover the rate of incorporation of glycine-2-^{14}C into bilirubin is not increased. It is unlikely that this surprising finding is due to a functionally significant drug-related alteration in the distribution or metabolism of the injected labeled precursor (glycine-2-^{14}C) because no detectable differences were noted in the measured parameters of the kinetics of $^{14}CO_2$ formation (Table IV).

AIA-treated animals not only produced less labeled bilirubin than would have been expected, but the relative incorporation of the ^{14}C label into bilirubin was considerably smaller than into CO (Figure 7, Table III) resulting in a molar CO/bilirubin ratio in excess of 2. This is the only condition so far observed in which the breakdown of heme results in formation of larger amounts of CO than of bilirubin. Several possible mechanisms have to be considered to explain this apparent discrepancy. First, excess ^{14}CO could be derived from peroxidation of lipids that were labeled by the glycine-2-^{14}C (48). This process might be facilitated by the reduced hepatic catalase activity that is present in AIA-treated animals (44, 45). This explanation appears unlikely because of the finding that in rat P treated with AIA and injected with glycine-1-^{14}C, which is an excellent precursor of fat (49) but not of heme (22), isotope appeared neither in CO nor in bilirubin, though the magnitude and kinetics of $^{14}CO_2$ formation were comparable to those in animals given glycine-2-^{14}C. Another possibility may be that in AIA-treated rats significant amounts of bilirubin are broken down to di- and monopyrrolic derivatives; this would explain the reduced recovery of labeled bilirubin relative to ^{14}CO, perhaps with formation of additional CO from labeled carbon bridges other than the α-methene bridge. This explanation is rendered unlikely by the finding in three rats that treatment with AIA did not impair the recovery in the bile of labeled bilirubin injected intravenously.

[4] Hammaker, L., and R. Schmid. Unpublished observations.

In rat O, similarly injected with bilirubin-^{14}C labeled in the methene bridge carbon atoms, no isotope was detectable in the breath CO.

A third, and most likely, explanation is that treatment with AIA profoundly alters the breakdown of heme in the liver. This appears to involve not only enhanced fission of the ferriprotoporphyrin ring to metabolites other than bilirubin and CO, but also an additional anomaly occurring probably in the further metabolism of the tetrapyrrole formed on ring cleavage. It is possible that the green pigments regularly found in the liver and bile of AIA-treated animals (31) are the result of this metabolic defect. This concept of defective heme catabolism is supported by the findings in four rats treated with AIA and injected with hematin-^{14}C. Under these conditions not only was there relatively less bilirubin-^{14}C formed than ^{14}CO (Fig. 6, Table II), but the absolute yield of labeled bilirubin was *consistently* less than in five untreated and in four phenobarbital-treated rats. By contrast, the total recovery of isotope in the bile was comparable in all three groups of animals.

These observations suggest that in AIA-treated rats the liver contains a heme fraction with a very rapid rate of turnover which is degraded, in part, through pathways that do not lead to formation of bilirubin and CO, or by mechanisms that permit conversion of the intermediary pyrroles to breakdown products other than bilirubin. The very short half-life of this heme fraction is reflected in the observation that a greater fraction of the total bilirubin-^{14}C formation tended to occur earlier (42) and that on normalization of ^{14}CO production rate curves to constant area, the rate of ^{14}CO production decreased more rapidly (Fig. 8) than in control animals. Parenterally administered hematin appears to gain access to this endogenous heme pool as it is also degraded in part through these alternate channels (Fig. 6, Table II). It remains to be determined whether these pathways of heme catabolism are qualitatively unique in being specifically dependent on treatment with AIA, or whether they simply represent an exaggeration of alternate routes of heme degradation that may be functional under normal conditions. It is tempting to speculate that to a limited extent, heme compounds formed in the normal liver may be subjected to similar presumably nonenzymatic degradation; the reduced hepatic catalase activity in experimental porphyria may enhance this process (44, 45). Finally, mention should be made of the possible relevance of these observations to the pathogenesis of experimental hepatic porphyria, which is characterized by inappropriate derepression of ALA synthetase (20, 50). Since hematin participates in the repression of this rate-controlling step (51), it is possible that the aberrant breakdown of hepatic heme may be related to the defect in this important control mechanism.

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