THE PREPARATION OF CRYSTALLINE BILIRUBIN-C^{14} *

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Conventional methods for measuring small amounts of bilirubin in biological material, based on the diazo reaction (1–3), have frequently proved unsatisfactory because of relative insensitivity at low pigment concentrations (2, 4–6), problems encountered in standardization (7–9), and loss of pigment through attachment to precipitated protein (1–3). Furthermore, while a small group of tetrapyrrolic chromogens derived from bilirubin can be estimated by their color reaction with *p*-dimethylaminobenzaldehyde (Ehrlich reagent) (10, 11), methods of analysis and quantitation are almost completely lacking for Ehrlich-negative pyrrole compounds (12, 13).

These methodological shortcomings have hindered adequate study of several important aspects of bile pigment metabolism including: the mode of bilirubin transport in the serum at physiological concentrations (14–18), the mechanism of bilirubin exchange between serum and tissue proteins (19, 20), the rate of conversion of bilirubin to non-urobilinoid catabolites (13, 21), and the nature and magnitude of the enterohepatic circulation of bile pigments (22, 23). Clarification of these problems has awaited preparation of radioisotopically labeled bilirubin of high specific activity.

Possible approaches for introducing a radioactive label into the bilirubin molecule include chemical synthesis from opsopyrrolecarboxylic acid-C^{14} (24), tritiation by the Wilzbach technique (25), and biosynthesis from glycine-2-C^{14} (26). Of these, only the last method seemed technically feasible.

The present paper describes a biosynthetic preparation of radiochemically pure bilirubin-C^{14} of high specific activity. It involves administration of glycine-2-C^{14} to rats with enhanced heme production, and subsequent isolation of bilirubin-C^{14} from the bile. Satisfactory yields are achieved by means of a new, relatively simple method for obtaining small amounts of crystalline bilirubin from bile. In connection with these studies, it was necessary to perform preliminary experiments concerning the rate at which bilirubin-C^{14} is excreted in rat bile after injection of glycine-2-C^{14}, and the stability of pure bilirubin in various solutions, both in the dark and on exposure to light. Finally, conventional techniques were adapted for radioassay of bilirubin-C^{14} and for determination of radiochemical purity.

PROCEDURE AND METHODS

Principle. In the biosynthesis of heme, 8 of the 34 carbon atoms of protoporphyrin are derived from the α-carbon of glycine, namely the 4 methene bridge carbon atoms and 1 of the α-carbons of each pyrrole ring (27). In the conversion of heme to bilirubin, 1 methene bridge carbon atom is lost (28), probably in the form of carbon monoxide (29, 30). Thus, after *in vivo* administration of glycine-2-C^{14}, the excreted bilirubin should contain 7 labeled carbon atoms of equal radioactivity (Figure 1).

Studies in man have indicated that, after administration of glycine-N^{15} (31–33) or glycine-2-C^{14} (34), the stercobilin excreted in the feces exhibits two distinct and widely separated peaks of specific activity. The major portion of the radioactive bile pigment is excreted between Days 100 and 140 after administration of the isotope, i.e., at the time when the labeled circulating erythrocytes reach the end of their physiological life span. Another narrower peak, usually of equal height, occurs during the first 20 days after administration of the isotopic glycine. The source of the labeled bile pigment excreted during this "early labeling period" is disputed (21, 31, 35–37) but it appears to be related to the erythropoietic activity of the bone marrow, since, in instances of stimulated erythropoiesis (34, 38) or of qualitatively abnormal red cell formation (32, 39–44), this peak may be greatly magnified.

Circulating erythroyctes in the rat, in contrast to those in man, may not have a uniform life span, but appear in part to be destroyed in random fashion (45, 46). This

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would indicate that sequestration and destruction of labeled murine red cells may extend over a relatively long period of time, resulting in prolonged low-grade labeling of excreted bile pigments, rather than in the distinct late peak of radioactivity observed in man. Therefore, bile collected during the period of physiological breakdown of rat erythrocytes seemed unsuitable for the purpose of obtaining bilirubin with high specific activity. On the other hand, it was deemed likely that rats would resemble man in excreting highly labeled bile pigments during the first few days following the administration of radioactive glycine, and also that the incorporation of the isotope into bilirubin could be enhanced by stimulating erythropoiesis.

Preparation of the experimental animals. In the preliminary studies, erythroid hyperplasia was induced in adult rats by repeated hemorrhage (47) or by injection of phenylhydrazine (47, 48). Two ml of blood per 100 g body weight was removed by cardiac puncture every other day for 7 days. In other animals phenylhydrazine hydrochloride was administered subcutaneously as a neutralized 2 per cent aqueous solution in an initial dose of 20 mg, followed by 3 doses of 10 mg each, injected every 36 hours. On the fourth day after the last treatment, both groups of animals were given a single injection of 0.2 mc glycine-2-C^{14} and the bile was collected through a catheter in the common bile duct.

Figure 2 shows the results obtained in two male rats of identical weight, each treated by one of the two regimens outlined above. In the rat subjected to repeated hemorrhage, the specific activity of the bilirubin excreted during the first day was 67 per cent higher than that in the animal prepared with phenylhydrazine. On the other hand, the bile of the phenylhydrazine-treated rat contained 40 to 60 mg of labeled bilirubin per 100 ml, while in the bled animal the bilirubin concentration averaged only 5 mg per 100 ml. In both instances, peak specific activity of bilirubin-C^{14} was attained within the first 18 hours after administration of the labeled substance.

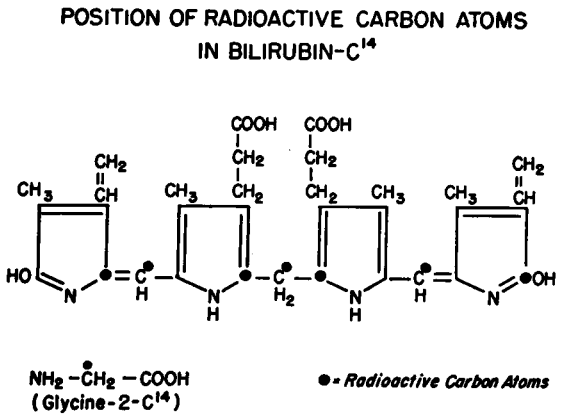

FIG. 1. STRUCTURAL FORMULA OF BILIRUBIN, SHOWING THE POSITIONS OF THE RADIOACTIVE CARBON ATOMS DERIVED FROM GLYCINE-2-C^{14} (27, 28).

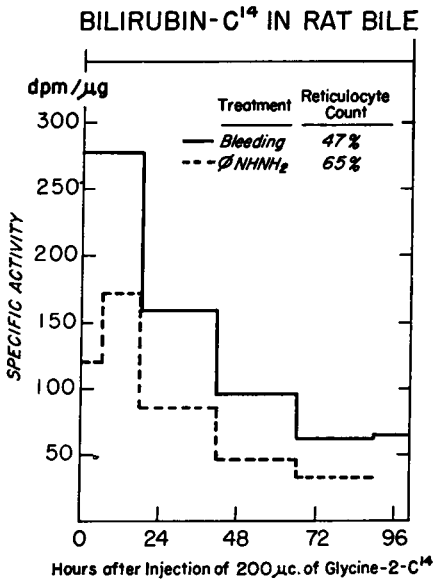

FIG. 2. SPECIFIC ACTIVITY (DISINTEGRATIONS PER MINUTE PER MICROGRAM) OF BILIRUBIN-C^{14} IN FISTULA BILE OF TWO 380 G RATS, AFTER EACH RECEIVED A SINGLE INJECTION OF 0.2 MC GLYCINE-2-C^{14}. Erythropoiesis was stimulated in one animal by repeated bleeding, and in the other with phenylhydrazine. Reticulocyte counts were determined at the time of isotope administration.

Based on these preliminary findings, the experimental animals were thereafter prepared in the following way.

Male Sprague-Dawley rats of 330 to 380 g body weight were given 20 mg phenylhydrazine subcutaneously on Day 1, followed by three injections of 10 mg each on Days 2, 4 and 5. On Days 8, 10, 12 and 14, 2 ml blood per 100 g body weight was removed by cardiac puncture. On Day 15, the rats were anesthetized with Nembutal (pentobarbital) and ether and an external bile fistula was produced. A PE-10 polyethylene catheter [1] (ID 0.011 inches) was anchored in the proximal portion of the common bile duct (49) and the free end was brought out through a stab wound in the right flank. The animals were placed in loose restraining cages and bile drained into plastic tubes containing approximately 50 mg ascorbic acid (50). These tubes were kept on ice in the dark, changed every 6 hours, and stored in the deep freeze at $-20°$ C immediately after each collection period. Bile was collected for 5 to 9 consecutive days, during which time the rats were given access to a drinking solution containing 5 per cent glucose, 0.45 per cent NaCl and 0.05 per cent KCl in 5 per cent (vol/vol) aqueous ethanol. If bile flow fell below 0.7 ml per hour, 4 to 5 ml of an aqueous solution containing 0.45 per cent NaCl and 2.5 per cent glucose was given by subcutaneous injection.

Following successful biliary cannulation, glycine-2-C^{14}

[1] Clay-Adams, Inc., New York 10, N. Y.

with a specific activity of 17.8 mc per mmole [2] was dissolved in 0.85 per cent saline and injected subcutaneously. After an initial dose of 0.4 mc, 0.2 mc was administered every 30 minutes to a total dose of 2.0 mc. Collection of bile for isolation of bilirubin-C^{14} began 60 minutes after the initial injection of radioactive glycine.

Isolation of crystalline bilirubin-C^{14} from bile. Previously reported methods for isolating and crystallizing bilirubin from bile (51–62) did not appear suitable for the present study because they required large quantities of bile, gave a poor yield of pigment of questionable purity, or called for prolonged extraction procedures. The technique to be described obviates these shortcomings, since it is applicable to as little as 0.2 mg bilirubin, consistently yields over 40 per cent of the pigment content of the bile as pure crystalline bilirubin, and can be completed in 3 to 4 hours.

Bile specimens were thawed and ascorbic acid was added to a final concentration of 20 mg per ml. Bilirubin glucuronide was precipitated as the lead salt by addition of one-fifth vol of 5 per cent aqueous lead acetate [$Pb(C_2H_3O_2)_2 \cdot 3H_2O$] (63). The precipitate was packed by brief centrifugation and the supernatant fluid discarded. The surface of the precipitate and the inside of the centrifuge tube were washed once with water and three times with absolute ethanol. The precipitate was then dispersed in 4 vol of absolute ethanol, centrifuged, and the yellow supernatant fluid discarded. After addition of a few milligrams of ascorbic acid, the glucuronide was hydrolyzed by stirring the precipitate with 4 vol of 1 N aqueous sodium hydroxide for 20 minutes at room temperature in the dark (64). Glacial acetic acid was added to bring the pH of the mixture to 5.0, and the unconjugated bilirubin was extracted into 4 vol of chloroform. The precipitate, forming at the interphase, was separated and re-extracted with a 3:1 mixture of chloroform and glacial acetic acid until the extracts were almost colorless. Any precipitate remaining on the surface of the pooled chloroform extracts was then floated off with a small amount of water.

The combined chloroform extracts were washed six times with one-fifth vol of 1 per cent aqueous ascorbic acid, once with an equal volume of 10 per cent saline, and four times with water. The chloroform solution was filtered through Whatman no. 40 paper which had been moistened with chloroform, and the filtrate then evaporated to a small volume in a boiling water bath. After adding one-half vol of methanol and boiling off the remaining chloroform, bilirubin readily crystallized as orange needles. These were separated by centrifugation, washed with 0.5 ml of cold methanol and dried *in vacuo* in the dark over anhydrous $CaSO_4$ and paraffin shavings. For recrystallization, the bilirubin was dissolved in a few milliliters of chloroform and again crystallized as above. With amounts of bilirubin less than 0.3 mg, addition of methanol to the chloroform solution occasionally resulted in formation of a white colloidal precipitate instead of orange crystals. This apparently consisted of aggregates of bilirubin (65–68) which redissolved readily in chloroform.

[2] Lot no. 35-219, New England Nuclear Corporation, Boston, Mass.

TABLE I

Yields of crystalline bilirubin from bile

No. of samples	Source of bile	Volume	Bilirubin		Yield	
			Concentration	Content	Mean	Range
		ml	*mg%*	*mg*	%	%
15	Rat	0.4–0.6	37–76	0.18–0.39	59.8	(44–93)
9	Rat	2.3–30	4.5–50	0.83–2.32	68.7	(48–82)
4	Human	10–60	45–70	5.5–41.6	68.0	(61–74)

For quantitation, chloroform solutions of the crystals were prepared and the bilirubin concentration measured by the diazo reaction (2) or by direct spectrophotometric determination at 450 mμ (68). Standard curves were prepared for each method with weighed amounts of commercially available bilirubin.[3]

In most instances more than half of the pigment content of the initial bile sample was recovered as crystalline bilirubin (Table I). Similar yields were obtained from human bile collected by drainage of the common bile duct (Table I).

Radioassay of bilirubin-C^{14}. Bilirubin crystals were dissolved in chloroform and 1 to 5 μg was counted at infinite thinness (69) on stainless steel planchets in a windowless gas-flow proportional counter with an efficiency of 58 to 64 per cent. Samples were also counted in a Packard Tri-Carb liquid scintillation spectrometer, using toluene-C^{14} as an internal standard.[4] The chloroform solution, containing 1 to 9 μg of bilirubin-C^{14} was evaporated in low-potassium glass counting vials,[5] the bilirubin was redissolved at 50° C in 0.5 ml 0.5 M Hyamine in methanol [6] (70) and 17 ml of scintillator solution [7] was added (71). Counting efficiency ranged from 44 to 50 per cent. Results were expressed as disintegrations per minute (dpm) per μg bilirubin-C^{14}.

Analysis of crystalline bilirubin-C^{14}. Crystals of bilirubin-C^{14} were dissolved in chloroform or in 0.1 N NaOH and the absorption spectra, as determined in a Beckman DU spectrophotometer, were compared with those reported in the literature (67, 72). Elemental analysis was carried out on 10 mg of thrice recrystallized bilirubin-C^{14}.[8]

Thirty-two samples of bilirubin-C^{14} were recrystal-

[3] Pfanstiehl Laboratories, Inc., Waukegan, Ill.

[4] Lot no. 25-273, New England Nuclear Corp., Boston, Mass.

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

[6] Prepared from Hydroxide of Hyamine 10X, Packard Instrument Co., La Grange, Ill.

[7] 2,5 Diphenyloxazole, 4 g per L, and 50 mg per L 1,4 *bis*-2(5-phenyloxazolyl)-benzene, dissolved in dry toluene. Pilot Chemical Company, Watertown, Mass.

[8] Performed by J. F. Alicino, P. O. Box 267, Metuchen, N. J.

lized three to five times each and the specific activity of the radioactive bilirubin was determined on the last two to four crystallizations.

Three individual samples of approximately 0.5 mg of thrice recrystallized bilirubin-C^{14} were subjected to countercurrent distribution, using a manually operated 100-tube glass apparatus.[9] The solvent system consisted of 5 vol freshly distilled chloroform, 3 vol *n*-heptane purified by passing through silica gel, and 4 vol dry propylene glycol. The system was equilibrated for 24 hours in a dry, dark glass bottle, forming an upper phase consisting mainly of *n*-heptane and chloroform and a lower phase of propylene glycol and chloroform. During the distribution, the apparatus was protected from direct sunlight. After 50 transfers, the 20-ml solvent system (10 ml upper; 10 ml lower) in each of the tubes was extracted with 0.5 vol of distilled water to remove the propylene glycol. In preliminary studies with bilirubin-C^{14}, it was ascertained that the colorless propylene glycol-water extract contained no significant radioactivity. An aliquot of the remaining chloroform-*n*-heptane mixture was used for determination of pigment concentration by comparing the optical density at 455 mμ with a standard curve prepared with commercially available bilirubin. Another aliquot was evaporated in low-potassium glass counting vials in a boiling water bath, the *n*-heptane being removed as an azeotrope with ethanol. The residue was dissolved in Hyamine-methanol and counted in a liquid scintillation spectrometer as described above. From the data obtained, the partition coefficient, K, of bilirubin in this solvent system was calculated to be 3.0 and the theoretical distribution curve was constructed (73). The specific activity data were treated mathematically as outlined by Baggett and Engel (74).

Studies of the stability of bilirubin solutions. Previous reports have indicated that in organic or in alkaline aqueous solutions bilirubin is unstable and may decompose or become oxidized to biliverdin (50, 72, 75, 76), particularly if exposed to light (7). It was therefore necessary to obtain detailed information on the behavior of bilirubin in those media used during the isolation, crystallization, radiochemical analysis and quantitation of bilirubin-C^{14}. In addition, solutions of bilirubin in 0.1 N NH_4OH or 0.1 N NaOH were investigated, because these are commonly used vehicles for introducing bilirubin into experimental biological systems.

Approximately 1.0 mg recrystallized bilirubin was dissolved in 100 ml of each of the following media: 0.1 N NaOH, 0.1 N NH_4OH, benzene, chloroform, or chloroform-methanol mixtures in the proportions of 1:7, 1:1 and 7:1. Half of the solution was kept in the dark, while the Erlenmeyer flask containing the remainder of the solution was exposed to an unfiltered G. E. mercury lamp at a distance of 25 cm. Immediately after dissolving the bilirubin and at intervals thereafter, aliquots from the light-protected and light-exposed solutions were removed for spectral analysis in the range from 325 to 700 mμ, for the diazo reaction and for the pentdyopent reaction. The concentrations of bilirubin in organic solvents were determined by the Malloy-Evelyn method (2), whereas for aqueous alkaline solutions, the diazo reagent was prepared in 0.6 N HCl and the azopigment estimated at 560 mμ. For the pentdyopent reaction, 1.5 ml of aqueous alkaline bilirubin solution was mixed with 1.5 ml of 2.0 N NaOH and 1 to 2 mg of sodium hydrosulfite ($Na_2S_2O_4$). After heating in a boiling water bath for 5 minutes, the optical density was determined at 525 and 530 mμ (77) against a blank of 1.0 N NaOH containing a comparable amount of hydrosulfite. With bilirubin in organic solvents, 1.5 ml of the solution was extracted with 3.0 ml of 1 N NaOH, and the pentdyopent reaction was performed on the undiluted extract.

A sample of crystalline bilirubin-C^{14} dissolved in 0.1 N NH_4OH was exposed to a G. E. mercury lamp for 5 hours, resulting in a virtually colorless solution giving a negative diazo and a strongly positive pentdyopent reaction. The water and ammonia were evaporated *in vacuo* and the residue was dissolved in propylene glycol and subjected to countercurrent distribution in the solvent system described above.

Freshly collected rat bile was stored in plastic tubes in the deep freeze at $-20°$ C. At intervals of several weeks to several months the bile was thawed and aliquots were removed for bilirubin determination (2). Radiochemically pure crystalline bilirubin-C^{14} was stored for 1 year in a dark vacuum desiccator containing $CaSO_4$ and paraffin shavings. The specific activity of the radioactive bilirubin was determined before and after the storage period.

RESULTS

Yield and specific activity of bilirubin-C^{14}. Rats prepared as described maintained a bile flow of 0.7 to 1.3 ml per hour with biliary bilirubin concentrations ranging from 8 to 13 mg per 100 ml. This permitted isolation of 1.2 to 1.9 mg of twice recrystallized bilirubin-C^{14} per day. Bilirubin-C^{14} excreted during the first 24 hour period following the initial injections of labeled glycine exhibited a mean specific activity of 2,500 to 3,800 dpm per μg or 0.66 to 1.0 mc per mmole (Figure 3). During the second and third 24-hour collection periods, the specific activity decreased to about one-half and one-third, respectively, of these peak values, and thereafter levelled off at approximately one-fourth of this value (Figure 3). From the preliminary studies (Figure 2) it is apparent that maximal specific activity of bilirubin-C^{14} was reached between 6 and 18 hours after isotope administration.

[9] H. O. Post Scientific Instrument Company. The authors are grateful to Drs. I. Weliky and L. L. Engel, Huntington Laboratory, Massachusetts General Hospital, Boston, for permitting the use of their equipment and for their help in carrying out these analyses.

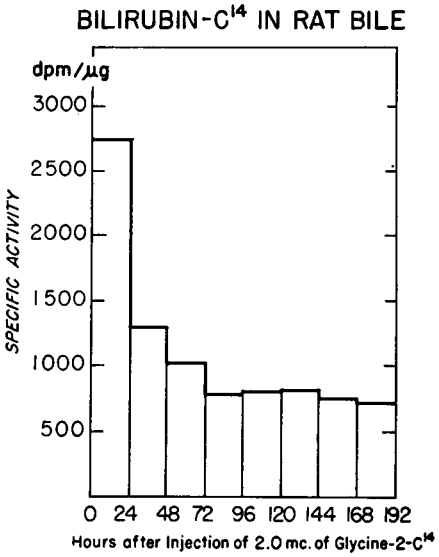

FIG. 3. SPECIFIC ACTIVITY OF BILIRUBIN-C^{14} IN FISTULA BILE OF A 350 G RAT AFTER INJECTION OF 2.0 MC GLYCINE-2-C^{14} OVER A PERIOD OF 4.5 HOURS. The animal was prepared with both phenylhydrazine and bleeding as outlined under Procedure and Methods.

Radiochemical purity of bilirubin-C^{14}. Solutions of crystalline bilirubin-C^{14} in chloroform and in 0.1 N NaOH showed absorption spectra corresponding to those previously reported for bilirubin (67, 72) with maxima at 450 and 420 mμ, respectively. Elemental analysis agreed closely with the calculated composition of bilirubin (78)—calculated: C, 67.79; H, 6.21; N, 9.58; found: C, 67.70; H, 6.29; N, 9.39. Within the limits of the experimental methods employed, the specific activity of each sample of bilirubin-C^{14} remained constant after the first recrystallization.

Analysis of the countercurrent distribution of crystalline bilirubin-C^{14} (Figure 4) yielded curves for radioactivity and for bilirubin closely corresponding with each other and with the theoretical distribution curve for a partition coefficient, K 3.0 (73). Secondary peaks due to radioactive impurities were not observed. When the logarithms of the specific activity in tubes 32 to 43 were plotted (Figure 4), no significant regression existed ($p > 0.2$), indicating that over the span of $\pm 2\theta$ (74), no separation of label and bilirubin had occurred.

Deterioration of bilirubin in aqueous alkaline solution. Solutions of crystalline bilirubin in 0.1 N NaOH or 0.1 N NH_4OH kept in the dark, showed slow deterioration of the pigment as indicated both by progressive reduction in optical density in the 420 to 440 mμ band and diminished formation of the diazo derivative (Figure 5). With a 1 mg per 100 ml solution of bilirubin in 0.1 N NH_4OH, optical density at 440 mμ decreased by 20 per cent in 2 hours and by 60 per cent in 20 hours, associated with progressive fad-

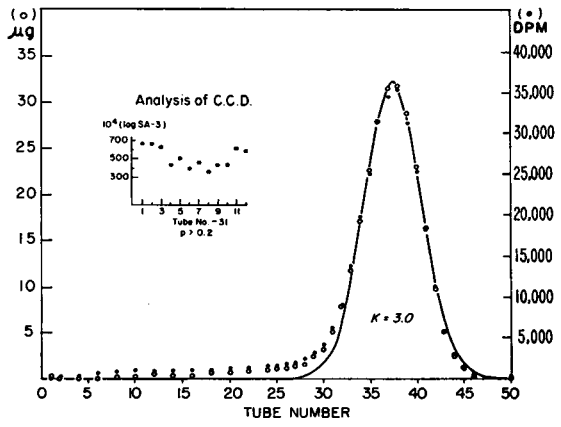

FIG. 4. FIFTY-TRANSFER COUNTERCURRENT DISTRIBUTION OF CRYSTALLINE BILIRUBIN-C^{14}, USING AS SOLVENT SYSTEM CHLOROFORM: *n*-HEPTANE: PROPYLENE GLYCOL (5:3:4). Open circles indicate bilirubin content, closed circles radioactivity, and the solid line the theoretical distribution curve for K 3.0. The slope, calculated from the logarithms of the specific activity for tubes 32 to 43 (insert), was not significantly different from zero ($p > 0.2$).

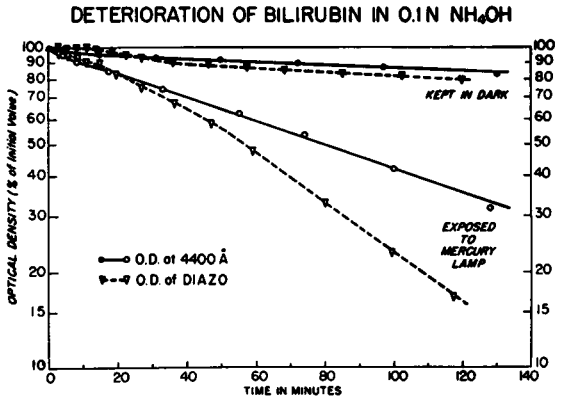

FIG. 5. DETERIORATION OF BILIRUBIN IN 1.0 MG PER 100 ML SOLUTION IN 0.1 N NH_4OH, KEPT IN THE DARK OR EXPOSED TO LIGHT. The values obtained for optical density at 4,400 Å (solid line) and with the diazo reaction (dotted line) are plotted as percentage of the values at zero time.

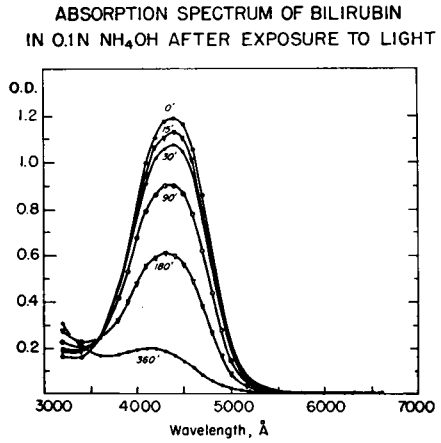

FIG. 6. SERIAL ABSORPTION SPECTRA OBTAINED DURING EXPOSURE OF A 1.0 MG PER 100 ML BILIRUBIN SOLUTION IN 0.1 N NH_4OH TO A G. E. MERCURY LAMP AT A DISTANCE OF 25 CM. The progressive decrease in optical density in the 4,400 Å region was associated with fading of the yellow color. The pentdyopent reaction was strongly positive after 2 and 4 hours of exposure, and weakly positive after 7 hours.

ing of the yellow color. Moreover, the initial absorption maximum at 440 mμ shifted to a peak at 420 mμ after 20 hours. No green or blue discoloration was observed at any time nor did significant absorption appear in the 600 to 700 mμ band. However, a positive pentdyopent reaction, with peak absorption between 525 and 530 mμ, first developed after 2 hours of storage and steadily increased to a maximum at 25 hours.

Exposure of these same aqueous alkaline solutions of bilirubin to intense illumination resulted in deterioration which was qualitatively similar, but occurred at an accelerated rate (Figures 5 and 6). Under these conditions the solutions became virtually colorless in 6 to 7 hours, with the decrease in concentration of diazo-reacting pigment proceeding more rapidly than the reduction in optical density between 420 and 440 mμ (Figure 5). As in the dark, no significant absorption in the 600 to 700 mμ band was observed (Figure 6). Moreover, the pentdyopent reaction became strongly positive after 2 hours, but was only weakly positive at 7 hours and negative thereafter.

When a sample of bilirubin-C^{14} dissolved in 0.1 N NH_4OH was illuminated for 5 hours and the resulting colorless solution evaporated *in vacuo*, the countercurrent distribution of the residue differed strikingly from that found with bilirubin-C^{14}. Virtually all radioactivity was present in the first four tubes, while in tubes 35 to 40, where bilirubin ordinarily accumulates, no significant radioactivity could be detected. Furthermore, at the completion of the countercurrent distribution all radioactivity in the collecting tubes remained in the aqueous propylene glycol extract, while none was present in the chloroform-heptane mixture.

Deterioration of bilirubin in organic solvents. Solutions of bilirubin in benzene, chloroform, or chloroform containing up to 50 per cent (vol/vol) methanol were stable for at least 2 hours if kept in the dark or in ordinary room light protected from direct sunlight (Figure 7). On the other hand, direct illumination by a mercury lamp resulted in rapid decomposition of the bilirubin as indicated by concomitant reduction in optical density at 450 mμ and in ability to form a diazo derivative (Figure 7). In contrast with the findings in aqueous alkaline solution, the original yellow color changed to an olive green exhibiting absorption maxima at 380 and 650 mμ, typical of biliverdin (79, 80). After 60 to 90 minutes of exposure to the mercury lamp, this green color gradually faded, the solution eventually becoming colorless and strongly pentdyopent-positive.

Bilirubin dissolved in a 7 : 1 (vol/vol) mixture of methanol and chloroform decomposed in a fashion similar to that observed with aqueous alkaline solutions, except that, on exposure to a mercury lamp, transient formation of small

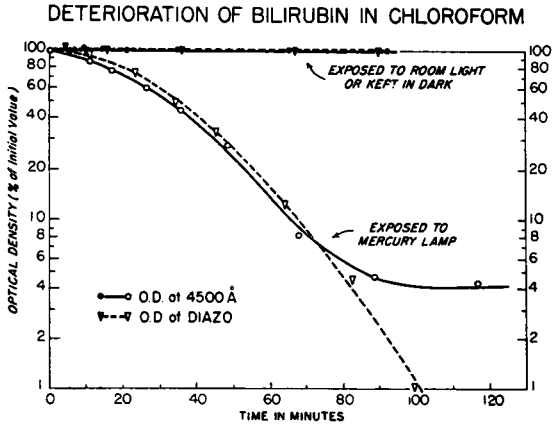

FIG. 7. BEHAVIOR OF A 2.0 MG PER 100 ML SOLUTION OF BILIRUBIN IN CHLOROFORM IN THE DARK, IN ROOM LIGHT, AND ON INTENSE ILLUMINATION. Data are plotted as in Figure 5.

amounts of biliverdin was observed during the initial 2 hours of illumination.

Storage of rat bile in deep freeze at − 20° C, and of crystalline bilirubin-C^{14} in a light-protected desiccator did not result in significant loss of diazo-reacting pigment, nor in change of specific activity of the radioactive bilirubin.

DISCUSSION

The above biosynthetic method permits preparation of several milligrams of radiochemically pure bilirubin-C^{14} with a specific activity adequate for most experimental work. By selecting a larger animal than the rat, or by administering more than 2 mc of glycine-2-C^{14}, one could presumably obtain larger quantities of radioactive bilirubin or achieve still higher specific activities, but, for technical and economic reasons, the procedure as outlined appeared to be most advantageous.

Using the isolation procedure described, crystalline bilirubin has been prepared from both rat and human fistula bile with relative yields well above those obtained by previously reported methods (51–62). This improvement results largely from the following modifications of technique: quantitative precipitation of bilirubin glucuronide from bile as the lead salt (63); rapid and complete hydrolysis of the glucuronide at alkaline pH (64); repeated addition of ascorbic acid to minimize oxidation of bilirubin in aqueous solution (50), and crystallization of the pigment from methanol, in which it is much less soluble than in chloroform (68). An additional factor contributing to the increased yield probably is the relatively short duration of the isolation procedure, resulting in less deterioration of pigment.

Elemental analysis and absorption characteristics in the visible range indicated that the substance crystallized from the bile was identical with bilirubin. The constant specific activity on repeated recrystallization and the result of the countercurrent distribution demonstrated that the compound was of high radiochemical purity. In crystalline form, bilirubin-C^{14} could be stored in a light-protected desiccator for at least a year without decomposition.

As seen in Figure 2, bilirubin-C^{14} appeared in the bile within 6 hours following administration of the labeled glycine and highest specific activity of the excreted pigment was reached between 6 and 18 hours. For comparison of these observations with similar experiments in man (31, 34, 38) allowance must be made for the fact that, in the human, isotope concentration was determined in stercobilin excreted in the feces rather than in biliary bilirubin. After making appropriate adjustments for the delay in pigment excretion and the dilution of the label caused by intestinal transit and enterohepatic recirculation (22, 23), the results of the murine and the human studies are in close agreement. The present observations do not further clarify previously expressed concepts regarding the origin of bile pigments excreted during the "early-labeling period" (21, 31, 34–38).

The lower isotope concentration found in the bilirubin from phenylhydrazine-treated rats (Figure 2) resulted from dilution of excreted bilirubin-C^{14} with increased amounts of nonlabeled pigment (81) derived from continued destruction of erythrocytes which had previously been injured by the toxin (82). In the definitive procedure adopted for preparation of the experimental animals, erythropoiesis was initially stimulated with phenylhydrazine and the erythropoietic hyperactivity then maintained by repeated hemorrhage. This permitted removal of virtually all injured erythrocytes before the labeled glycine was administered, both by splenic sequestration (83) and by bleeding. Consequently, excretion of nonlabeled pigment was substantially reduced, resulting in higher specific activity of the isolated radioactive bilirubin (Figure 3).

In confirmation of previous reports (50, 72, 75, 84–86), aqueous alkaline solutions of unconjugated bilirubin were found to be unstable, even when protected from light. Contrary to a widely held belief (75, 87), however, the major oxidation product(s) was not biliverdin, but a diazo-negative yellow intermediate(s) (88, 89). This yellow color gradually faded and the solution became colorless. Concomitantly, a strongly positive pentdyopent reaction developed, which indicated that some of the bilirubin had been converted to dioxy-dipyrrylmethanes or propentdyopent (Figure 8) (88, 89). Unlike bilirubin (90), these colorless breakdown products are very soluble in water (86, 88), as illustrated by the appearance of most of the radioactivity in the propylene glycol-water phase after countercurrent distribution of the products obtained by illumination of

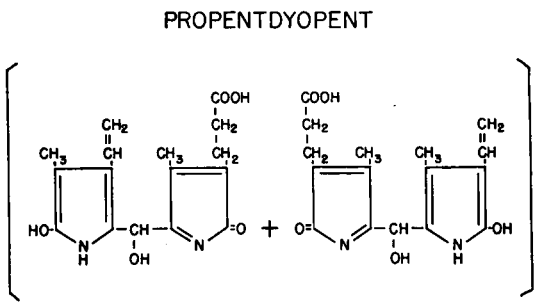

FIG. 8. STRUCTURAL FORMULAE OF THE TWO ISOMERIC FORMS OF PROPENTDYOPENT ACCORDING TO SIEDEL (13).

bilirubin-C^{14} in dilute ammonia. Decomposition of bilirubin in aqueous alkaline solution was qualitatively similar in the dark and in the light, but occurred far more rapidly under the latter condition. These findings re-emphasize that, in order to prevent decay, exposure of bilirubin to aqueous alkali should be avoided. When this is not feasible, decomposition of the pigment should be minimized by addition of ascorbic acid, by exclusion of light, and by restriction of duration of exposure to a high pH.

If protected from intense illumination, bilirubin was stable for at least a few hours in chloroform (7, 87) or benzene. This permitted preparation of the appropriate organic solutions for crystallization, countercurrent distribution and quantitation, without significant decomposition. By contrast, in methanolic solution containing only small amounts of chloroform, bilirubin was unstable even in the dark (7, 91). Therefore, after crystallizing bilirubin from methanol, it is desirable to separate quickly the formed crystals from the mother liquor by centrifugation.

Bilirubin in organic solvents, when exposed to direct illumination, deteriorated very rapidly but, in contrast to aqueous alkaline solutions, some biliverdin could be detected. Conceivably, biliverdin formation is also the first oxidation step in dilute alkali, but this phase may be so transient as to escape spectrophotometric detection. As in aqueous alkali, illuminated organic solutions of bilirubin eventually became colorless, diazo-negative, and pentdyopent-positive.

Formation of propentdyopent (Figure 8), which yields red pentdyopent on reduction in alkali, has been shown to be a group reaction given by a variety of tetrapyrroles on treatment with hydrogen peroxide (13, 77). In the present studies, however, dioxy-dipyrrylmethanes were formed in aqueous alkaline (88, 89) and organic solutions of bilirubin in the absence of added hydrogen peroxide or other oxidizing agents (77). This suggests the possibility that a similar breakdown of bilirubin to dipyrrolic compounds may occur in the organism (77, 92) or in the excreta (93, 94), particularly after exposure to light (95, 96).

SUMMARY

1. A procedure is described for preparing radiochemically pure bilirubin-C^{14} which involves administration of glycine-2-C^{14} to rats with stimulated erythropoiesis, and subsequent isolation of the radioactive pigment from fistula bile.

2. A new and rapid method is presented for obtaining crystalline bilirubin from bile with yields consistently in excess of 40 per cent.

3. With these techniques, crystalline bilirubin-C^{14} was prepared, with a specific activity ranging from 0.66 to 1.0 mc per mmole. Radiochemical purity of the labeled pigment was demonstrated by spectrophotometry, elemental analysis, recrystallization to constant specific activity, and countercurrent distribution.

4. In rats injected with glycine-2-C^{14}, the "early labeling peak" of the excreted radioactive bile pigment was shown to occur between 6 and 18 hours following administration of the isotope.

5. Bilirubin dissolved in benzene, chloroform or in chloroform containing up to 50 per cent methanol, was stable when kept in the dark but rapidly deteriorated on illumination. In alkaline solution bilirubin was unstable even in the dark. The breakdown products formed included yellow diazo-negative pigments and colorless propentdyopent.

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