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Reduction of Biliverdin-C^{14} to Bilirubin-C^{14} *in vivo*.* (29666)

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Bilirubin is the major end-product of heme catabolism. The intermediates in this degradative process are not well defined, but it has long been assumed that biliverdin is the immediate precursor of bilirubin(1). The structural relationships between heme, biliverdin, and bilirubin support this concept, and enzymatic conversion of biliverdin to bilirubin has been performed *in vitro*(2,3). Surprisingly few attempts have been made to demonstrate experimentally that mechanisms permitting conversion of biliverdin to bilirubin do, in fact, function *in vivo*(4,5). In view of recent reports describing an enzyme system which facilitates conversion of hemoglobin-haptoglobin to a biliverdin precursor(6), characterization of the physiologic relationship between biliverdin and bilirubin has become increasingly important. For this purpose, C^{14}-labeled biliverdin was prepared from bilirubin-C^{14}, and investigations of its metabolism and excretion were performed.

Materials and methods. Bilirubin-C^{14}(7) dispersed in methanol was refluxed for 30 minutes in the presence of ferric chloride and hydrochloric acid. The reaction mixture was neutralized, and the green pigment was extracted successively into ethyl ether, 1 N hydrochloric acid, and chloroform. The final solution was washed with water and dried by filtration through chloroform-moistened paper(8). Biliverdin-C^{14} was crystallized by displacement of chloroform with methanol; recrystallization was accomplished by dissolving the primary crystals in hot methanol and permitting the solution to cool at —5°C. The crystals were stored in the dark *in vacuo* until immediately prior to use.

In order to establish purity of the bili-

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TABLE I. Spectroscopic Data for Biliverdin.

MeOH	λmax	$10^{-3}\epsilon$	λmax	$10^{-3}\epsilon$	E_2/E_1
Biliverdin*	640–650	12.9	375–380	40.5	3.13
Biliverdin-C^{14}	632–668	13.1	370–376	42.0	3.21
$CHCl_3$					
Biliverdin*	640–650	13.4	378–380	41.7	3.12
Biliverdin-C^{14}	632–656	13.4	376–380	46.4	3.46
2.8 M HCl in MeOH					
Biliverdin*	660–680	22.9	375	45.5	2.00
Biliverdin-C^{14}	664–692	27.0	372–376	54.6	2.02

* Values reported by Gray *et al*(8).

verdin-C^{14} preparations, crystals were dissolved in chloroform or acidic methanol and spectral absorption curves were obtained in a Beckman DB spectrophotometer. In addition, biliverdin-C^{14} in methanolic zinc acetate was examined for fluorescence when subjected to ultraviolet irradiation(8). The specific radioactivity of the crystals was measured during repeated recrystallization by radioassay in a Packard Tri-Carb liquid scintillation spectrometer(7).

Metabolism and excretion of biliverdin-C^{14} was studied in 3 rats prepared with an external biliary fistula, and with a plastic perianal receptacle(9). Radioactive pigment was dissolved in 0.15 ml of glacial acetic acid and pipetted into 2 ml of 5% human albumin; the protein solution was then neutralized and centrifuged. Although care was exerted to avoid protein denaturation or pigment precipitation during these preparative procedures, the biliverdin-C^{14} solutions in albumin were unstable, and a fine precipitate formed on standing for prolonged intervals. Radioactive pigment solutions were, therefore, administered by intravenous infusion immediately after preparation. Thereafter, the rats were maintained in a modified Bollman restraining cage and were permitted to recover from anesthesia. Bile and urine were collected at intervals and radioactivity in these excreta was assayed by techniques described previously(9). Bilirubin was crystallized from the bile samples for determination of its specific activity; the percent conversion of biliverdin-C^{14} to bilirubin-C^{14} was calculated from the bilirubin concentration in the bile and the specific activity of the bilirubin crystals. Bile samples were analyzed spectrophotometrically for detection of biliverdin.

Results. Yields of biliverdin-C^{14} equalled approximately 20% when 12 mg of bilirubin-C^{14} was utilized as the starting material. The wavelengths of the 2 absorption maxima, obtained when biliverdin-C^{14} was dissolved in chloroform or acidic methanol, were in close agreement with values for biliverdin reported by Gray *et al*(8)(Table I). Similarly, the millimolar extinction coefficients ($10^{-3}\epsilon$) and the relative absorption (E_2/E_1) at the maxima were comparable to established values. Impure preparations of biliverdin contaminated with oxidation products exhibit a characteristic red fluorescence when complexed with zinc(8). No fluorescence was visible when freshly prepared solutions of crystalline biliverdin in methanolic zinc acetate were subjected to ultraviolet irradiation. As anticipated, fluorescence developed rapidly when these solutions were permitted to stand in light and air. In accord with previous experience with bilirubin-C^{14}(7), the specific activity of the biliverdin-C^{14} preparations diminished between the primary crystallization and the first recrystallization. Thereafter, on repeated recrystallizations, the specific activity remained at a constant level.

The results of studies on biliverdin-C^{14} disposition in 3 rats are depicted in Table II. In each experiment radioactivity appeared in the bile during the first 30 minutes after intravenous injection of the labeled pigment, and continued to be excreted during the ensuing 20 hours. In all, 54 to 93% of the radioactive dose was recovered in bile, while less than 1% appeared in the urine. Invariably, most of the biliary radioactivity was

TABLE II. Disposition of Biliverdin-C^{14} After Intravenous Administration to Rats.

Rat No.	Biliverdin-C^{14} administered μg	dpm	Radioactivity excreted in bile (dpm)	% Radioactivity excreted in bile as intact bilirubin-C^{14} glucuronide
1	250	24,000	14,000	100
2	190	18,000	10,000	65
3	150	30,000	28,000	100

present in the form of conjugated bilirubin-C^{14}. In one experiment (No. 2, Table II) a small fraction of the administered biliverdin-C^{14} was degraded, and was excreted in the bile in the form of labeled biliverdin derivatives of undetermined structure; *intact* biliverdin was not detectable in any bile samples.

Discussion. In the experiments described above, radiochemically pure biliverdin-C^{14} was prepared and administered to rats with external biliary drainage. Amounts of biliverdin-C^{14} equivalent to 1/6 to 1/4 of the estimated daily bile pigment production were injected as a single intravenous dose. A major fraction of this material was reduced to bilirubin-C^{14}, conjugated, and subsequently excreted in the bile. Reduction and excretion occurred rapidly and the process neared completion within the first several hours of pigment administration.

The disposition of greater quantities of biliverdin-C^{14} could not be studied accurately because of the limited solubility of the pigment in albumin solution. Nevertheless, preliminary investigations utilizing quantities of biliverdin-C^{14} roughly equivalent to the total estimated daily pigment output gave results comparable to those obtained with lesser doses. Despite gross pigment precipitation in the solutions prepared for these studies, 4/5 of the administered material was converted to bilirubin-C^{14} during the 20 hours of observation.

In one of the rats studied (No. 2, Table II), in addition to bilirubin-C^{14}, lesser amounts of diazo negative C^{14}-labeled biliverdin derivatives were present in the bile. It could not be determined whether these were formed while dissolving biliverdin-C^{14} prior to intravenous administration or as the result of pigment metabolism.

The results establish that, in the rat, mechanisms exist for the rapid transformation of biliverdin to bilirubin *in vivo*. These experiments are, therefore, consistent with the concept that biliverdin is an intermediate in the degradation of hemoglobin to bilirubin.

Summary. Radiochemically pure biliverdin-C^{14} was administered intravenously to rats prepared with an external biliary fistula. A major fraction of the pigment was rapidly reduced to bilirubin and was recovered from the bile as conjugated bilirubin-C^{14}. The results are consistent with the view that biliverdin may be an intermediate in the degradation of hemoglobin to bilirubin.

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