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EVIDENCE FOR THE PARTICIPATION OF THE LIVER IN THE FORMATION OF "SHUNT" BILIRUBIN*

William J. R. Novak, M.D.

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I. INTRODUCTION

The majority of bilirubin excreted daily is derived from the destruction of senescent circulating red blood cells and the degradation of the contained hemoglobin. The separate works of London, West, Shemin and Rittenberg in America (1) and Gray, Neuberger and Sneath in England (2) with glycine N^{15} presented convincing evidence that in normal man 5 to 16% of the total daily production of stool stercobilin was derived from sources other than the circulating red cell. This fraction which appeared during the 4 to 5 day interval following the ingestion of the isotope is known as the "early peak" or "early labelled fraction" of stercobilin.

Since then, several studies have shown that this "early peak" may be quantitatively altered in various conditions including pernicious anemia (3), congenital porphyria (2, 4), erythropoietic porphyria (5), thalassemia major and minor (6, 7), a congenital type of jaundice termed "Shunt hyperbilirubinemia" (8, 9) and several isolated case reports of a similar type of hyperbilirubinemia (10,11). All these cases are characterized by abnormalities of erythropoiesis which led observers to postulate a relationship between the "early peak" and red cell production. This concept was further strengthened by the studies of Gray and Scott (12) who demonstrated an increase in the "early" fraction following stimulation of erythropoiesis by bleeding.

A case reported by James and Abbott (13) with "virtual erythroid aplasia" had a normal appearing "early peak" which suggested this

fraction was at least in part derived from a non-erythroid source. Subsequently, Israels, Yamamoto, Skanderbeg and Zipursky provided conclusive evidence for the existence of a non-erythropoietic source and thus the presence of at least two components (14, 15).

The purpose of this communication is to present further evidence for the presence of a non-erythropoietic source of the "early peak" and more specifically evidence pointing to the liver as a major contributor.

II. METHODS AND MATERIALS

1. DUCKS

(A) Operative Procedure

(a) Bile Fistula

White Pekin ducks under light nembutal anesthesia were subjected to laparotomy. The common bile duct was isolated, cannulated with polyethylene tubing (Clay-Adams N.Y., N.Y. PE:260) and secured. An accessory bile duct situated at the apex of the gallbladder was tied off. The cannula was then brought out through a stab wound in the abdominal wall. Bile was collected in rubber balloons containing ascorbic acid and the duck was fitted with a cloth jacket to keep the balloon in place. Upon recovery, the ducks were given water and grain ad libitum.

(b) Hemoglobin Infusion

White Pekin ducks were prepared with a bile fistula and when

good bile flow was attained, 50 ml. of a prepared pure 2-3% hemoglobin solution was infused intravenously over a period of 15 minutes. The hemoglobin solution was prepared by taking blood from a duck that had received 20 microcuries of glycine-2C^{14} 10 days previously, adding an equal volume of double distilled water, repeatedly freezing and thawing, and finally centrifuging at 18,000 r.p.m. for one hour. The hemoglobin concentration was determined and the amount infused calculated.

Following the infusion, bile collections were made, biliverdin and bilirubin were prepared and the specific activity estimated.

(B) Radioactive Material

In the bile fistula preparations when good bile flow was attained, the ducks were given either 20 microcuries of glycine 2C^{14} or 5 microcuries of delta-aminolevulinic acid 4-C^{14} (Δ- ALA - 4 - C^{14}) into a foot vein. Bile was subsequently collected at suitable intervals, frozen and stored. Blood for hemin was removed from a large vein on the leg.

(C) Purification of Bilirubin

Pure bilirubin from the bile was prepared by a modification of the method described by Ostrow, Hammaker and Schmid (16). To a thawed bile specimen was added approximately 20 mg. of ascorbic acid and a one-fifth volume of 5% aqueous lead acetate, the latter precipitating bilirubin glucuronide as the lead salt. The precipitate was packed by brief centrifugation at 3000 r.p.m., the supernatant discarded, the surface of the precipitate rinsed gently with double

distilled water and drained. Four volumes of 95% ethyl alcohol was added, mixed well with the precipitate, recentrifuged and the supernatant discarded. After the addition of a few milligrams of ascorbic acid, 4 volumes of 1.0 N sodium hydroxide was added, mixed well and allowed to stand in the dark for 20 minutes to hydrolyse the glucuronide. Glacial acetic acid was then added to bring the pH to 5.0. The unconjugated bilirubin in the solution was extracted with 4 volumes of a freshly prepared solution of 3:1 chloroform and acetic acid, a total of three times or until the yellow color was gone. The chloroform extracts were pooled, washed six times with 1/5 volume of fresh 1% ascorbic acid and once with 10% sodium chloride. Finally, the extract was washed four times with a 1/2 volume of double distilled water. The extract was filtered through Whatman No. 1 filter paper premoistened with chloroform. The filtrate was collected in an Erlenmeyer flask and evaporated slowly to dryness.

Chromatography of this crude unconjugated bilirubin preparation was then carried out. The precipitate was reconstituted in chloroform and gently applied to a well packed aluminum oxide column. When the bilirubin was adsorbed onto the column it was washed with three volumes of chloroform and then eluted with a 1% glacial acetic acid in chloroform solution. After the yellow band of pure bilirubin descended to the bottom of the column, it was collected in a test tube, washed once with double distilled water and filtered through Whatman No. 1 filter paper premoistened with chloroform. The bilirubin solution was evaporated to dryness in a boiling water bath.

Previous observations have shown that when the above prepared bilirubin is rechromatographed with a Kieselguhr column as described by Cole and Lathe (17), the specific activity of bilirubin is constant (18). This was thought to indicate a high degree of purity of the bilirubin eluted from the aluminum oxide column and this was the only chromatography utilized throughout the study.

After redissolving the bilirubin in a known volume of chloroform, the concentration of bilirubin was determined by the diazo method described by Malloy and Evelyn (19). One milliliter was placed onto each of two planchets, dried and counted in a gas flow counter (Nuclear Chicago Model C115) with an efficiency of 20%. We were unable to determine the bilirubin concentration in duck bile with the diazo method and thus could not calculate the total amount of bilirubin excreted in the bile in each collection period.

(D) Calculations

The amount of bilirubin on each planchet could, however, be calculated and thus the specific activity.

$$\text{Specific activity} = \frac{\text{counts per minute}}{\text{mg. bilirubin per planchet}}$$

(E) Preparation of Biliverdin

Bilverdin, which is found in large amounts in duck bile, was isolated by a modification of the procedure outlined by Garay and Argerich (20). To 5 ml. of duck bile was added five volumes of a 10% barium chloride solution and 1.5 ml. of saturated ammonium sulfate $(NH_4)_2.SO_4$). The mixture was agitated vigorously, allowed to stand

for five minutes, centrifuged at 3000 r.p.m. for 15 minutes, the supernatant discarded and the precipitate was washed with double distilled water and recentifuged for 30 minutes.

The biliverdin precipitated as barium salt was added to 1 ml. of a 3% ascorbic acid solution, 20 ml. of a 5% hydrochloric acid in methanol reagent, mixed well and centrifuged at 3000 r.p.m. for 15 minutes. The green biliverdin-containing supernatant was decanted and this procedure repeated until the supernatant contained very little green color. The supernatants were pooled and added to a separatory funnel containing 50-100 ml. of diethyl ether, 5 ml. of 3% ascorbic acid and 50 ml. of 2% hydrochloric acid. After shaking, the lower green layer was removed and the upper yellow ether layer was discarded. This extraction was repeated until the ether layer was clear or contained no visible bilirubin.

The green fraction was again added to the separatory funnel to which was added 100 ml. of 2N sodium acetate and enough chloroform to produce a blue-green lower layer. This extraction was also repeated until most of the biliverdin appeared to be extracted from the upper layer into the chloroform. The chloroform extract was then washed once with a 1/2 volume of 2N sodium acetate, once with double distilled water and filtered through chloroform moistened Whatman No. 1 filter paper. The filtrate was evaporated to a small volume on a boiling water bath. One ml. of the biliverdin-chloroform solution was plated at infinite thinness and counted.

The purity of the biliverdin was established by determining the absorption curve compared to pure commercial biliv erdin.

(F) Red Cell Hemin Isolation

Hemin was prepared from washed red cells by a modification of the method of Delory (21) described previously (22). The examination of the crystals under ultra-violet light after the addition of 3N hydrochloric acid showed no fluorescence, indicating the absence of contaminating porphyrins. The hemin was plated after dissolving in 0.1 N sodium hydroxide and counted.

2. LIVER PERFUSION

To ascertain whether liver can synthesize bilirubin de novo an isolated rat liver was prepared for perfusion.

(A) Operative Procedure

Under ether anesthesia male albino rats weighing approximately 400 g were subjected to laparotomy. The common bile duct was cannulated with a small polyethylene cannula (Clay-Adams, N.Y., N.Y. PE:10) and secured. The portal vein was then cannulated with a larger polyethylene catheter following which the chest cage was quickly opened and the hepatic vein cannulated with a catheter of the same size (Clay-Adams N.Y., N.Y. PE:260). Each cannula was irrigated with heparinized Ringer's solution and the liver removed as rapidly as possible without disturbing the inserted cannulae. The liver was then attached to the perfusion apparatus as designed by Miller et al (23).*

*Manufactured by Metaloglass, Inc., Boston, Mass.

The inflow tube containing oxygenated blood was attached to the portal vein cannula and the hepatic vein cannula was directed into the reservoir below. The liver itself rested on a glass dish and was covered with a gauze strip soaked in warm Ringer's solution. The distal end of the bile duct cannula was placed in a collecting vessel.

Blood was pumped continuously from the reservoir to the multibulbed oxygenating tube and then to the portal vein cannula. Blood for the experiment was obtained by cardiac puncture from 8 to 10 rats of the same strain. The blood was diluted with Ringer's solution to a final hematocrit of 30% and a volume of approximately 120 ml. The partial pressure of oxygen of the oxygenated blood ranged between 400 and 500 mm. of mercury. Air was circulated continuously within the apparatus-containing cabinet and the temperature kept at 37° C with a regulating device.

Optimal blood flow through the liver under an inflow pressure of 5-10 cm. of blood was 200-300 drops per minute. The liver maintained a relatively normal color throughout and the bile flow reached a maximum of 0.7 ml. per 9 hour perfusion. Bile flow was hampered by kinking of the biliary outflow apparatus. Considerable hemolysis occurred after a 9 hour period reducing the hematocrit to 20-25%. Histological sections of the liver at the endof the perfusions revealed no disruption in the normal architecture of the liver.

(B) Radioactive Material

After the blood flow was established, one microcurie of ALA 4-C^{14} was injected directly into the portal vein cannula. The bile collected was frozen and stored for later bilirubin isolation.

(C) Bilirubin Preparation

As the total volume of bile produced was small, it was necessary to use unlabeled carrier bile obtained from a normal bile fistula rat to facilitate isolation of the bilirubin. The small volume made it impossible to determine the concentration of bilirubin in the bile. It was therefore assumed that the concentration of bilirubin in the sample was the same as that in the carrier bile. Bilirubin was isolated and purified as described. The isolated bilirubin was plated at infinite thinness and counted. The specific activity of the sample bilirubin plus carrier was determined. The specific activity of the liver perfusion bilirubin C^{14} was then approximated as follows:

$$\text{Corrected specific activity} = \frac{\text{carrier bilirubin plus perfusion bilirubin (mg)}}{\text{perfusion bilirubin (mg)}} \times \text{specific activity of carrier plus perfusion bilirubin.}$$

3. RATS

(A) Bilirubin Experiments

(a) Operative Technique

To study the excretion pattern of radiolabeled bilirubin, bile fistula rats were prepared and injected with a radioactive tracer. Male Sprague-Dawley rats of 300 g weight under light ether anesthesia

were subjected to laparotomy. At laparotomy the common bile duct was isolated, ligated distally and a fine polyethylene catheter (Clay-Adams N.Y., N.Y. PE:10) was placed in the proximal end and secured. The tube was then brought out through a stab wound in the abdominal wall. After wound closure the rat was confined in a cylindrical wire cage designed after Bollman (24). Good bile flow was usually immediate and enough bile was collected from each rat to serve as carrier for subsequent bilirubin isolation. The rats were allowed water and food in the form of pellets ad libitum. Fluid supplements were given when required by subcutaneous injections of isotonic saline.

Following this, one microcurie of Δ ALA 4-C^{14} was injected intravenously into a tail vein. Bile collections were then made at frequent intervals over a 24 hour period and frozen as taken.

(b) The bilirubin concentration of each bile specimen and of the carrier bile was determined by the method of Malloy and Evelyn (19). The total amount of bilirubin excreted could then be calculated.

(c) Bilirubin was isolated and purified from each specimen with the use of unlabeled carrier bile by the method described above.

(d) The corrected specific activity of the sample bilirubin was calculated as described above. The radiolabeled bilirubin was recorded by calculating the total number of counts excreted per collection period and thus the percentage of total injected counts. This calculation was achieved with the use of the following formula:

$$\text{Percentage of injected counts} = \frac{\text{total bilirubin excreted} \times \text{specific activity} \times 100}{\text{total counts injected}}$$

(B) Liver Heme Experiments

Again using rats, an attempt was made to correlate the appearance of radiolabeled liver heme with the appearance of radiolabeled bile bilirubin described in the previous section.

(a) Operative Procedure

Male Sprague-Dawley rats weighing approximately 300 g were given one microcurie of Δ ALA 4-C^{14} into a tail vein and then at predetermined intervals the rat was sacrificed, the liver removed and the heme content determined.

Under light ether anesthesia the liver was exposed, the portal vein isolated and tied distally, a polyethylene cannula inserted into the proximal end of the vein and secured. Thechest cage was then opened and the hepatic vein cut. With isotonic saline the portal veincannula was irrigated washing most of the blood from the liver. The liver was then removed, dissected free of non-hepatic tissue and frozen. After freezing, liver tissue is almost completely inactive with regard to heme synthesis (25).

(b) Preparation of Liver Hemin

The thawed liver was placed with approximately 20 ml. of double distilled water into a Waring blender and homogenized for 10 to 15 minutes. The mixture was then further homogenized for 10 to 15 minutes in a Potter-Elvejhm homogenizer. Liver heme could then be isolated by a method which evolved from that described by Fischer (26), Chu and Chu (27) and later modified by Labbe and Nishida (28).

One hundred mg. of carrier heme in the form of hemoglobin was used to facilitate isolation of the small amount of liver hemin.

A stock solution of 2% strontium chloride in glacial acetic acid was prepared. A mixture of one part of this solution plus one part acetone was made immediately before starting the isolation to avoid crystallization and the resultant solution was called the extraction solvent. To the liver homogenate and carrier mixture 12 volumes of the extraction solvent was slowly added. This combination was then heated in a water bath to boiling for no longer than three minutes to encourage precipitation of liver protein. The mixture was then allowed to stand at room temperature for 30 minutes. The settled protein was filtered twice through a double thickness of Whatman No. 2 filter paper. Fresh extraction solvent was used to wash remaining hemin from the precipitated protein.

The reddish-brown filtrate was heated with 2 or 3 boiling stones on an enclosed element until the temperature reached a level no higher than 101° C, then allowed to stand at room temperature overnight. Crystallization was increased if the mixture was agitated several times over the following six hours.

The next day, the hemin crystals were removed by centrifugation and washed twice with 50% glacial acetic acid and then twice with 50% ethyl alcohol. A final washing with absolute ethyl alcohol and then with diethyl ether was carriedout and the hemin crystals were dried at room temperature.

After the hemin crystals were weighed, 2 mg. were dissolved in 2 ml. of 0.1 N sodium hydroxide, 1 ml. of which was placed on a planchet, dried and counted. Sodium deoxycholate (DOC) was used in an attempt to increase the yield of heme from thehomogenate, as suggested by Higashi and Peters (29) but resulted in essentially the same yields as obtained with the above method.

To test the purity of the hemin and reproducibility of the specific activity, recrystallization of the hemin with pyridine by a method described by Shemin, London and Rittenberg (30) was carried out. The specific activity was foundto be constant and the hemin originally isolated was considered pure. The recrystallization method was only used periodically to test the isolation method.

(c) Calculations

i. A total of 100 mg of carrier hemin was used and the recovery of hemin was calculated from this figure, assuming the total amount of liver heminrecovered to equal 1 mg. The recoveries ranged from 70% to 90%.

ii. Specific activity (cpm/mg) was calculated and the total counts in liver hemin were obtained by multiplying by the total initial amountof hemin (100 mg).

iii. As there was some variation in liver weight the total counts were adjusted to a calculated mean liver weight.

$$\text{Corrected total counts excreted} = \frac{\text{weight liver}}{\text{mean weight}} \times \text{total counts excreted.}$$

The percentage of injected counts was then calculated.

4. ALTERED HEME METABOLISM

Drug-induced Porphyria

In order to study the bilirubin excretion and hepatic heme patterns during accelereated porphyrin synthesis, male Sprague-Dawley rats weighing approximately 300 g were fed allylisopropyl-carbamide* orally in suspension in propylene glycol. The suspension was fed to the rats while under light ether anesthesia with a small rubber catheter in doses of 300 mg per kg for three consecutive days. The animals were allowed water and pellets ad libitum but as the animals became intensely anorexic isotonic saline was given sub-cutaneously to maintain hydration.

The degree of porphyria was estimated by the intensity of red fluorescence of bile when exposed to ultra-violet light. The tracer was given when the bile showed bright red fluorescence.

Bile fistulae were done on one group of rats and after injection of ΔALA 4-C^{14}, bilirubin collections were made over a 24 hour period. Another group of rats was given the same tracer and the livers were removed at similar intervals for hemin isolation.

5. ULTRA-FILTRATION EXPERIMENTS

An attempt was made to determine if hepatic heme in a measurable amount existed in a "free" or "unbound" state before incorporation as the prosthetic group of the hemoprotein enzymes in the liver. It was postulated that if this fraction existed,

* Sedormid - courtesy of Hoffman-LaRoche, Inc., Basel.

perhaps this was a source of the "early" bilirubin fraction.

Male Sprague-Dawley rats weighing approximately 300 g were given 1 microcurie of ΔALA 4-C^{14} intravenously and one hour later the liver was removed. The liver was homogenized and centrifuged at 40,000 r.p.m. for 30 minutes, removing the cellular debris. The clear brownish upper fluid portion was decanted and placed in an ultrafiltration apparatus consisting of a cellophane filter under a pressure of 50 pounds per square inch. After 24 hours the clear, light green filtrate was pooled and frozen. With the use of hemin carrier the hemin in the filtrate was isolated, plated and counted. The total number of counts present in the original homogenate and the centrifugate was also determined. The procedure was repeated in allylisopropylcarbamide intoxicated rats.

6. 3-AMINO-1, 2, 4, TRIAZOLE ADMINISTRATION

Using the above compound to inactivate and thus decrease liver catalase as described by Heim, Appleman and Pyfrom (31) an attempt was made to determine the possible relationship between liver catalase and the "early" bilirubin fraction.

Male Sprague-Dawley rats weighing 300 g under light ether anesthesia were injected subcutaneously with a 5% aqueous solution of the 3-amino-1,2,4,-triazole in a dose of 1 g per kg daily for two successive days. Eighteen hours after the second dose a bile fistula was done following which the rat was injected with 1 microcurie of Δ ALA 4-C^{14}. Bile was collected and bilirubin isolated as described.

III. RESULTS

1. Ducks

(A) Glycine 2-C^{14}

The appearance of radiolabeled bilirubin and biliverdin representative of ducks 1, 2 and 3 is shown in Figure I (a). Specific activity was used for comparison as it was not possible to estimate the total counts excreted as biliverdin. Labeled bilirubin reached a peak activity within 12 hours after injection, falling to a nadir at 72 hours and subsequently rose to a second peak at 96 hours, falling to a low level thereafter. Biliverdin reached a peak specific activity at 24 hours, falling to a nadir by 36 hours and maintaining the fall in activity through 9 days. The specific activity of the bilirubin was higher than biliverdin during the entire 8 days of the experiment. The specific activity of hemin of circulating red cells is shown in Figure I (b), with maximum increment of activity occurring between 4 and 10 days synchronous with the appearance of the second bilirubin peak.

(B) Aminolevulinic acid- 4-C^{14}

The representative results obtained in ducks 4 and 5 given ALA 4-C^{14} can be seen in Figure I (c). It is evident that the specific activity of both bilirubin and biliverdin has risen abruptly to a peak activity within 8 hours, falling as rapidly to a low level within 12 to 24 hours and remaining at low levels thereafter. In both ducks the biliverdin achieved a greater activity than bilirubin

and the specific activity of both was much greater than that seen after the injection of glycine.

(C) Hemoglobin Infusion

The specific activity of the heme of the injected hemoglobin solution was constant at approximately 67 cpm/mg by 10 days. Figure II shows the appearance of bilirubin C^{14} and biliverdin C^{14}. Bilirubin activity reached a peak at 36 hours and biliverdin by 24 hours, both falling to near zero levels by 96 hours. The bilirubin activity was nearly double the biliverdin at peak levels.

2. RAT LIVER PERFUSION

The volumes of bile produced and the specific activities of radiolabeled bilirubin are shown in Table I. Athough the volumes were small, ranging from 0.065 to 0.63 ml of bile, the specific activity of the bilirubin obtained was very high, reaching a maximum of 142,000 counts per minute per mg or 4.7% of the total injected counts. A value similar to background radioactivity was obtained when bilirubin was isolated from unlabeled or "cold" bile containing an added 1 microcurie of $\triangle$ALA 4-C^{14} indicating that the bilirubin isolated from the liver perfusion was not contaminated with infused radioactivity in the form of C^{14} labeled $\triangle$ ALA.

3. NORMAL RATS

(A) Liver Heme C^{14}

The appearance of labeled liver heme following the

injection of $\triangle$ALA 4-C^{14} into a series of normal rats is represented by figure III (a). There was a rapid rate of incorporation of the isotope first observed at 15 minutes after injection which rapidly rose to a maximum mean value of 12.4% of the injected counts within 1 to 2 hours. The specific activity slowly declined from this point maintaining a relatively high level of activity until 12 hours and thereafter fell more rapidly reaching a low value by 24 hours.

The calculated arithmetic mean and standard deviation of 8 different series of rats is shown in Figure III (b). Peak activity was reached within 2 hours and fell gradually over the following 22 hour period. There are no secondary peaks in the 24 hour period.

(B) Bile Fistula Bilirubin C^{14}

The radiolabeled bilirubin appeared very soon after the injection of $\triangle$ALA 4-C^{14} and a representative result is shown in Figure III (c). No less than 1.6% of the injected counts were excreted as early as 30 minutes after injection. Maximum incorporation of the isotope into bilirubin was achieved in all 6 rats studied within 2 hours. Thereafter the activity fell sharply reaching low levels by 48 hours. The bilirubin curves were remarkably consistent and the mean and standard deviation of the 6 rats are shown in Figure III (d). Within 4 hours the cumulative percentage of injected counts excreted in the bile reached a level of 16%.

4. PORPHYRIC RATS

(A) Liver Heme C^{14}

The mean values for the percentage of counts injected which were incorporated into the heme of porphyric livers of 3 series of rats is shown in Figure IV (a). The maximum value of 4% incorporation is 1/3 that recorded in the normal animals. This cannot be accounted for by loss of liver weight as it was generally maintained. The other values are similarly severely depressed.

(B) Bile Fistula Bilirubin C^{14}

Satisfactory experiments were completed in 2 porphyric rats with bile fistulas. The maximum incorporation of radioactivity occurred at 2 hours reaching values of 5.6% and 6% of the total injected counts. Both these observations are comparable to those found in the normal rats and a representative result as shown in Figure IV (b).

5. 3-Amino-1,2,4,-Triazole Experiment

One rat given the above compound excreted bilirubin C^{14} following the injection of Δ ALA$4C^{14}$ as shown in Figure V. There was, as in the normal rats, a rapid incorporation of radioactivity reaching a maximum of 7.1% at 2 hours and falling rapidly to a nadir at 8 hours. The cumulative excretion of counts in the first 4 hours was 17.2%, slightly greater than the mean excretion in normal animals.

6. Ultra-Filtration Experiments

An attempt was made to isolate a "free" or "unbound" heme from the protein-free liver ultra-filtrate of 2 normal rats and

1 sedormid intoxicated rat that had received ΔALA 4-C^{14}. This heme component, if present, was in amounts too small to measure by this technique as no radioactivity was found in this fraction.

IV. DISCUSSION

Until recently the "early" bilirubin fraction was thought to be a single, homogeneous peak, probably emanating from a single source. Overcoming some of the methodological problems involved in this work, Israels and co-workers (14) provided evidence that the peak was in reality not single but composed of at least 2 separate components. It was postulated that perhaps each peak was derived from a different source.

This interesting observation, for reasons cited below, supported the theory that one peak was of the non-erythropoietic and the other of erythropoietic origin. However, some observers still feel that there is little significant non-erythropoietic bilirubin (32). Most observers share the opinion of Israels supporting the presence of two components. The first component, because of its early appearance following the injection of the precursor and before the occurrence of a significant amount of erythropoiesis, was thought to be of non-erythropoietic origin and conversely the second peak seemed to occur at a time when maximal erythropoietic activity was taking place thus suggesting a relationship to red cell formation.

Each of the erythropoietic and non-erythropoietic sources almost certainly consists of several sub-components and some of the suggested possibilities are discussed below.

(A) Erythropoietic Components

(a) London and his co-workers in 1950 (1) first suggested that normally some erythrocytes or erythrocyte precursors may be destroyed either within or very shortly after leaving the bone marrow. This process was termed "ineffective erythropoiesis" by Giblet et al in 1956 (33) and is thought to be an important mechanism for the large increase in the "early" peak found in pernicious anemia (3), congenital porphyria (2) and thalassemia major (6) and minor (7). Studies by Malmos, Belcher, Gyftaki and Binopoulos (34) have provided more conclusive evidence for the occurrence of this mechanism in thalassemia major and minor. There is unfortunately no direct method of measuring the precise amount of bilirubin contributed by such a mechanism.

(b) Bessis, Breton-Gorius and Thiery (35) demonstrated that some hemoglobin is present in the cytoplasmic remnant that accompanies the nucleus upon extrusion from the normoblast and bears a close temporal relationship to the second component.

(c) Heme or heme precursors such as protoporphyrin IX formed in excess in red cells or red cell precursors may contribute to this bile pigment component. An excess of protoporphyrin IX is known to exist (36), although an excess of heme itself in young or old red cells, normal or abnormal has not been demonstrated (37). Tschudy (38) has suggested that increased heme synthesis probably accounts for the increase in the "early" fraction in congenital erythropoietic

porphyria. He further states that the nuclei of the normoblasts in these patients contain heme which is released on extrusion of the nucleus which may be a source.

(d) Oxidative metabolism exists in erythrocyte precursors and to a lesser extent in reticulocytes. The heme-containing enzymes including the cytochrome system may be degraded to bilirubin as the mature red cell is converted to anaerobic metabolism (39).

(e) A direct anabolic pathway from a common precursor pool in the bone marrow without first synthesis of heme was first postulated by Israels, Suderman and Ritzmann as the possible mechanism in 4 cases described as "shunt hyperbilirubinemia" (8). However, the possibility that the important mechanism in these cases is one of ineffective erythropoiesis was later established in these patients (15).

(B) Non-Erythropoietic Components

(a) Myoglobin, the second largest heme-containing substance in man is an obvious possibility as a source of the "early" bilirubin fraction. However, attempts to measure turnover of myoglobin by Theorell, Beznak, Bonnichsen, Paul and Akeson (40) revealed slow incorporation of Fe^{59} and suggested that the turnover was too slow to merit consideration. Later, a more sophisticated study by Akeson, Ehrenstein, Hevesy and Theorell (41) suggested the presence of two populations of myoglobin molecules, one with a half-life of 20 days and the other with a half-life of 80-90 days. However, glycine 2-C^{14} was rapidly incorporated into myoglobin and careful

measurements during the first 24 hours would have to be done to eliminate participation of myoglobin.

(b) The next important consideration is the liver as a major source of the non-erythropoietic "early" bilirubin component as it is the largest non-erythropoietic site of porphyrin metabolism. There are at least three possible contributing intrahepatic pathways.

i. There may be a direct anabolic pathway or "shunt" from liver heme precursors to bilirubin.

ii. The possible existence of a "free" or "unbound" heme before it is incorporated as the prosthetic goup of the various hemoprotein enzymes in the liver. Several investigators have shown that heme in the form of the chloride, hematin can be converted into bilirubin in both animals and man (42, 43). Hematin has recently been shown to be an effective precursor of bilirubin in the rat (44). Nakajima and associates (45) have recently described an enzyme they have called heme alpha-methenyl oxygenase which may catalyse the reaction. Kench (46) was unsuccessful in converting either protoporphyrin or any other porphyrin into bile pigments in vitro and Nakajima et al (45) have had similar difficulty with protoporphyrin IX.

iii. The next most obvious source is the hemoprotein enzyme system of the liver; catalase, the cytochromes, peroxidase and succinic dehydrogenase. These substances have been generally dismissed by most workers because the amounts were thought to be too small and the turnover rate too slow to be a sigificant contribution. It is of

interest that Theorell in 1951 (40) however, stated that although the heme of catalase in the liver was small in amount, less than one three-hundredths of the total amount of heme of hemoglobin, the turnover rate was fast and was a possible source of the "early" fraction.

The present study is mainly concerned with the contribution of the liver to the non-erythropoietic part of the "early" peak and consideration is given to all three possible avenues suggested above.

It is generally assumed that biliverdin, an intermediate product in the degradation of heme, is reduced to the final product, bilirubin (47). However, the exact steps are not well defined (48, 49). Support for this conversion in vivo has recently been reported by Goldstein and Lester in rats (50) by the isolation of bilirubin C^{14} in bile after the intravenous injection of biliverdin C^{14}.

The duck provides an excellent experimental model in which to study the relationship of bilirubin and biliverdin. In duck bile 80% of the bile pigment is in the form of biliverdin and 20% as bilirubin and both are in recoverable quantities. If the conversion of biliverdin to bilirubin is a random process which takes place at a hepatic or post-hepatic site then the various hemes regardless of source will be partitioned between these two pigments in the same proportion. That their specific activities should be the same regardless of the precursor employed be it heme-C^{14}, Δ ALA 4-C^{14} or glycine 2C^{14}

with their different distribution among the various heme pools. It is of interest then that C^{14} labeled hemin gives rise to a bilirubin of higher specific activity than the biliverdin recovered from the same experiment. A similar relationship is present in the ducks given glycine-$2C^{14}$. In those ducks given $\triangle$ALA-4-C^{14}, however, the specific activity of the recovered biliverdin exceeded that of the bilirubin. These differences suggest that there may be some selectivity in the formation of bilirubin or biliverdin from different hemes or from hemes coming from different sites. It is apparent that infused hemin C^{14} is converted to both pigments. The lower activity of the biliverdin may indicate a preferential conversion to bilirubin or dilution in a relatively larger biliverdin pool. In either case this would represent conversion to the bile pigments at a pre-hepatic site and not post-hepatic reduction of the biliverdin to bilirubin in the biliary tree. That the specific activity of biliverdin exceeds that of bilirubin when $\triangle$ ALA-$4C^{14}$ is the precursor may be evidence for the preferential conversion of those early labeled hemes formed from $\triangle$ ALA to biliverdin. This is not easy to identify in t he duck as the circulating nucleated red cells of birds are capable of converting $\triangle$ALA to heme (51).

It is clear that before one can state that the liver is playing a predominant role in the production of the "early" pigment fraction, it is necessary to ascertain whether the liver is capable, with the exclusion of all other tissue, of synthesizing bilirubin

de novo. This can be done indirectly by hepatectomizing an animal and studying the non-erythropoietic peak. This has met with little success because of the difficulty in keeping the animal alive. The answer can be provided by the isolated liver perfusion. It is possible to keep such a preparation "alive" for several hours and the results obtained in the present study provide conclusive evidence that the liver is so capable.

The bilirubin produced in such a preparationwas of a highly radioactive nature indicating synthesis directly from the Δ ALA 4-C^{14} provided. This also contributes further evidence for the high degree of permeability of the liver cell to Δ ALA. The erythropoietic system was eliminated due to the poor permeability of red cells to Δ ALA (52), in addition to the fact that the liver in the adult rat plays little or no role in normal hematopoiesis. The possibility of non-specific adsorption of ΔALA to "cold" bilirubin produced by the liver was excluded by the addition of the isotope to "cold" bile and isolating the bilirubin. The prepared pigment possessed radioactivity no greater than background.

The liver is also capable of producing bilirubin- C^{14} with such a preparation utilizing glycine C^{14} as the precursor as was demonstrated by Robinson, Owen, Flock and Schmid (53). Although the quantitation of radioactivity was not stated in their report, the results were said to be comparable in magnitude to that seen in the in-vivo bile fistula rats. The specific activity of bilirubin

produced with △ ALA 4-C^{14} would thus be much greater and this provides further evidence for the greater ability of △ ALA to act as a precursor of hepatic heme.

With the knowledge that the liver is capable of synthesizing bilirubin, it remains to demonstrate which of the above suggested possible pathways is the most important. Several experiments have been designed in an attempt to further knowlege in this regard.

Information regarding the precise quantity of hepatic heme is not available. In these experiments it has been assumed that the amount approximates 1 mg and it is clear that in order for heme to be an important contributor to the "early" bilirubin fraction the turnover rate of heme must be rapid. The incorporation of △ ALA 4-C^{14} into hepatic heme occurred quickly reaching a maximum mean value of 12% of the injected counts within 1 to 2 hours of injection.

The rate of incorporation and turnover of hepatic heme is shown in Figure III (b). This of course is a mean value of all the hepatic hemes. It is to be expected that rates of turnover of catalase, the cytochromes, etc., will differ and this is shown in the study of Loftfield and Bonnischen (54). The incorporation of △ ALA 4-C^{14} into hepatic heme reaches the maximum of 12.4% at one hour with a slow fall to 11% at 4 hours and thereafter a gradual decrease to 4.4% at 24 hours.

Preliminary studies of a similar nature in dogs have been recorded by Schwartz and Cardinal (55). They found the maximum of

22.2% of the injected dose in liver heme following Δ ALA 4-C^{14} injection which occurred between 1/2 and 2 1/2 hours after injection. This is approximately 10% higher than that attained in our study which can possibly be explained by the difference in experimental animals and the use of intramuscular Δ ALA 4-C^{14}. This would allow slower absorption rather than a single intravenous "pulse" used in the rat experiments. Heme isolated from the kidney at this time possessed 7.3% of theinjected counts and fell to 11.8% and 1.5% in liver and kidney respectively at 29 hours. In another study, Schwartz and coworkers (56) stated that the number of counts in liver heme was "somewhat greater" than necessary to account for those excreted in the "early" bilirubin fraction.

In the present study, the maximum incorporation of radioactivity in bilirubin had a mean value of 6.25% of injected counts at 2 hours. The total number of counts excreted in 4 hours accounted for 16% of the total injected dose. These figures compare favorably with results obtained in dogs (18, 57) and man (14). Comparing the total counts excreted as bilirubin C^{14} with the total radioactivity present in heme at 1 and 4 hours indicates that not all the bilirubin excreted can be accounted for by liver heme alone and perhaps other non-erythropoietic sources also contribute. The findings of Schwartz would indicate the kidney to be an important source.

Studies using glycine 2-C^{14} as the heme precursor (58) have shown peak activity in rat liver heme 3-5 hours after injection but

the striking feature was the very low incorporation of radioactivity which was in the range of 0.06 to 0.07% of the injected dose. A discrepancy arises here regarding the appearance of bilirubin-C^{14} in bile fistula rats using glycine 2-C^{14} in which labeled bilirubin was maximal at 1 1/4 hours (53).

Several studies on the turnover of hepatic hemoprotein enzymes have been conducted. Theorell and associates in 1951 (40) excluded all these enzymes except liver catalase as possible contributors to the early fraction and described a catalase half-life of 4-5 days. Loftfield and Bonnichsen in 1956 (54) with Fe^{59} provided evidence that catalase has a more rapid turnover exhibiting maximum activity within 4 hours. The catalase radioactivity was first recorded at 1 hour. Similar results were obtained with the cytochromes b and c. More recently more refined techniques have provided further answers. Higashi and Peters (29) using rat liver sub-cellular fractions with leucine-C^{14} to label the protein moiety of catalase, showed a peak specific activity within 30 minutes followed by a similarly rapid fall-off. This interesting study provides evidence that at least 1 molecular type of catalase has a rapid turnover and it suggests a possible relationship to the early bilirubin fraction. Price et al (59) suggested that there may be two populations of catalase molecules, one with a half-life of a few hours and the other of 4-5 days. There have been no studies to determine the outcome of injected labeled catalase, but it is interesting to re-call the work of Kench (46)

who converted catalase and peroxidase into pure biliverdin but was unsuccessful in coverting cytochrome c into bile pigments. It is also of interest that the heme moiety of catalase is the same as and can be used to synthesize hemoglobin (60). Presumably the same degradation reaction could occur. A rapidly synthesized catalase may be partly responsible for the rapid rise in radio-labeled heme in the present study. In view of their small quantity it is unlikely that the other enzymes play a significant role in the production of the non-erythropoietic component.

The evidence presented thus far has mainly supported a catabolic source of the "early", non-erythropoietic component. As previously mentioned, a direct anabolic pathway to bilirubin is also possible. This could occur from heme precursors or heme itself formed in excess of the carrier proteins. The difficulty in obtaining bile pigments from any of the heme precursors including protoporphyrin IX by other investigators has been mentioned. However, Schwartz (61) has recently achieved this in bile fistula dogs which produce large amounts of bilirubin-C^{14} from intravenously administered protoporphyrin-C^{14}.

If the early labeled bilirubin arises primarily from hepatic heme then changes in heaptic heme synthesis shouH be reflected in the early labeled bilirubin. Total hepatic heme synthesis as measured by FE^{59}, 2, $3C^{14}$-succinate and 2-C^{14}-acetate was found to increase in allylisoproplyacetamide (AIA) intoxicated rats (58). A similar increase

in the incorporation of glycine-2C^{14} into hepatic heme in Sedormid rats was found by Schmid and Schwartz (62). These workers however, noted a marked decrease in catalase (63). In the Sedormid animals in this study given Δ ALA-4C^{14} the following was observed:--

(1) No alteration in total bilirubin excretion as compared to normal rats (Figure IV (b)).

(2) Incorporation of C^{14} into bilirubin was similar to the control group.

(3) Incorporation of C^{14} into hepatic heme was less than in the control group.

In interpreting these results one must remember that there is a markedly increased synthesis of Δ ALA in Sedormid porphyria as one of the primary biochemical lesions is the increase of ALA synthetase (64). Thus the lower incorporation into hepatic heme when it is known that hepatic heme synthesis is increased is probably due to dilution in a larger heme precursor pool. Thus the bilirubin labeling is relatively greater in the Sedormid animals as compared to hepatic heme in contrast to the normal. The ratio of incorporated counts (hepatic heme: bilirubin) at one hour in the normal is 2:1, in the porphyric rat, it is 1:2. This is consistent with a relatively greater production of bilirubin from hepatic heme and heme precursors in these animals.

Three-amino 1, 2, 4 triazole (AT) has been shown to inactivate liver catalase apparently without interfering with total

liver heme or catalase synthesis (31, 59). In Price's study (59) there was a delay of approximately 18 hours in which little or no catalase synthesis occurred after the injection of AT. In the present study, injection of ΔALA 4-C^{14} in this 18 hour post amino trizole period did not alter the amount nor pattern of C^{14} labeled bilirubin.

The studies presented indicate that:--

1. The early labeled bilirubin fraction I which appears within minutes of giving 4-C^{14} Δ ALA is in part of hepatic origin and can be synthesized in an isolated rat liver perfusion system.

2. The amount of this pigment produced is greater than can be accounted for by the turnover of hepatic heme.

3. In Sedormid induced porphyria there is a relative increase in this bilirubin fraction as compared with hepatic heme.

4. A protein free hepatic heme which might act as a bilirubin precursor was not found.

5. In ducks there may be some preferential conversion of hemes of different sources to either bilirubin or biliverdin.

*Closing Remarks

I wish to sincerely thank Dr. Lyonel G. Israels for his thoughtful direction and helpful criticisms in the investigation of the above problem. I would also like to thank Mrs. P. Smith for her valuable technical assistance.

Liver Perf.	Bile Vol. (cc)	Time (hrs.)	CPM /mg	% Incorp.
1	0.33	3	91	0.0004
2	0.25	2.5	480	0.0009
3	0.15	3	23,182	0.18
4	0.63	9	142,000	4.7
5	0.065	3	125,510	0.2

Table I. Data on 5 rat liver perfusions.

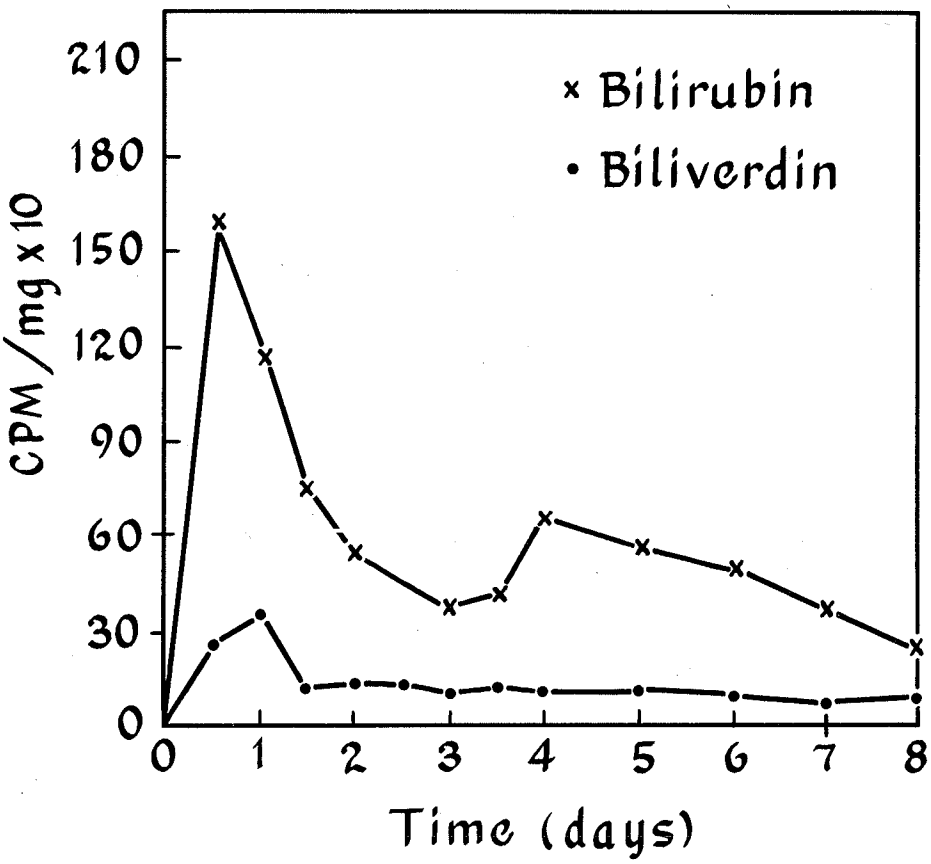

Fig I(a). The specific activity of bilirubin and biliverdin isolated from duck bile following the injection of 20 microcuries of glycine 2-C^{14} at time zero.

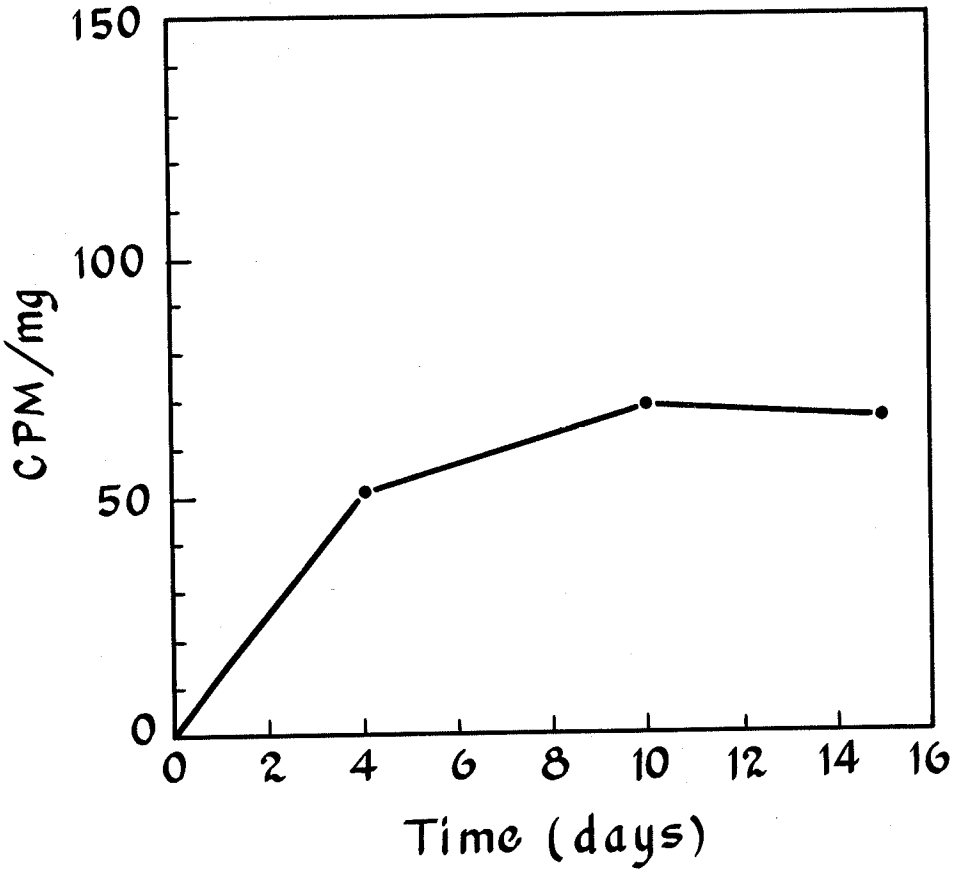

Fig I(b). The specific activity of hemin isolated from duck circulating red blood cells following the injection of 20 microcuries of glycine-2-C^{14} at time zero.

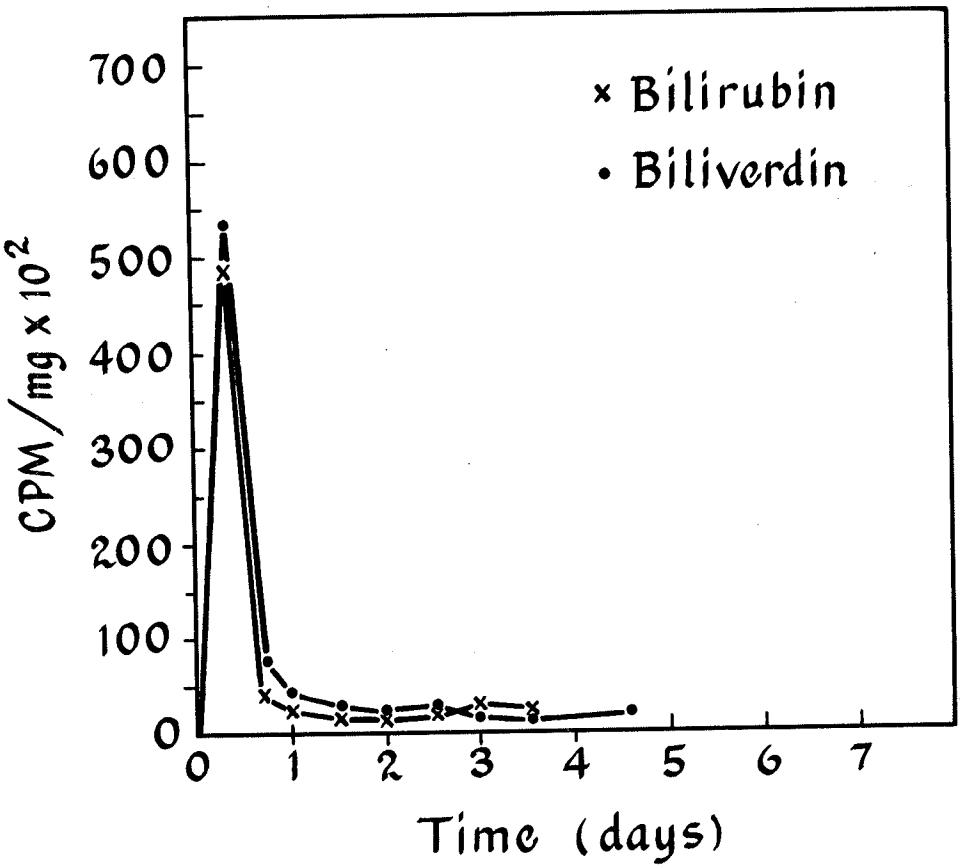

Fig I(c). The specific activity of bilirubin and biliverdin isolated from duck bile following the injection of 5 microcuries of △ ALA 4-C^{14} at time zero.

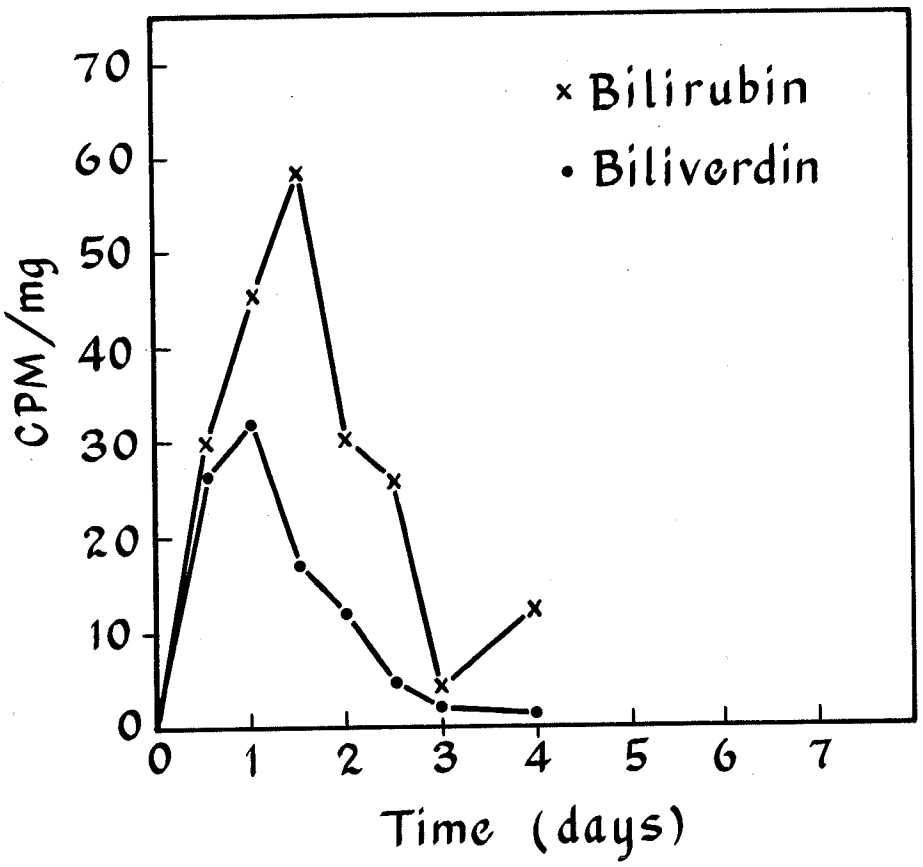

Fig II. The specific activity bilirubin and biliverdin in duck bile following the infusion of a pure hemoglobin-C^{14} solution at time zero.

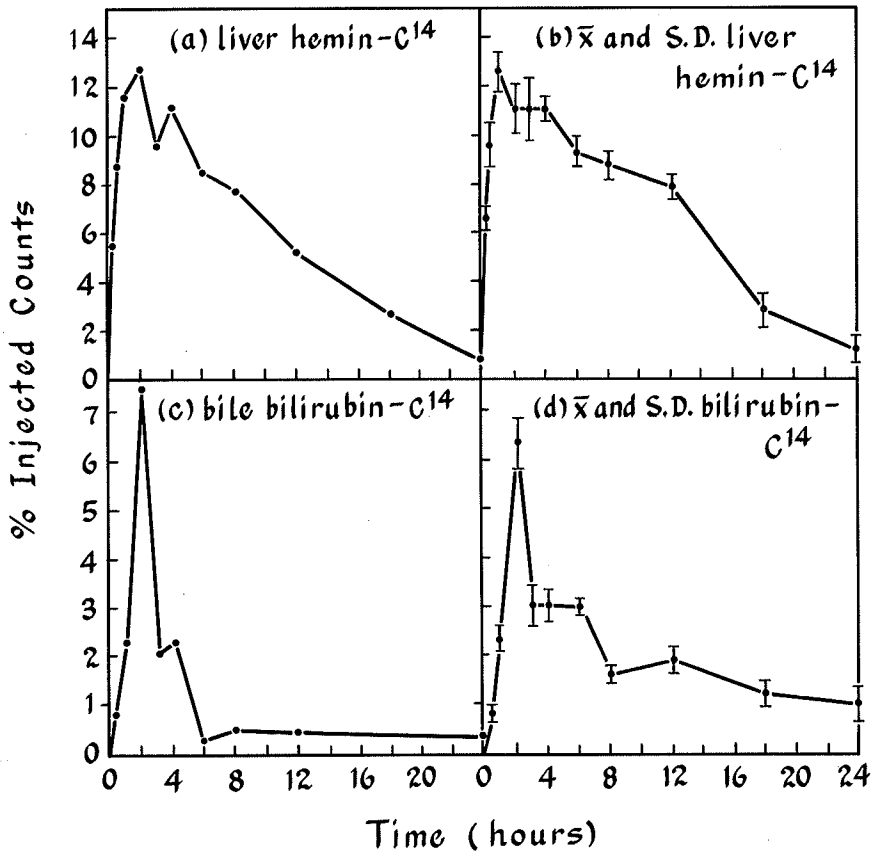

Fig III(a). Ratliver hemin-C^{I4} following the injection of I microcurie ofΔ ALA 4-C^{I4} at time zero.

(b). Mean and standard deviation of rat liver hemin-C^{I4}.

(c). Rat bile bilirubin-C^{I4} following the injection of I microcurie ofΔALA 4-C^{I4} at time zero.

(d). Mean and standard deviation of rat bile bilirubin-C^{I4}.

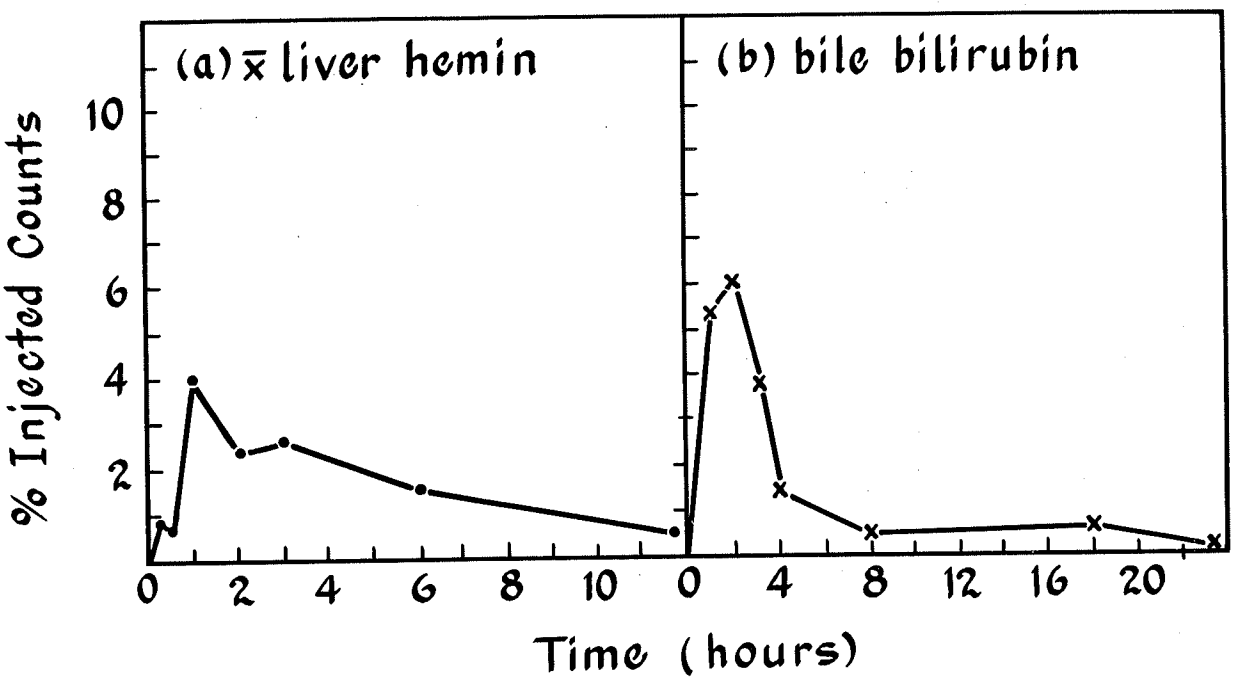

Fig IV (a). The mean percent of injected counts in liver hemin-C^{14} following the injection I microcurie of Δ ALA 4-C^{14} into Sedormid intoxicated rats at time zero.

(b). The percent of injected counts in bile bilirubin-C^{14} of a Sedormid intoxicated rat following the injection of I microcurie of Δ ALA 4-C^{14} at time zero.

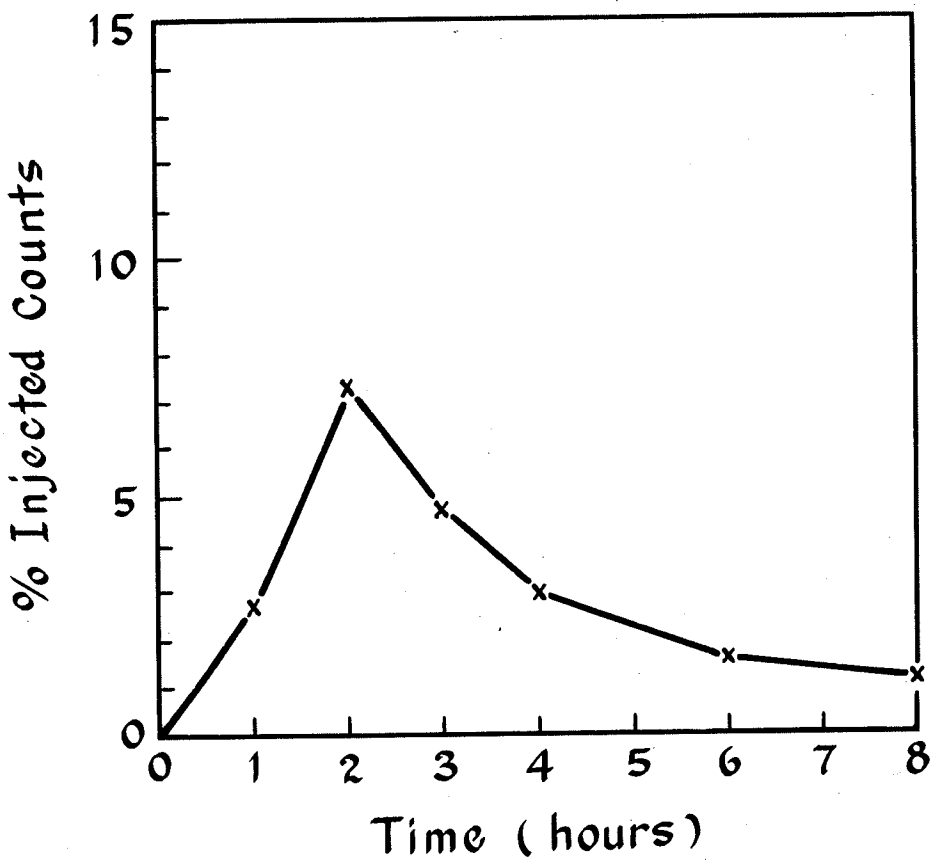

Fig V. The percent of injected counts incorporated into bile bilirubin of a rat fed 3-amino-I,2,4-triazole following the injection of I microcurie of ΔALA 4-C^{14} at time zero.

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