A QUALITATIVE AND QUANTITATIVE STUDY OF THE EARLY LABELLED FRACTION OF BILE PIGMENT

A Thesis

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SECTION I

INTRODUCTION

Bilirubin is a yellow tetrapyrrole which is primarily derived from the degradation of hemoglobin. That bilirubin is a product of heme degradation was first suggested by Virchow in 1847 when he showed that hematoidin, the orange pigment found at the site of old blood extravasations was similar to bilirubin. In 1874 Tarachanoff, using animals with biliary fistulae showed that the injection of hemoglobin caused an increased excretion of bile pigment. That bilirubin and hematoidin were identical was proved by Fisher in 1923 when he was able to synthesize bilirubin and compare its chemical properties with those of hematoidin.

Hawkins, Sribhishaj, Robscheit-Robbins and Whipple in 1931 showed that the prosthetic group of hemoglobin is almost quantitatively converted to bilirubin. This was done by injecting hemoglobin into bile fistula dogs kept at a reduced but relatively stable hemoglobin level for several months by repeated bleeding and measuring the increase in bile pigment excretion following each intravenous injection of hemoglobin. These experiments were repeated by Hawkins and Johnson in 1939 and the same results were noted. In dogs with biliary fistulae

given acetylphenylhydrazine to cause intravascular hemolysis an average of 88 per cent of heme liberated from destroyed red cells was recovered as bilirubin (Cruz, Hawkins and Whipple, 1942).

James, Abbott, Norberg, Birkeland and Evans in 1953 showed that erythrocytes containing N^{15} labelled hemoglobin injected into normal man had a life span of 119 days and that the N^{15} label at the time of cell destruction was detectable only in the fecal stercobilin. Its absence from plasma protein and urinary nitrogen indicated that the heme moiety of hemoglobin was not reutilized. More recently Ostrow, Jandl, and Schmid in 1962 administered ^{14}C labelled hemoglobin as well as sensitized red cells containing ^{14}C labelled hemoglobin to rats with bile fistulae. With sensitized red cells 63-80 per cent of the administered heme was recovered as ^{14}C labelled bilirubin. Similar bile pigment recovery was obtained after injection of large amounts of hemoglobin. When the hemoglobin did not exceed the binding capacity of plasma haptoglobin, conversion to ^{14}C labelled bilirubin was nearly complete. In 1961 Yamaguchi, Nakajima, and Yamaoka, using an in vitro beef liver system were able to convert hemoglobin to bile pigment in vitro.

At the end of the red cell life span of 120 days, the erythrocytes are taken up by the reticuloendothelial system and conversion of the protoporphyrin moiety of hemoglobin to bilirubin takes

place in reticuloendothelial tissue but the relative contributions to this process by the spleen, liver, bone marrow and other organs has not been defined (Harris, 1963). The conversion of hemoglobin to bilirubin proceeds rapidly as labelled bilirubin is detectable in the bile three hours after the injection of ^{14}C labelled hemoglobin (Ostrow, Jandl and Schmid, 1962).

Bilirubin is transported in plasma bound to protein (Ostrow and Schmid, 1963) and the pigment is almost insoluble in protein free aqueous solutions at physiological pH (Burnstine and Schmid, 1962). It has been shown with isotopically labelled bilirubin that the pigment is bound tightly to albumin (Ostrow and Schmid, 1963) and that both albumin and bilirubin exhibit similar patterns of distribution between the intravascular and the extravascular space (Schmid and Hammaker, 1963). The maximum binding capacity is 2 moles of bilirubin per mole of albumin which corresponds to a plasma unconjugated bilirubin of 60-80 mg/100 ml in the normal adult. Thus saturation virtually never occurs. The mode of binding of conjugated bilirubin is not understood although albumin may play an important part (Klatskin and Bungards, 1956).

Bilirubin is taken up from the plasma by the liver and excreted into the gut. The unconjugated bilirubin must be transferred from the plasma across the cell membrane into the hepatic cell,

conjugated, secreted into the lumen of the biliary canaliculus and passed through the biliary tree into the intestine.

The two carboxyl groups of bilirubin are esterified with glucuronic acid forming an alkali labile acylglucuronide (Billing, Cole and Lathe, 1957, Talafant, 1957, and Schmid, 1957) which is polar and water soluble. The reaction is as follows:

Bilirubin + 2 uridine disphosphate glucuronic acid $\longrightarrow$

Bilirubin diglucuronide + uridine diphosphate.

Conjugated bilirubin gives a direct Van den Berg reaction yielding an azo pigment which can be separated by paper chromatography from the azo derivative of the unconjugated pigment (Schmid, Hammaker and Axelrod, 1957). Two direct reacting pigment fractions referred to as Pigment I and Pigment II were found using reverse phase chromatography (Billing, Cole and Lathe, 1957) and it was thought that Pigment I was a monoglucuronide and Pigment II a diglucuronide conjugate of bilirubin, or that Pigment I was a complex of unconjugated bilirubin and Pigment II. Recent evidence suggested that Pigment I is really a complex of unconjugated bilirubin and bilirubin diglucuronide (Weber, Schalm and Witmens, 1963; Gregory, 1963). Approximately 75 per cent of bilirubin is conjugated as glucuronide and about 15 per cent is thought to be conjugated with sulfate. Azo derivatives of bilirubin labelled with ^{35}S have been demonstrated by radioautography and paper

chromatography. The sulfate conjugate is alkali stable, is not hydrolyzed by glucuronidase and gives a direct diazo reaction. About 10 per cent of bilirubin is conjugated with other substances, one of which may be glycine (Isselbacher and McCarthy, 1959).

The transfer of bilirubin from the plasma through the liver and into the bile is not understood but the rate-limiting step seems to be the secretion of pigment from the liver cells into the biliary canaliculi (Arias, Johnson and Wolfson, 1961) and in mammals, excretion depends on prior conjugation (Schmid, Axelrod, Hammaker and Swarm, 1958). Little bilirubin glucuronide is absorbed as conjugation converts the pigment to a water soluble molecule which on reaching the bowel is transformed to colorless urobilinogens by the intestinal flora through a stepwise enzymic reduction. Unconjugated bilirubin is readily absorbed from the bowel (Gilbertsen and Watson, 1962; Lester and Schmid, 1963) and gallbladder (Ostrow and Schmid, 1963). As almost all bilirubin is excreted in the conjugated form by mammals, there is probably little enterohepatic circulation of bilirubin.

Other sources of bilirubin might be the prosthetic group of heme-containing enzymes and myoglobin.

That a pathway of bile formation existed other than that derived from the heme of circulating red cells was first described by

London, West, Shemin and Rittenberg (1950) and Gray, Neuberger and Sneath (1950). When N^{15} labelled glycine was administered orally to human subjects, it was found that the N^{15} glycine was rapidly incorporated into heme reaching plateau activity at about 25 days. This N^{15} concentration began to decline at about 80-120 days as the red cells bearing the label came to the end of their life span. At that time, they noted a rapid rise in the N^{15} concentration of stercobilin which was the derived product of degraded heme. The unexpected finding, however, was the appearance of an early labelled fraction of stercobilin which was noted prior to the appearance of maximal activity in the circulating heme. This fraction was referred to as the early labelled bile pigment, which, because of its early appearance, must have originated from a source other than the heme of circulating red cells. In the normal human subject 11-20 per cent of the bile pigment excreted in the stool as stercobilin was early labelled pigment. This alternate pathway of bile pigment production was found to be greatly increased in certain diseases and London and West,(1950) showed it to be responsible for at least 40 per cent of the bile pigment excreted in one case of pernicious anemia. In congenital porphyria, it may account for 30 to 85 per cent of the bile pigment excreted (London, West, Shemin and Rittenberg, 1950, and Gray, Neuberger and Sneath, 1950). Subsequent studies showed a similar process to be operative in thalassemia (Grinstein, Bannerman, Vavra and Moore, 1960).

It was suggested that this bile pigment arose from one or more of the following sources:

1. Hemoglobin of newly formed red cells destroyed before reaching the peripheral circulation (so-called "ineffective erythropoiesis").
2. Intracorpuscular degradation of hemoglobin in the erythrocyte precursors within the marrow.
3. Heme formed in excess of globin and rapidly converted to bile pigment.
4. A direct synthetic pathway from a common precursor pool that does not require the previous synthesis of heme as an intermediate compound.
5. Other heme containing compounds such as myoglobin, catalase, peroxidase and the cytochromes.

This pathway of bilirubin production has been of such magnitude in some cases as to result in an overproduction type of hyperbilirubinemia in the absence of a reduction in circulating red cell life span. Such cases include a family reported by Israels, Suderman and Ritzman, (1959) as well as cases reported by Arias, (1962), Robinson, Vanier, Desforges and Schmid, (1962) and Berendson, Lowman, Sundberg and Watson, (1964). A similar mechanism may be operative in those cases of primary liver disease as reported by Kalk, (1955) and

Seide, (1957). That this fraction of bile pigment was found to be increased in various disorders of erythropoesis such as pernicious anemia, congenital porphyria and thalassemia suggested it was in some way related to red cell formation and the findings of Gray and Scott, (1959) that this fraction increased following bleeding gave additional support to this concept. A similar increase, however, was reported by James and Abbott, (1961) in patients with erythroid aplasia and they suggested that it may in part have arisen from a non-erythroid source. This study is an attempt to more clearly quantitate and define the site of origin of this early labelled fraction of bile pigment.

SECTION II

THE PROBLEM

The subject matter of this thesis deals with:

1. The quantitation of the early labelled bile pigment.
2. The accurate timing of the excretion of early labelled bile pigment.
3. The relationship of erythropoiesis to the early labelled bile pigment.
4. The effects of agents which alter erythropoiesis or heme synthesis on the early labelled bilirubin fraction.

Previous studies were based on the administration of glycine labelled with either N^{15} or ^{14}C and the subsequent detection of the label in stercobilin in stool. The quantitative study of bile pigment production based on the activity and rate of appearance of the isotope label in stercobilin is hampered by difficulties of stool collection, the difficulties of isolating pure stercobilin in quantity and the delay in its appearance because of the transit time in the gut. In addition the recovery of stercobilin from the stool does not quantitatively reflect the total formation of bile pigment. Given a normal red cell life span of 120 days, with a loss of 1/120 of the red cell mass per day, approximately seven grams of hemoglobin are liberated daily in the

average adult man. This would give rise to 245 mg of stercobilin. Watson (1937) found a daily excretion of 40-280 mg. This loss of stercobilin may be due in part to resorption and in part to degradation via unknown pathways (James, 1955). This has made it difficult to study the early-labelled fraction of bile pigment quantitatively under varying conditions of bilirubin production and red cell life span using stercobilin as the recovered bile pigment. In an attempt to elucidate the production of this early-labelled fraction of bile pigment under varying conditions of erythropoiesis, it was decided to carry out a series of experiments in bile fistula dogs and in human subjects with a draining T-tube in the common bile duct. These studies would allow quantitative collections of bile pigment under controlled conditions. The use of an experimental animal also permits the alteration of erythropoiesis either by stimulation through bleeding or by depression with Busulfan (1,4 dimethanesulfanoxybutane) or total body radiation.

To measure the time and pattern of excretion of early-labelled bile pigments, radioactive compounds which are utilized in the biosynthesis of pyrroles, porphyrins and heme such as $2^{14}C$ glycine and $4^{14}C$ delta aminolevulinic acid (Δ ALA) were used. Figure 1 outlines heme biosynthesis and degradation and shows the labelling pattern for $2^{14}C$ glycine and $4^{14}C$ Δ ALA used in this study.

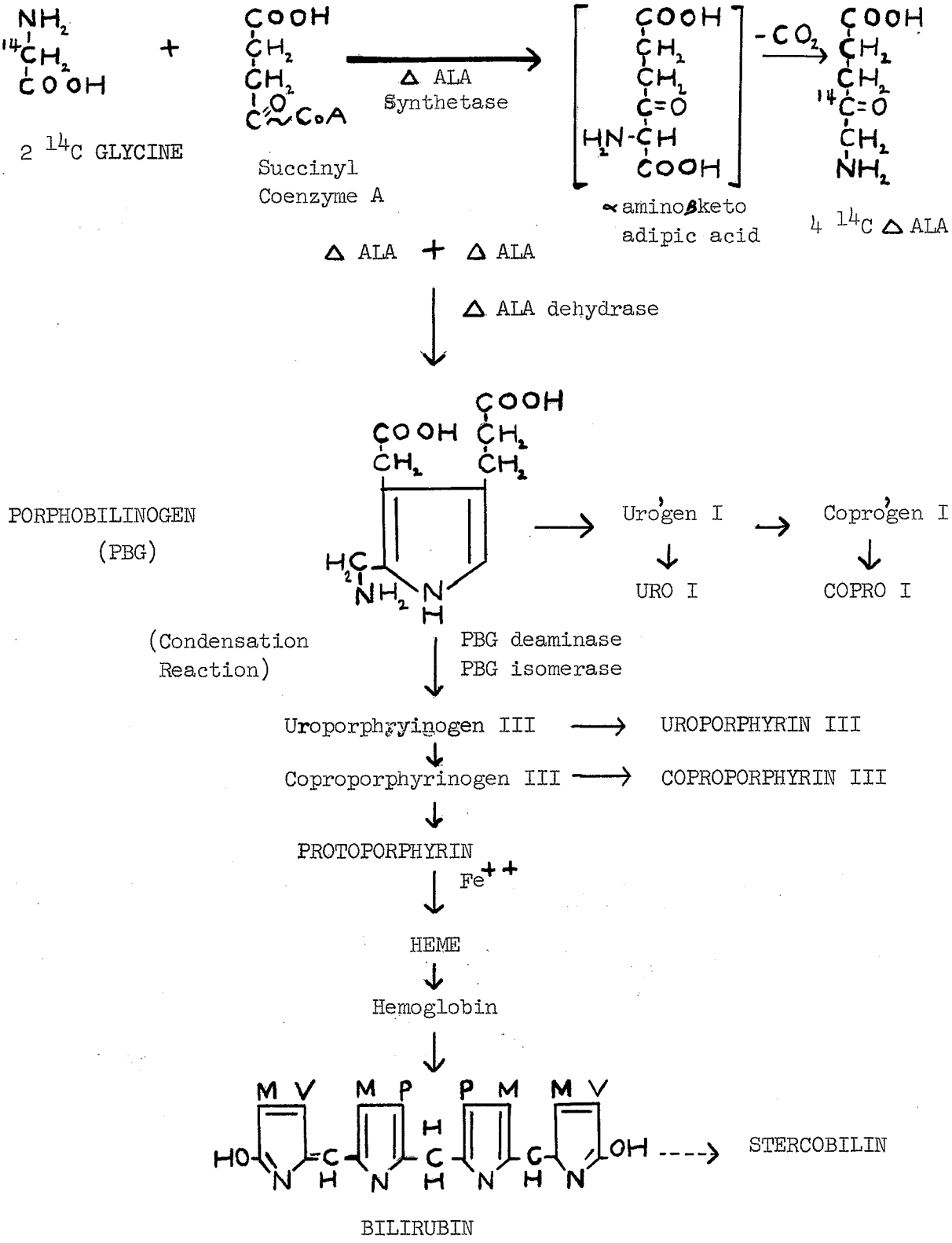

FIGURE 1. An Outline of Heme Synthesis and Degradation.

SECTION III

METHODS AND MATERIAL

Human Subjects

The clinical data on the five human subjects, four of whom had a draining T-tube in the common bile duct, is given in Table I.

Animals

The animals used were healthy mongrel dogs passively immunized against distemper and hepatitis. The total hemoglobin, hemotocrit, reticulocyte count and the total leukocyte count were determined periodically in each dog throughout the experimental period.

Preparation of Biliary Fistulae

In each dog, the common bile duct was ligated and cut, and a polyethylene catheter was introduced into the proximal end. The catheter was led externally through a stab wound in the abdominal wall and the bile was collected continuously in rubber balloons. The dogs were maintained on a meat diet, fed bile salts daily and given 10 mg of 2 methyl-1,4 napthaquinone (Vit. K) intravenously at weekly intervals. The dogs were given ^{14}C labelled glycine or delta amino-levulinic acid three to five days post surgery, when normal bile flow and feeding habits had returned. In some cases it was necessary to

TABLE I

Clinical Data of Human Subjects

	Subject 1	Subject 2	Subject 3	Subject 4	Subject 5
Diagnosis	Hypertension	Cholelithiasis	Cholelithiasis	Cholelithiasis	Cholelithiasis
Sex	Male	Male	Male	Male	Female
Age	82	82	70	38	18
Weight, kg.	112	54	72	75	60
T-tube	No	Yes	Yes	Yes	Yes
Hemoglobin, g/100 ml	15.0	13.4	15.6	15.0	13.2
Bilirubin, mg/100 ml					
Direct	0.2	0.5	0.2	0.8	0.5
Indirect	0.8	0.7	0.7	0.5	0.5

administer fluids subcutaneously or intravenously in order to maintain adequate hydration and bile flow.

Preparation and Administration of Labelled Compounds

The 2^{14} glycine was dissolved in 5 ml of sterile saline. A small aliquot was retained to determine the total number of counts injected into the animal. The $4^{14}C$ delta aminolevulinic acid (ΔALA) was dissolved in sterile saline and the total number of counts present was determined in the same manner as for glycine. The labelled glycine or ΔALA was administered intravenously to avoid differences in intestinal absorption between experimental subjects and to deliver a single rapid pulse of radioactivity to permit accurate timing of the appearance of the label in the isolated products.

Quantitative Determination of Bilirubin

A calibration curve from which concentrations of total bilirubin could be determined was prepared by the following method: approximately 20 mg of bilirubin obtained from the Homburg Chemical Company of Frankfurt, Main, Germany were boiled twice with chloroform and filtered while hot into a small weighed evaporating dish. The chloroform was then evaporated and a known weight of bilirubin, completely soluble in chloroform, was dissolved in 100 ml of chloroform. A series of dilutions ranging from 2 mg to 20 mg per 100 ml was then prepared with absolute methanol and was treated with diazo reagent

according to the method of Malloy and Evelyn, (1937). One ml aliquots of the serial dilutions of the standard were diluted to 10 ml with absolute methanol and 4 ml aliquots pipetted into two tubes containing 5 ml of absolute methanol. One ml of diazo reagent, consisting of 0.3 ml of 0.5 per cent sodium nitrite and 10 ml of a solution containing 1 gm of sulfanilic acid and 15 ml concentrated hydrochloric acid in 1000 ml of distilled water, was then added. Blanks were prepared for each point on the graph by adding 1 ml of the blank containing 15 ml concentrated hydrochloric acid per litre of distilled water. Color development was allowed to occur for 30 minutes and then the solutions were read on a Beckman (Model DU) spectrophotometer at a wave length of 540 mu and at a slit width of 0.05 mm. (Figure 2).

Isolation of Bilirubin from Bile

Bilirubin was isolated from bile by two methods.

Method A (Hawk, Oser and Sommerson, 1947).

At least 5 ml of bile was used. The bile was diluted with two to three volumes of water and an equal volume of five per cent barium chloride was added with stirring plus a few drops of 10 per cent sodium hydroxide. When the precipitate settled the supernatant was removed. The precipitate was poured onto a filter, washed with water, dried and pulverized in a mortar. The powder was extracted successively with warm alcohol, ether and chloroform and then air dried. The precipitate was transferred to a 50 ml centrifuge tube, moistened with 10 per cent

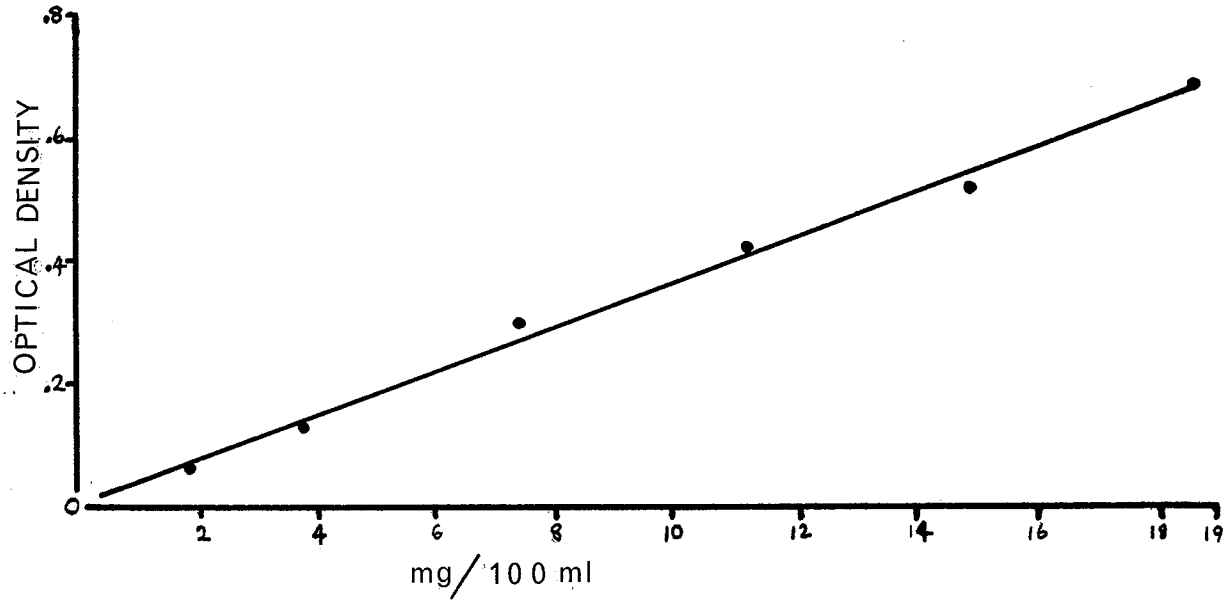

FIGURE 2. Calibration Curve for Total Bilirubin Content.

sulfuric acid and washed three times with small volumes of absolute alcohol. The residue was twice treated in a flask of boiling chloroform, filtered and the chloroform evaporated, avoiding overheating. The bilirubin thus obtained was dissolved in a small volume of chloroform and applied to an alumina column (Schwartz and Watson, 1942). The column was prepared by making a slurry of alumina (BDH) and chloroform which was then poured into a 30 cm chromatographic tube to a depth of 4 cm, tamped with a plunger or vigorously tapped to pack the alumina. The absorbed bilirubin was then washed with chloroform and eluted with one per cent glacial acetic acid in chloroform. The eluted bilirubin in chloroform was then evaporated to dryness and the bilirubin set aside for determination of concentration and specific activity.

Method B (Ostrow, Hammaker and Schmid, 1961)

This method is applicable to small volumes of bile containing as little as 0.2 mg of bilirubin. About 50 mg of ascorbic acid was added to the collecting vessels and the bile samples frozen until the time of analysis.

Bile specimens were thawed and ascorbic acid added to a final concentration of 20 mg/ml. Bilirubin glucuronide was precipitated as the lead salt by addition of one fifth volume of five per cent aqueous acetate. The precipitate was packed by brief centrifugation and the supernatant 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 four volumes of absolute ethanol, centrifuged and the yellow supernatant discarded. After addition of a few milligrams of ascorbic acid the bilirubin glucuronide was hydrolysed by stirring the precipitate with four volumes of 1 N sodium hydroxide and allowing the mixture to stand for twenty minutes at room temperature in the dark.

Glacial acetic acid was added to bring the pH of the mixture to 5.0 and the unconjugated bilirubin was extracted into four volumes 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 which remained 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 volume of one per cent ascorbic acid, once with an equal volume of 10 per cent sodium chloride and four times with water. The chloroform solution was then filtered and applied to an alumina column as described in the previous method of bilirubin isolation.

Isolation of Bilirubin from Plasma

Thirty ml of blood or more depending on the plasma bilirubin and hematocrit were taken using heparin as the anticoagulant. The

blood was centrifuged and the plasma removed. Bilirubin was then isolated from plasma in the following manner.

To the plasma were added 0.18 volumes saturated ammonium sulfate and 2.5 volumes of ethanol. The mixture was shaken well and allowed to stand in the dark at 4°C for one hour. The mixture was then centrifuged at 3000 rpm for twenty minutes and the supernatant decanted. This solution was then evaporated in vacuo at 35° - 40°C. The residue was taken up in chloroform and filtered through chloroform moistened Whatman No. 1 filter paper. The filtrate was then applied to an alumina column packed to a depth of 4 cm. The column holding the adsorbed bilirubin was washed several times with chloroform and the bilirubin was then eluted with chloroform containing one per cent acetic acid. The specific activity and the concentration of the eluted bilirubin were determined immediately.

Determination of the Purity of the Isolated Bilirubin

Since it is known that the main pigment of human bile is present as a diglucuronide conjugate, additional purification methods were undertaken to determine if the radioactivity was in the bilirubin itself or in the conjugating substances. It is known that glycine can be converted to glucuronic acid and that glycine itself may be a conjugating substance. It was thus necessary to determine if the bilirubin prepared from the alumina column was of constant specific activity and

that all the label resided in the bilirubin molecule. This was done by using a column chromatography system which separated conjugated bilirubin from unconjugated bilirubin. Then the specific activity of the bilirubin obtained from serial fractions of the second system was determined and compared with the specific activity of the bilirubin originally obtained from the alumina system. Samples of bilirubin prepared from alumina columns were fractionated on reverse phase Kieselguhr columns according to the method of Cole and Lathe, (1953). As Isselbacher and McCarthy, (1959) indicated that the major portion of human conjugated bile pigment is alkali labile, preliminary alkaline hydrolysis with 0.1 N sodium hydroxide for 30 minutes was performed to hydrolyze any alkali labile conjugates of bilirubin which were present and release pure bilirubin. Thus pure unconjugated bilirubin was obtained in the slow moving non-polar phase of the Kieselguhr column, while any remaining alkali stable bilirubin conjugates were eluted in the fast moving polar or aqueous phase. The fractionated samples were then dissolved in chloroform and one ml aliquots were used for total bilirubin content and plated for determination of ^{14}C activity.

Reverse Phase Chromatography of Bilirubin (Cole and Lathe (1953)

a) Preparation of Kieselguhr

Kieselguhr was treated according to the directions given by Howard and Martin, (1950). Hyflo Super Cel was dried at 110°C and when

cool was allowed to stand in a dessicator containing dichloro-dimethly-silane. The Kieselguhr was then aerated in a fume chamber for 24 hours with frequent stirring. The material was then removed and washed with methanol to a pH of 6.0. Finally it was dried at 110°C and stored in an air-tight container. The material floated when placed in water.

b) Phases Used on Column

The phases to be used on the column were prepared according to the method of Cole and Lathe, (1953). The solvent system used contained chloroform 25 volumes, carbon tetrachloride 25 volumes, absolute methanol 38 volumes and 6 volumes 0.025 M phosphate buffer pH 6.0 plus 6 volumes of water. The phosphate buffers were made according to the method of Hawk, Oser and Summerson, (1949). These constituents were well shaken, stored overnight in a separatory funnel and separated. The bottom phase is the stationary, non-polar one and the top layer is the polar mobile phase.

c) Preparation of Kieselguhr Column

The columns were made ready for use by mixing six grams of Kieselgur with 3 ml of stationary phase, 17 ml of the mobile phase were added rapidly and the material tamped into the column by means of a stainless steel plunger. The column itself was a 1.8 cm diameter glass tube about 15 cm in length with a constriction at the bottom. The bottom of the column was plugged with cotton wool. The bilirubin to be chromatographed was dissolved in a small volume of top phase (polar),

applied to the kieselguhr column and then eluted using top phase.

d) Purity of Prepared Bilirubin

Studies were then carried out to determine the purity of the bilirubin prepared from the alumina column system. In the first phase of this work, the specific activity was determined on the following:

1) bilirubin after alumina column purification;
2) bilirubin not soluble in aqueous top phase of the kieselguhr column after preliminary alkaline hydrolysis;
3) bilirubin after a first chromatographic separation on a kieselguhr column;
4) bilirubin after chromatographic separation on a second kieselguhr column.

Table II shows the results of this study. It may be seen that the relative specific activity of this bilirubin sample remained constant throughout all these procedures, even after chromatographic separation and fractional collection from the second kieselguhr column. This constancy of specific activity suggested that chromatographic separation achieved by the alumina column system was satisfactory and yielded a pure product.

To test this hypothesis, simultaneous determinations of the relative specific activity of bilirubin prepared by both the alumina and kieselguhr columns were performed on four dogs. Figure 3 shows

TABLE II

Constant Specific Activity of Bilirubin Through Various Stages of Purification

Bilirubin Product	Specific Activity cpm per mg
Alumina Column Purification	21.4
Bilirubin Not Soluble in Aqueous Top Phase	20.7
Bilirubin After First Separation by Kieselguhr Column	19.2
Bilirubin After Second Kieselguhr Column	Fraction I - 18.0 Fraction II- 21.5 Fraction III-20.0

the result of this study. It may be seen that the relative specific activity of unconjugated bilirubin prepared by chromatographic separation on the kieselguhr column was only slightly higher than that of bilirubin prepared by the alumina column. In all dogs the shape of the two curves was almost identical. These results indicated that the initial precipitation of bilirubin in alkaline solution and subsequent treatment with chloroform had converted most of the bilirubin to the pure unconjugated form by the time it was eluted from the alumina column. On the basis of these results, it was decided to omit the difficult chromatographic separation and to accept the specific activity of the bilirubin as prepared from the alumina column system.

Plasma bilirubin specific activity was also found to remain constant from the stage of elution from the alumina column through its recovery from the kieselguhr system and the initial alumina column preparation was used throughout this study. Because of the low yield of bilirubin from the plasma in normal subjects, it was not possible to crystallize this bilirubin. To further examine the method, serum was obtained from patients given 2^{14}C glycine with high levels of indirect reacting bilirubin (two patients with shunt hyperbilirubinemia and one with thalassemia). The bilirubin as eluted from the alumina column was dissolved in chloroform and crystallized from cold methanol by the method of Ostrow, Hammaker and Schmid, (1961). The specific activity

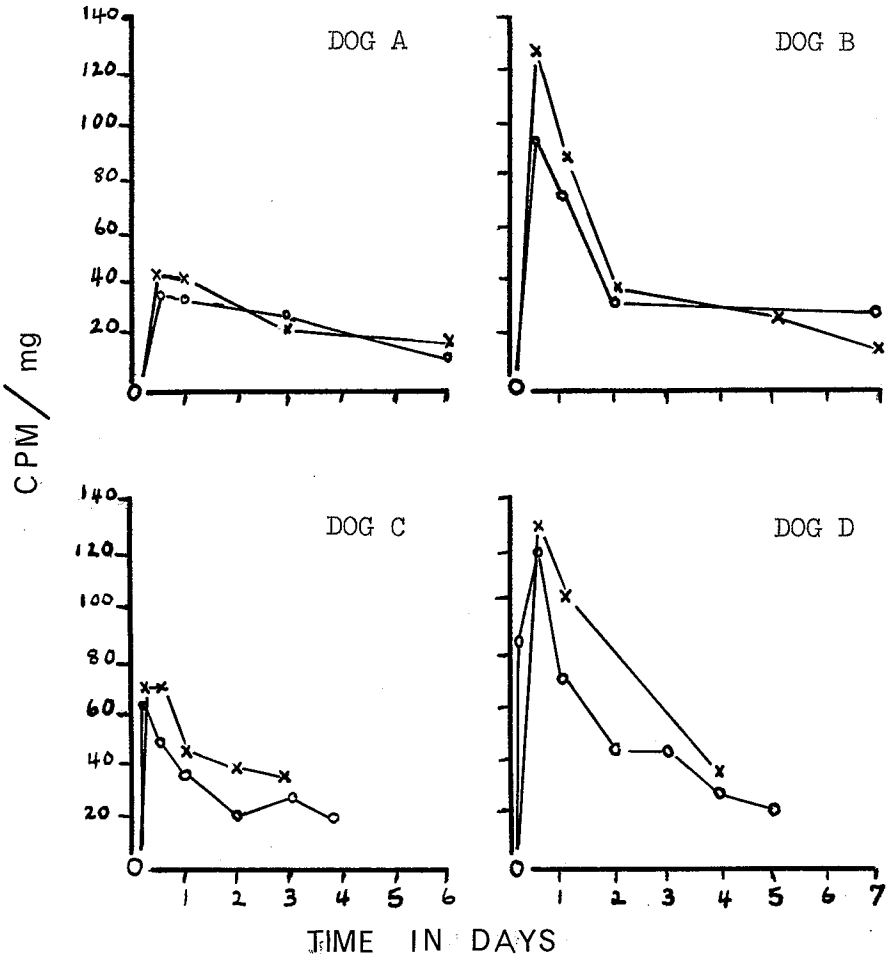

FIGURE 3. Comparison of Specific Activity of Bilirubin Prepared by both Alumina Column and Kieselguhr Column.
Bilirubin Prepared by Kieselguhr Column (x-x)
Bilirubin Prepared by Alumina Column (o-o)

of the samples before and after crystallization was as follows: before (cpm per mg) A, 58; B, 70; C, 56; after (cpm per mg) A, 66; B, 73; C, 40.

Determination of Total Radioactivity Present in Bilirubin

The total bilirubin excretion in the bile was determined from the total volume of bile produced and the quantitative determination of the bilirubin content as measured by the diazo reaction. Knowing the total bilirubin content as well as the specific activity of the individual samples, the total radioactivity appearing in the bilirubin of the bile was easily determined. It was found that the dogs, under the conditions of this study, excreted an average of 4 mg/kg per day of bilirubin. This figure was used to estimate the bilirubin excretion in dogs for which actual quantitative data was not available. The total number of counts excreted in the bilirubin isolated from bile is the summation of the number of counts in each sample.

Hemin Isolation (Delory, 1943)

Twenty ml of heparinized venous blood was centrifuged at 3000 rpm for ten minutes, and the plasma removed. The remaining red cells were diluted to a volume of thirty ml with 0.85 per cent sodium chloride. Ten mg of sodium chloride dissolved in 4 ml of water plus 80 ml glacial acetic acid were heated to 90°C in a water bath. The saline suspension of red cells was filtered through cheesecloth and added slowly with stirring to the above solution. The mixture was kept

at 90°C for fifteen minutes, then allowed to cool and stand overnight. The supernatant was then removed and the crystals resuspended in five per cent acetic acid and allowed to stand for several hours at room temperature. The supernatant was again removed and distilled water was added. The crystals were filtered using a Buchner funnel, washed twice with five per cent hydrochloric acid, three times with 3 N hydrochloric acid to remove free porphyrins and three times with water. As the hydrochloric acid extract exhibited no fluorescence in ultra-violet light, the hemin was considered to be free of contaminating porphyrins. Repeated crystallization produced no change in the specific activity of the hemin. The specific activity of the isolated hemin was determined by dissolving a weighed amount of hemin in 1 ml of 0.1 N sodium hydroxide and plating two 0.5 ml aliquots using stainless steel planchets which were then allowed to air dry before counting. The weight of heme on each planchet was always less than 5 mg as self-absorption of the counts present was exhibited above this weight. The effect of self-absorption is shown in Figure 4 and indicates that valid counting is achieved by plating quantities of 4 mg or less.

Total Radioactivity Incorporated into Hemin of Circulating Red Cells

The blood volume of all dogs used in this study was taken to be 90 ml per kilo. This value was based on that of Altman, (1959) as well as blood volume studies carried out previously in this laboratory.

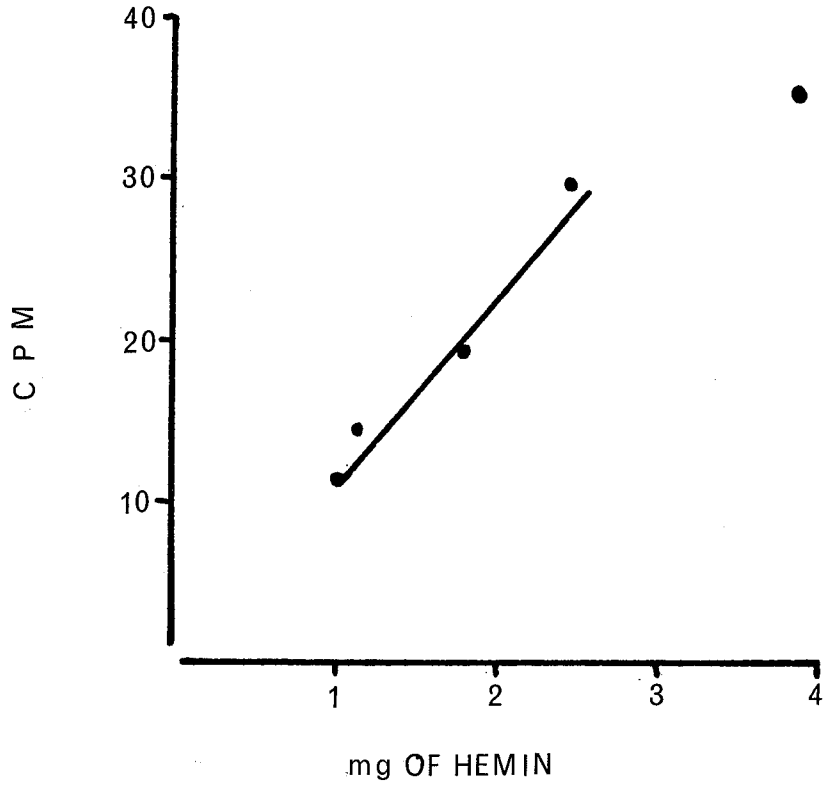

FIGURE 4. The Relationship of Specific Activity to Quantity of Hemin.

This approximation was adequate for the present study.

The molecular weight of hemoglobin is taken as 68,000 and the heme content as 2,608. Thus the total circulating heme in any dog is equal to:

$$\text{Dog weight (kg)} \times 90 \times \frac{2608}{68{,}000} \times \text{Hemoglobin (mg/ml)}$$

The total radioactivity incorporated into circulating heme is then equal to the circulating heme in milligrams times the specific activity of the heme (cpm/mg). The specific activity is taken at the plateau value reached six to eight days following the intravenous injection of the $2^{14}C$ glycine or $4^{14}C\Delta$ ALA.

Size of "Shunt"

Because of their similarity in strucrure, bilirubin and heme probably arise from a common precursor pool. The sum of the total radioactivity appearing in the early labelled bilirubin and the circulating heme approximates the ^{14}C present in this pool.

The percentage of radioactivity found in the excreted bilirubin during the first five days after the injection of labelled glycine relative to the total radioactivity present in both the bilirubin and circulating hemin represents the percentage of this common precursor pool shunted directly to bile pigment. This will be referred to as the "shunt bilirubin". No correction was made for the difference in

molecular weight between bilirubin and heme or for the fact that hemin carries one more carbon atom derived from the alpha carbon of glycine than does bilirubin. The percentage of the common precursor appearing as early labelled bilirubin was based arbitrarily on the labelled bilirubin excreted during the first five days after the administration of labelled glycine. This interval was selected to reduce any possible contribution by short lived circulating erythrocytes containing labelled heme and because the high initial peak in bilirubin specific activity had occurred prior to this time.

Stercobilin Isolation

Stercobilin was isolated by the modified procedure of Watson, Lowry, Sborov, Hollingshead, Kohan, and Matte, (1953). Fifty grams of feces from a well-mixed stool sample were ground up in a mortar with 100 ml of distilled water. The mixture was poured into a one liter Erlenmeyer flask and 100 ml of freshly prepared 20 per cent ferrous sulfate were added and mixed well. Then 100 ml of 10 per cent sodium hydroxide were added slowly with constant swirling. The flask was corked and set aside for approximately three hours, or until the filtrate exhibited no urobilin absorption band at 508 mu. Fifty ml of this ferrous hydroxide filtrate were placed in a large separatory funnel, 100 ml petroleum ether (B.P. 30°C-60°C) were added, and the filtrate was acidified with approximately 5 ml of buffered glacial

acetic acid (four parts glacial acetic acid to one part saturated sodium acetate). After shaking well, the layers were allowed to separate. The aqueous layer was separated and extracted three more times with 50 ml of petroleum ether. The combined petroleum ether solution was filtered and a quantitative Ehrlich reaction was carried out on a 10 ml aliquot. This aliquot was treated exactly as the petroleum ether stage in the regular quantitative method. The 10 ml portion of ether solution was placed in a clean separatory funnel and washed once with a small amount of distilled water, which was discarded. Next were added 2 ml of a modified Ehrlich's reagent[1] followed by vigorous shaking. Six ml of a pure saturated aqueous sodium acetate solution were added and shaken well. The colored aqueous fraction, after complete separation, was removed into a 100 ml graduated cylinder. Repeated extractions of the petroleum ether with Ehrlich's reagent and saturated sodium acetate were continued until the aqueous phase was almost colorless. The combined urobilinogen-aldehyde solution in the graduated cylinder was made up to a convenient volume with distilled water and read in a Coleman Junior Colorimeter at a wave length of 565 mu, against a blank consisting of 3 ml of Ehrlich's reagent and

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[1]700 mg p-dimethylaminobenzaldehyde
150 ml concentrated hydrochloric acid
100 ml distilled water

9 ml of saturated sodium acetate. A standard curve was prepared using a Pontacyl dye standard. Calculation of the urobilinogen concentration is as follows:

$$\frac{\text{Total vol. of } H_2O + FeSO_4 + NaOH \text{ (ml)}}{\text{grams of stool}} \times \frac{\text{Vol. final urobilinogen-aldehyde (ml)}}{Fe(OH)_2 \text{ filtrate used (ml)}}$$

X conc.[2] = Urobilinogen (mg/100 mg of stool)

The entire petroleum ether solution was placed in a large separatory funnel and subjected to iodine dehydrogenation - water extraction as follows. The optimum amount of iodine was approximately 0.45 mg per mg of urobilinogen. The quantity of solution required was prepared by adding the appropriate amount of a stock one per cent ethanolic solution of iodine to 200 ml of distilled water. The petroleum ether solution was shaken with four 50 ml portions of this solution, these being drawn off and pooled. The aqueous solution was orange-yellow and exhibited intense urobilin absorption at 495 mu. After washing twice with ethyl ether, it was acidified to pH 1.0 by addition of 7.5 N hydrochloric acid, after which the urobilin hydrochloride was extracted by repeated shaking with small amounts of chloroform. The latter were combined, filtered through chloroform-moistened filter paper and concentrated under reduced pressure to a

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[2]Concentration in mg of urobilinogen per 100 ml is read off the standard pontacyl curve.

residual syrup of 1 to 2 ml. To this were added several mls of hot acetone which had been used to rinse the suction flask. Crystallization was then allowed to occur. The resulting material was dissolved in chloroform and plated on planchets which were weighed before and after plating and counted in a gas flow counter to determine the specific activity.

Absorption curves were run on a sample of urobilin prepared by the above method. The acid urobilin gave a maximum absorption peak at 490 mu, and the alkaline urobilin gave a maximum absorption peak at 510 mu. These peaks are characteristic of urobilin.

Protoporphyrin Isolation from Bile

Protoporphyrin was prepared from bile using the method of Schwartz and Wikoff, (1952). Five ml of bile were added to 15 ml of water in a separatory funnel. Total porphyrin was extracted using three 75 ml portions of a 4:1 ethyl acetate and acetic acid mixture. The combined extract was washed once with 0.005 per cent iodine in three per cent sodium acetate to reduce any porphyrinogens present. The extract was then washed three times with three per cent sodium acetate and once with water. The ethyl acetate solution was then extracted with 5 ml portions of 0.1 N hydrochloric acid until no fluorescence was present in the hydrochloric acid extract. This removed any coproporphyrin present in the solution. The ethyl acetate

was then extracted with several 5 ml portions of 1.5 N hydrochloric acid to remove the protoporphyrin. The 1.5 N hydrochloric acid solution containing the protoporphyrin was then neutralized with saturated sodium acetate and extracted with 50 ml of ethyl acetate. The ethyl acetate was then washed once with ten per cent sodium chloride, once with water, filtered and evaporated in vacuo.

Esterification of Porphyrins

Protoporphyrin was esterified using the method of Schwartz, Hawkinson, Cohen and Watson, (1947).

The protoporphyrin residue from above was dissolved in 50 ml of a 20:1 methanol and sulfuric acid mixture and allowed to stand overnight. Two volumes of chloroform were added and the solution was washed successively once with four volumes of cold two per cent sodium hydroxide, five times with one-half volume of seven per cent sodium chloride and twice with one volume of water. The chloroform solution and then filtered and evaporated to dryness in vacuo.

Chromatography of Protoporphyrin

Since porphyrins are notoriously difficult to isolate in a pure state, it was thought necessary to purify the protoporphyrin ester using chromatographic techniques. Column chromatography of the porphyrin esters was performed using aluminium oxide grade II as the adsorbent. Chloroform-light petroleum ether mixtures were used to

elute the porphyrin esters (Nicholas and Rimington, 1953).

a) Preparation of Grade II Aluminium Oxide

Aluminium oxide was suspended in water, filtered, dried at 120°C and cooled in a dessicator.

b) Preparation of Solvents

1) Petroleum ether redistilled between 40°C - 60°C.

2) Chloroform containing one per cent absolute methyl alcohol.

c) Preparation of Column

Glass tubes 5 mm in diameter which tapered at one end were provided with a glass wool plug. The lower end was closed by a stopper and the glass tube almost filled with solvent (an 8:1 mixture of petroleum ether and chloroform). The adsorbent was then sprinkled in and the column was lightly tapped to ensure adequate packing. The finished column was approximately 6 cms in height. The stopper was then removed and the column allowed to drain.

The protoporphyrin ester, dissolved in as small a volume as possible of chloroform containing one per cent methanol was then applied to the column. Elution of the adsorbed porphyrins took place as chloroform-petroleum ether solutions of increasing chloroform concentration were applied to the column. As we were interested only in protoporphyrin, which was eluted by a 6:1 mixture of the above solvents,

only this elutant was used, the other porphyrin esters remaining on the column, while the protoporphyrin ester was eluted. The elutant containing the protoporphyrin ester was then evaporated in vacuo. For determination of specific activity, the protoporphyrin ester was dissolved in a small amount of chloroform of which two 1 ml aliquots were plated and a 1 ml aliquot was retained to determine the concentration of protoporphyrin ester. The porphyrin concentration was assayed by determining the adsorbance at 408 mu using the Beckman DU spectrophotometer with reference to an experimentally determined calibration curve.

Preparation of Protoporphyrin Methyl Ester

As pure protoporphyrin could not be obtained commercially, it was prepared according to the method of Fischer and Peitzer, (1926). Three hundred grams of 95 - 100 per cent formic acid were added to six grams of hemin. After heating to boiling, a total of six grams of powdered (reduced) iron was added to one gram portions every five minutes. Heating was continued for fifteen minutes. After cooling, the solution was filtered and two to three volumes of water were added to precipitate the protoporphyrin. The yield was increased by the addition of ammonium acetate. The precipitated protoporphyrin was then esterified and chromatographed as described.

Calibration Curve of Protoporphyrin Concentration

The absorption maximum of this preparation of protoporphyrin methyl ester dissolved in chloroform was found to be 408 mu using the Beckman DU spectrophotometer. A weighed amount of protoporphyrin ester was dissolved in chloroform and the absorption of serial dilutions measured at 408 mu and a calibration curve constructed, as shown in Figure 5.

Bone Marrow Studies

Bone marrow aspirations were performed in some dogs and in one human subject at four to eight hours after the injection of the labelled glycine or ΔALA. The marrow was obtained from the posterior iliac crest using a Bierman needle. The buffy coat was removed and total and differential cell counts performed on both the buffy coat layer and packed red cell layer. Hemin was isolated from both layers by the techniques described and the specific activity determined.

Counting Techniques

Samples were counted using a Nuclear Chicago C-115 low background Gas Flow counter with an efficiency of 20 per cent. All samples were either counted at infinite thinness or corrected for self-absorption as described.

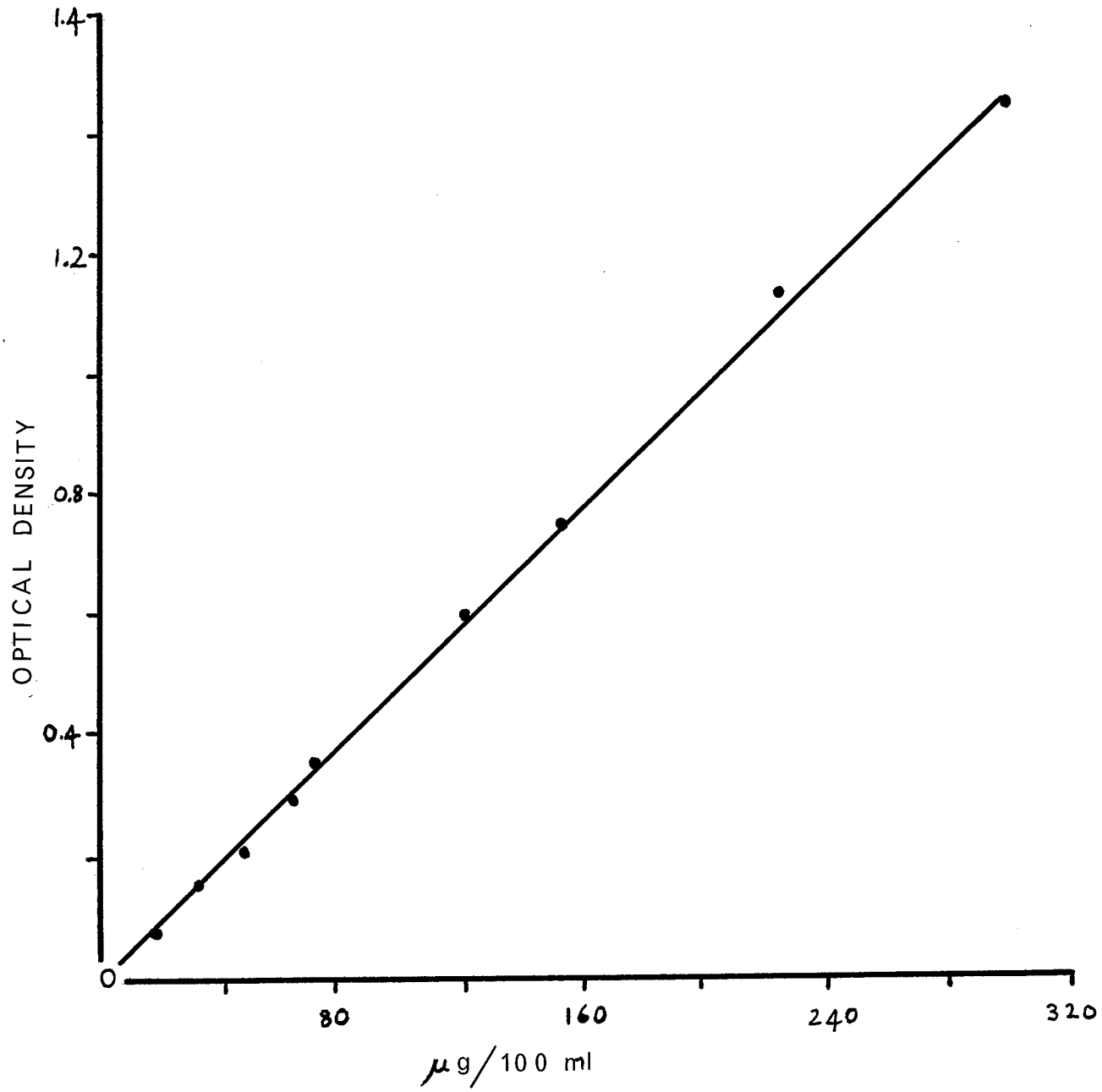

FIGURE 5. Calibration Curve for Total Protoporphyrin Methyl Ester Content.

SECTION IV

RESULTS

Normal Dogs

The appearance of radioactivity in the bilirubin and hemin of four normal dogs is shown in Figure 6 and Table III. It will be noted that the radioactivity appeared rapidly in the bilirubin and was present with a specific activity of 11 to 95 counts/min. per mg at 4 to 8 hours following the intravenous administration of 2 ^{14}C glycine. The maximum specific activity in all four dogs occurred within the first 24 hours and fell thereafter. The specific activity of the heme increased slowly over the first six to eight days and remained at a plateau value until the experiment was terminated. Radioactivity of hemin prepared from the circulating red cells at four and eight hours after the injection of 2 ^{14}C glycine was 0.8 and 0.16 counts/min. per mg in Dogs 1 and 2. In Dog 2, the hemin prepared from the buffy coat of the marrow at eight hours had a specific activity of 2.6 counts/min. per mg while the hemin prepared from the peripheral blood at eight hours had a specific activity of 0.16 counts/min. per mg. Dog 3 incorporated 0.08 per cent of the total injected counts into bilirubin and 2.42 per cent into circulating heme while Dog 4 incorporated .034 per cent of total injected counts into bilirubin and 0.49 per cent into circulating heme.

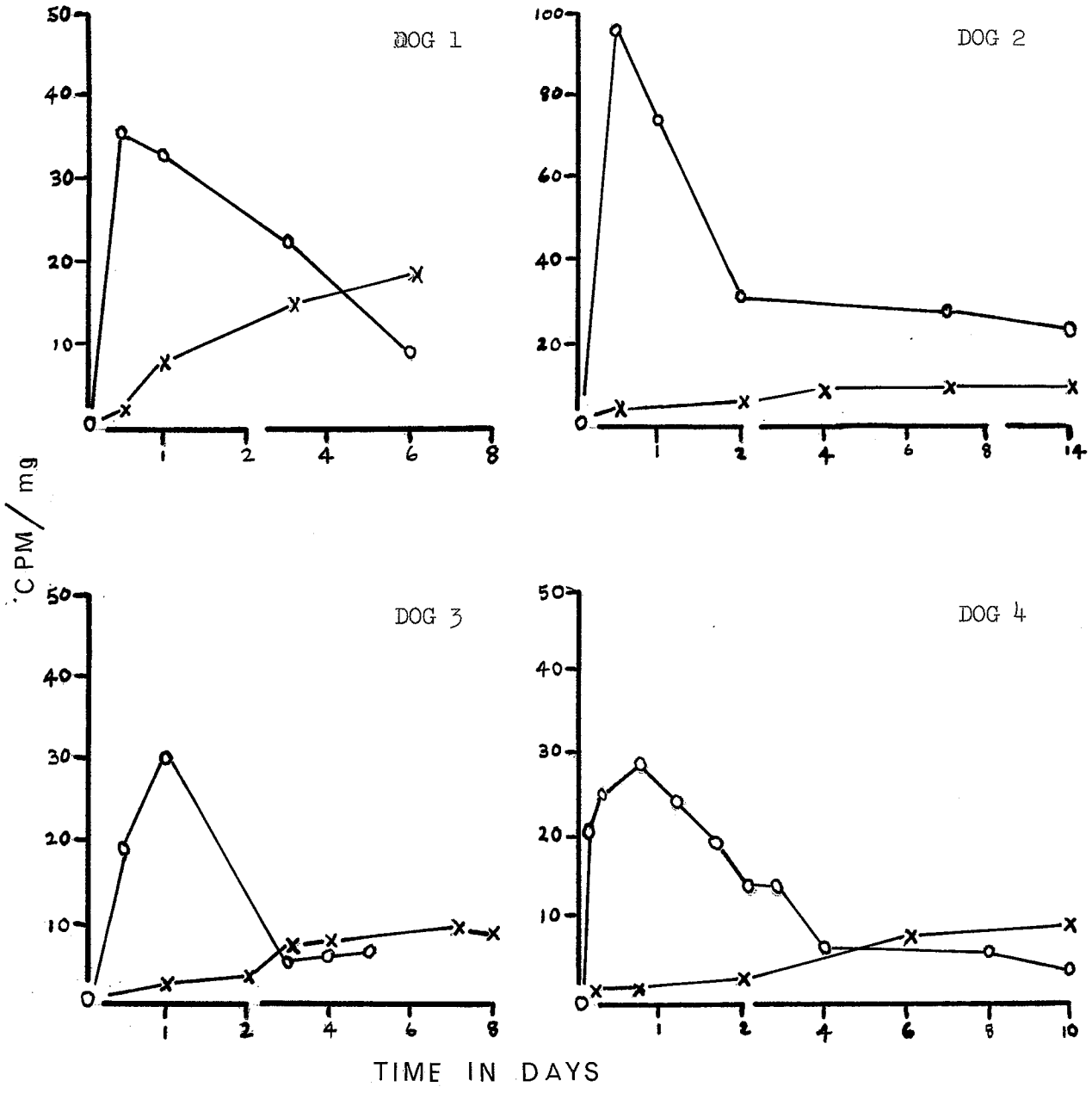

FIGURE 6. The Specific Activity of Bilirubin Prepared from Bile and Hemin from Peripheral Blood in Four Normal Dogs with Biliary Fistulae. The Dogs were Given 2 ^{14}C Glycine Intravenously at Zero Time. Bilirubin (o-o). Hemin (x-x).

TABLE III

Radioactivity in Bilirubin and Hemin of Normal Dogs and Human Subject After Intravenous Injection of 2 ^{14}C Glycine

Subject	Wt. (kg.)	Time after 2 ^{14}C Glycine (Hours)	Specific Activity CPM/mg			Total CPM ^{14}C Injected	Per Cent of Total Injected Counts		Shunt Size
			Hemin (Blood)	Hemin (Marrow)	Bilirubin		Bilirubin	Hemin	
Dog 1	14	8	0.8	-	34.6	-	-	-	5%
Dog 2	19	8	0.16	2.6	95	-	-	-	16%
Dog 3	32	4	-	-	11	5×10^6	.008	2.42	3.5%
Dog 4	10.6	3	-	-	20.4	-	.034	.49	6.5%
Patient M.O.	45	5	0	0	27	-	-	-	16%

The shunt size of Dog 3 was 3.5 per cent and that of Dog 4 was 6.5 per cent. Thus, even though Dog 4 incorporated less of the total injected radioactivity, the relative incorporation into heme and bilirubin remained the same. The shunt size of Dog 1 was five per cent and that of Dog 2 was sixteen per cent as shown in Table III.

In three of the four normal dogs studied, the bilirubin specific activity had dropped to one third or less of the peak value by day 4. The peak bilirubin specific activity in the four normal dogs studied occurred within 24 hours after the injection of 2 ^{14}C glycine. Although the data does not permit one to state the exact timing, in normal dogs it would appear to be between 12 and 24 hours after 2 ^{14}C glycine injection. The specific activity of the bilirubin then dropped to a plateau value of one third of the maximum by day 4 in Dogs 2, 3 and 4. Dog 1 may have reached a plateau but no sample was obtained on day 4.

Human Subject

The pattern of incorporation of 2 ^{14}C glycine in a human subject is shown in Figure 7. This 73 year old woman had previously had a cholecystectomy. A T-tube had been left in place but because of a stone in the common duct all the bile drained to the exterior with an average volume of 700 ml in 24 hours. Stools were clay colored. No biochemical evidence of hepatic dysfunction was present. Following the

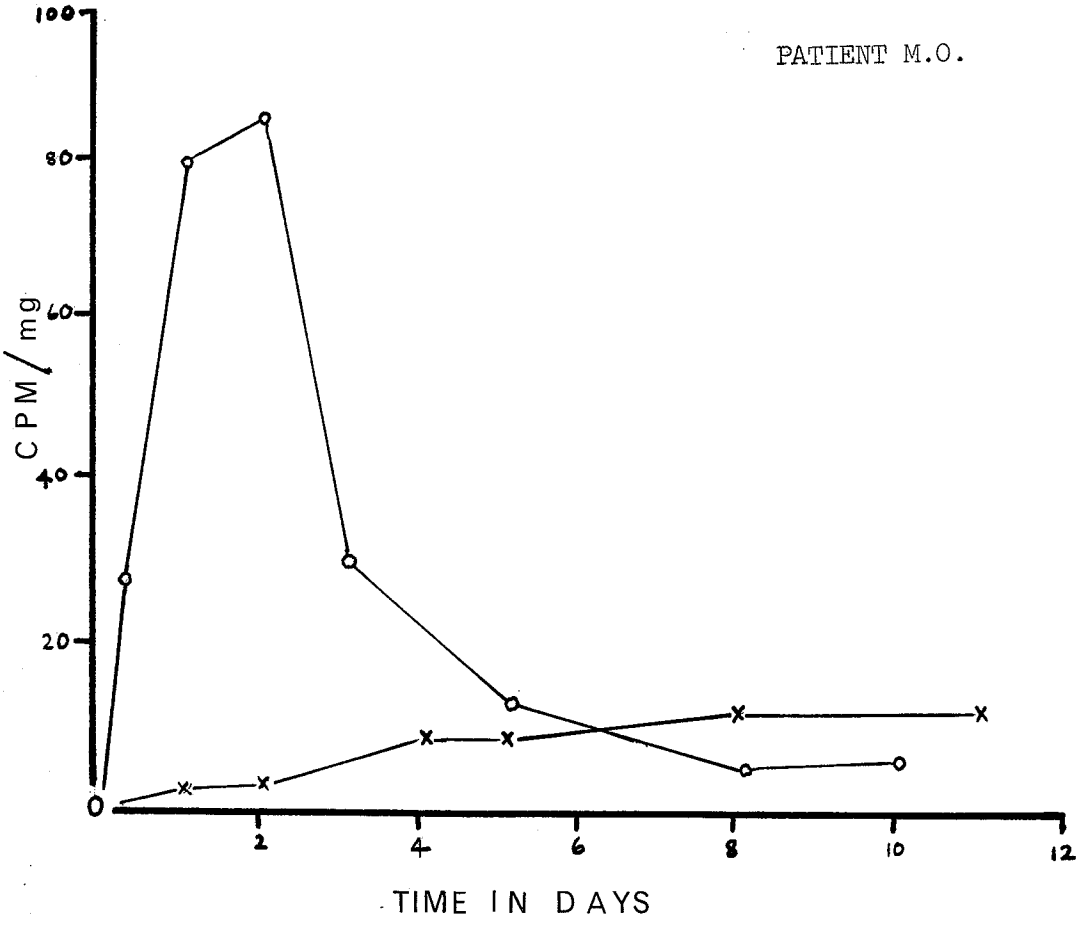

FIGURE 7. The Specific Activity of Bilirubin Prepared from Bile and Hemin Prepared from Peripheral Blood in a Normal Human Subject Given 2 ^{14}C Glycine Intravenously at Zero Time. Bilirubin (o-o). Hemin (x-x).

present study, the common duct was re-explored and the complete obstruction of the common duct confirmed. The calculated shunt size in this patient was 16 per cent and the pattern of early labelled bilirubin was similar to that shown in normal dogs although the peak specific activity occurred between 24 and 48 hours rather than at 24 hours or earlier as in the normal dogs studied. Note that at four hours, there was no activity in circulating red cells nor marrow heme but the bilirubin isolated from bile had reached a specific activity of 27 counts/min. per mg. The bilirubin specific activity then rose to a peak of 85 counts/min. per mg on day 2, and then decreased to 10 counts per minute per mg by day 4.

The incorporation of 2 ^{14}C glycine into heme reached plateau values of 18 counts/min. per mg by day 8. The shunt size was calculated to be 16 per cent which was comparable to that of the normal dogs studied.

Altered Erythropoiesis

I. Bleeding

To assess the effects of stimulating erythropoiesis on the rate of appearance and amount of early labelled bilirubin, two dogs were bled at the time of surgery. Table IV shows the weight of the animal, the volume of blood removed and the rise in reticulocyte count after bleeding. The reticulocyte count shown was that present on the day

TABLE IV

Reticulocyte Response of Bled Dogs

Dog	Weight	Blood Taken	Reticulocytes (Per Cent) Pre-bleeding	Post-bleeding
5	19 kg	550 ml	0.8	5.0
6	23 kg	685 ml	0.8	3.0

the 2 ^{14}C glycine was given. The incorporation of labelled glycine into the heme and bilirubin of these dogs is shown in Figure 8 and Table V.

Both dogs showed a rapid incorporation of 2 ^{14}C glycine into bilirubin within the first six hours. The specific activity of the bilirubin rose to a peak within 24 hours and then fell sharply to about one third of its original value by 48 hours and a shoulder was noted on the curve in both dogs. This activity was maintained for two to four days by Dog 5 and Dog 6 and the activity then fell to low values.

This curve of bilirubin specific activity differs from the normal curve in that none of the normal dogs show a pronounced shoulder of radioactivity after 24 hours as do the bled dogs. The active erythropoiesis drew 4.82 per cent of the injected dose of glycine into the hemin pool in Dog 5 and 3.8 per cent in Dog 6 as compared with 2.42 per cent in the normal (Dog 3). The hemin prepared from the buffy coat of a marrow aspirate done four hours after giving the labelled glycine, showed a relatively high specific activity of hemin in the buffy coat (7.0 counts/min. per mg in Dog 5). The amount of the injected 2 ^{14}C glycine appearing in the bilirubin was 0.18 per cent and 0.20 per cent of the injected activity accounting for 3.5 and 5 per cent of the calculated precursor pool. The active erythropoiesis then may have drawn more radioactive glycine into both circulating heme and bilirubin than in the normal but the percentage of the precursor pool

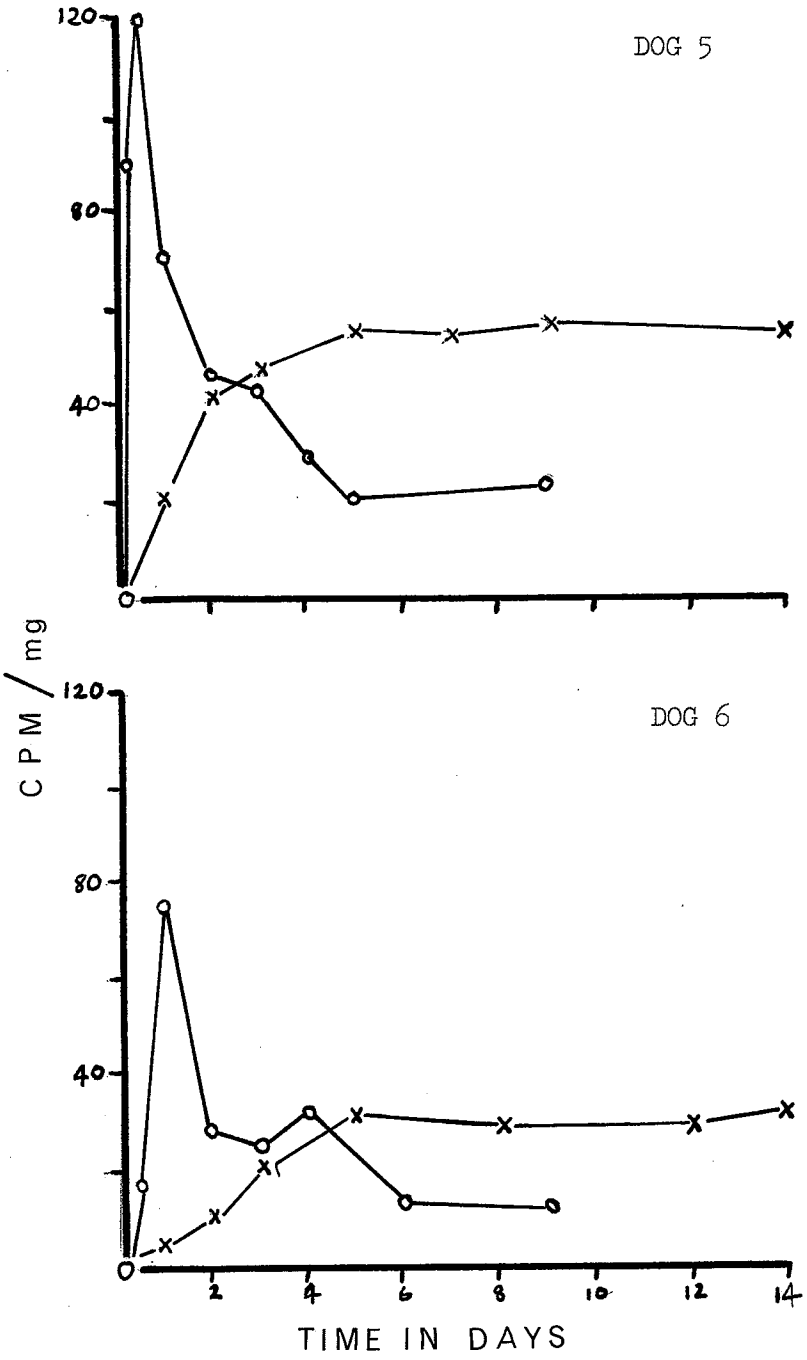

FIGURE 8. The Specific Activity of Bilirubin Prepared from Bile and Hemin Prepared from Peripheral Blood in Two Bled Dogs with Biliary Fistulae. The Dogs were Given 2 ^{14}C Glycine Intravenously at Zero Time. Bilirubin (o-o). Hemin (x-x).

TABLE V

Radioactivity in Bilirubin and Hemin after Intravenous Injection of 2 ^{14}C Glycine into Bled Dogs

Dog	Time after 2 ^{14}C Glycine in Hours	Specific Activity (CPM/mg			Per Cent of Injected Counts		Shunt Per Cent
		Hemin (blood)	Hemin (marrow)	Bilirubin	Heme	Bilirubin	
5	4	5	7	85	4.82	0.18	3.5
6	6	1.1	-	13	3.80	0.20	5.0

diverted through the shunt was comparable to that of normal dogs. Note that the shunt was quantitatively determined in Dog 5 while the shunt in Dog 6 was calculated using an assumed average bilirubin excretion of 4 mg/kg per day.

II. Busulfan

To assess the effect of marrow depression, Dogs 8 and 9 were given the alkylating agent Busulfan (20 mg/kg and 15 mg/kg respectively) subcutaneously in sesame oil immediately following surgery. The reticulocyte counts were followed until they reached zero per cent on day 5. The dogs were then given 2 ^{14}C glycine intravenously and the appearance of the label in the bilirubin and in the hemin was determined. The results are shown in Figure 9. Dog 8 received 20 mg per kg of Busulfan and sustained a marked depression in leukocyte and reticulocyte count. The depression in erythropoiesis and heme synthesis was also reflected in the marked impairment of ^{14}C incorporation into hemin which reached a maximum specific activity of only 1.5 counts/min. per mg on day 7. The label, however, appeared rapidly in the bilirubin, reached a peak activity of 61 counts/min. per mg at 8 hours and then declined. A bone marrow aspiration done at 8 hours following the administration of 2 ^{14}C glycine showed 0.45 counts/min. per mg in the hemin of the buffy coat. The calculated shunt in this dog was 37 per cent. Dog 9 received a somewhat lower dose of Busulfan and heme

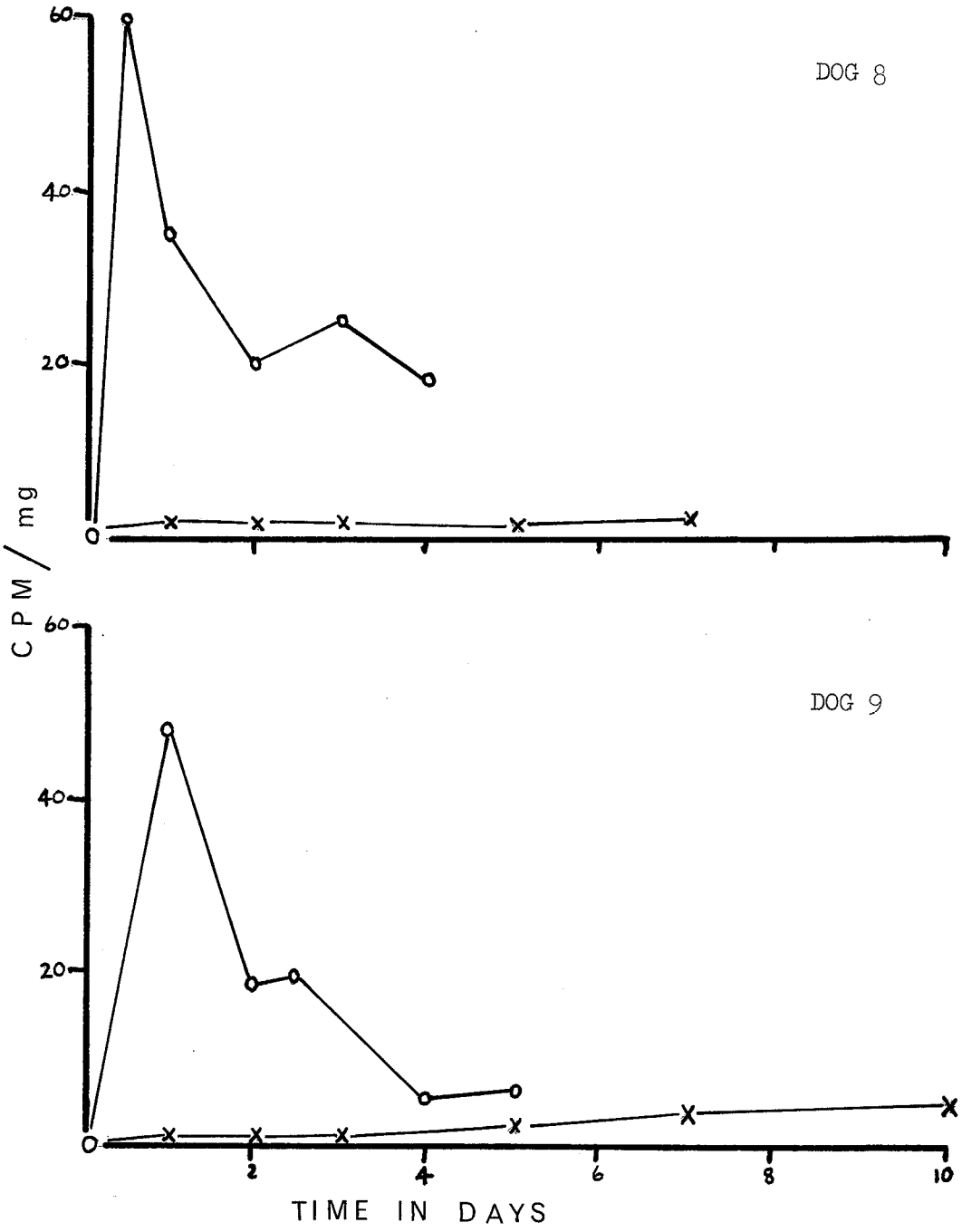

FIGURE 9. The Specific Activity of Bilirubin Isolated from Bile and Hemin Prepared from Peripheral Blood in Two Dogs Given Busulfan. Bilirubin (o-o). Hemin (x-x).
The Dogs were Given 2 ^{14}C Glycine Intravenously at Zero Time.

synthesis was less depressed reaching a plateau value of four counts/min. per mg on the tenth day. The bilirubin reached a maximum activity of 98 counts/min. per mg at four hours. In Dog 9, only 0.66 per cent of the total injected counts appeared in circulating hemin while 0.22 per cent went rapidly to bilirubin with a calculated shunt size of 25 per cent. In both dogs, the peak activity of bilirubin was present at four and eight hours respectively. As this is the first determination of bilirubin activity, the peak specific activity may have occurred sooner. Both dogs showed a rapid decrease in bilirubin specific activity eight hours after the 2 ^{14}C glycine pulse to a plateau value within two days. The more rapid appearance and disappearance of early labelled bilirubin is in contrast with that found in dogs with normal or stimulated erythropoiesis in which the bilirubin specific activity does not reach its peak until 12 to 24 hours after the injection of 2 ^{14}C glycine and does not decrease as rapidly.

III. Radiation

Dogs 10 and 11 were given 700 ♥ of total body radiation immediately following surgery. In both dogs, the reticulocyte count fell to zero on day 4 and reticulocytes did not reappear in the peripheral blood for the duration of the experiment. The 2 ^{14}C glycine was given on the seventh post-radiation day and the incorporation of ^{14}C into bilirubin and hemin determined as in the other animals. Dog 12

received 500 r two days post-surgery. The reticulocytes fell from 11.1 per cent to 0.8 per cent on the sixth day post-radiation and did not rise for the duration of the experiment. The radioactive glycine was administered on the sixth day post-radiation.

In Dogs 10 and 11, the total leukocyte count had fallen to 500 per cu. mm. and the reticulocytes to zero at the time the 2 ^{14}C glycine was given on the seventh day after radiation. The relative specific activity of the bilirubin rose to 170 counts/min. per mg in Dog 10 and 70 counts/min. per mg in Dog 11, within four hours and then declined rapidly as shown in Figure 10. In Dog 12, peak bilirubin specific activity occurred at 3.5 hours (Figure 10). The peak in this dog is well delineated as there are three points on the graph in the first five hours. The specific activity declined rapidly after 3.5 hours. The hemin reached a specific activity of two counts/min. per mg on day five. In Dogs 10 and 11, bone marrow aspiration yielded hypocellular marrow with fewer than 1000 nucleated cells per cu. mm. in the concentrated buffy coat. In Dog 10, the hemin prepared from the peripheral blood had a specific activity of 0.5 counts/min. per mg and that of the bone marrow 0.65 counts/min. per mg four hours after the administration of the 2 ^{14}C glycine. The absolute incorporation of the total injected counts into hemin was 0.031 per cent and into bilirubin 0.102 per cent with a shunt of 76 per cent. In Dog 11, no radioactivity

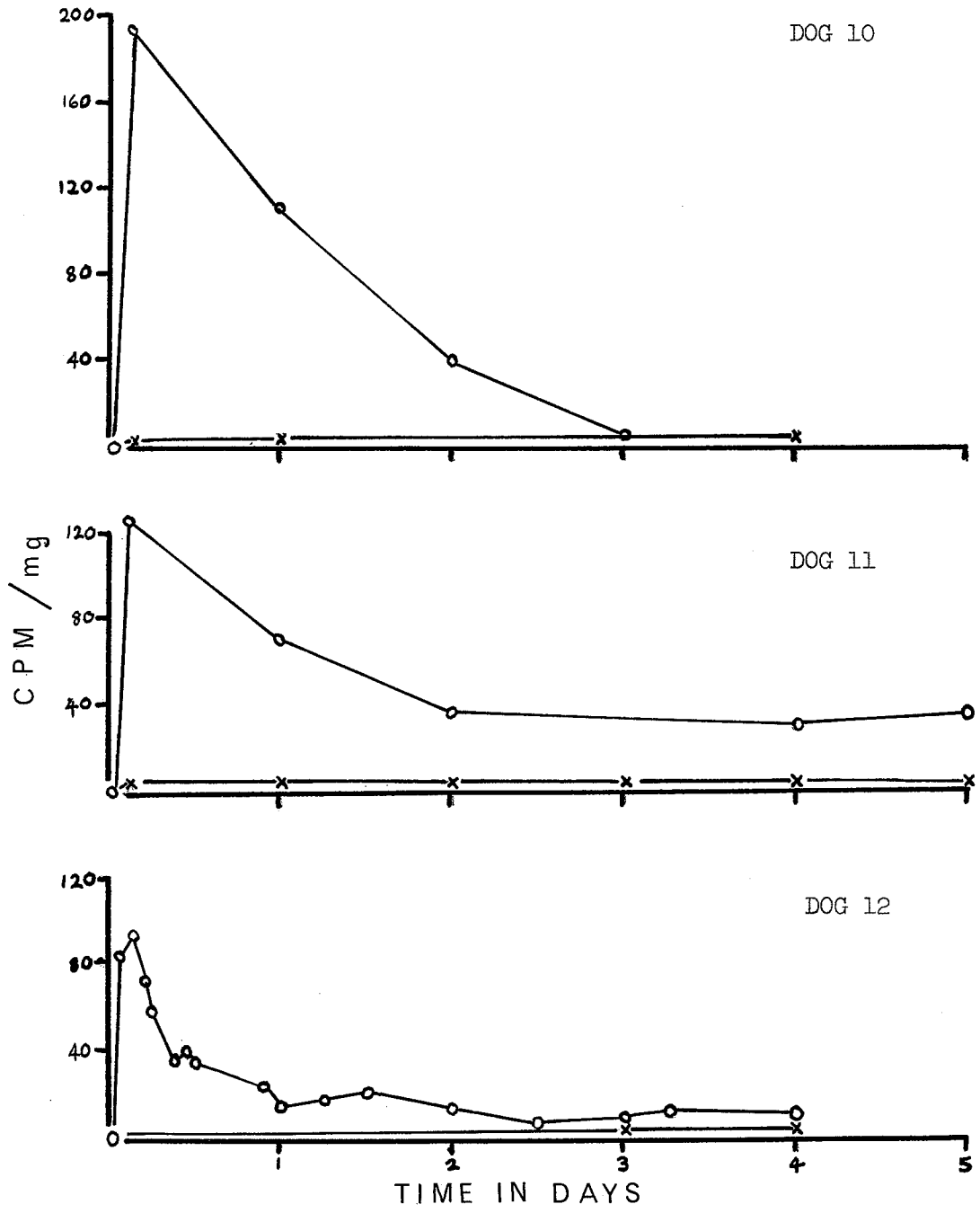

FIGURE 10. The Specific Activity of Bilirubin Prepared from Bile and Hemin Prepared from Peripheral Blood in Three Dogs Given Total Body Radiation Prior to the Intravenous Administration of 2 ^{14}C Glycine at Zero Time. Bilirubin (o-o). Hemin (x-x).

appeared in the circulating heme although the dog survived for six days after the 2 ^{14}C glycine was given. The shunt, therefore, was taken as 100 per cent. In Dog 12, 0.54 per cent of the total injected counts appeared in the early labelled bilirubin and 0.119 per cent appeared in the circulating heme. The shunt size was calculated to be 31.5 per cent.

Thus, in dogs whose marrow is severely depressed by radiation and in which the specific activity of the circulating heme remains very low after administration of radioactive glycine, the early labelled bilirubin is still present. Peak specific activity is attained within four hours after the radioactive glycine pulse is given and the specific activity of the bilirubin declines rapidly within 24 hours. This effect is similar but more pronounced that that seen in dogs treated with Busulfan.

Excretion of Labelled Bilirubin Following the Infusion of ^{14}C Labelled Heme

To determine the time required for conversion of hemin to bilirubin, hemin labelled with ^{14}C (prepared from a dog previously given 2 ^{14}C glycine) was given intravenously to a dog with a biliary fistula. Dog 7 weighed 13.6 kg and was given 200 mg of hemin with a specific activity of 60 counts/min. per mg dissolved in 0.1 N sodium hydroxide and brought to a pH of 8.9 with 0.1 N hydrochloric acid. Bilirubin was isolated from the bile and the specific activity determined. The first

bilirubin sample obtained at eight hours had a specific activity of 22 counts/min. per mg; this fell to five counts/min. per mg at 24 hours and 0 counts/min. per mg at 72 hours as shown in Figure 11. The radioactivity recovered was equal to five per cent of the total counts injected.

Normal Dogs Given 4 ^{14}C Delta Aminolevulinic Acid (Δ ALA)

Delta aminolevulinic acid is a good precursor of heme *in vitro* in red cell hemolysate systems as well as in the nucleated red cells of chickens (Dresel and Falk, 1954). However, it is a poor precursor of mammalian red cell heme *in vivo* and this has been explained on the basis of its rapid clearance by the kidney as Δ ALA and to the relative impermeability of the red cell precursors to Δ ALA in man (Scott, 1955). Because of this property it was decided to use Δ ALA as a potential bilirubin precursor. In the following experiments, Dogs 13 and 14 with biliary fistulae were injected with 25 uc and 10 uc respectively of 4 ^{14}C Δ ALA and the time of appearance of labelled bilirubin and heme determined. The results are shown in Figure 12. In Dog 13, the maximum specific activity of 74,500 counts/min. per mg of bilirubin occurred at seven hours and this dropped to 15,200 counts/min. per mg at 20 hours and 278 counts/min. per mg at 48 hours. In Dog 14 peak activity of 40,600 counts/min. per mg occurred at five hours and dropped to 2,160 counts/min. per mg by 24 hours. Thus, the maximum specific activity of

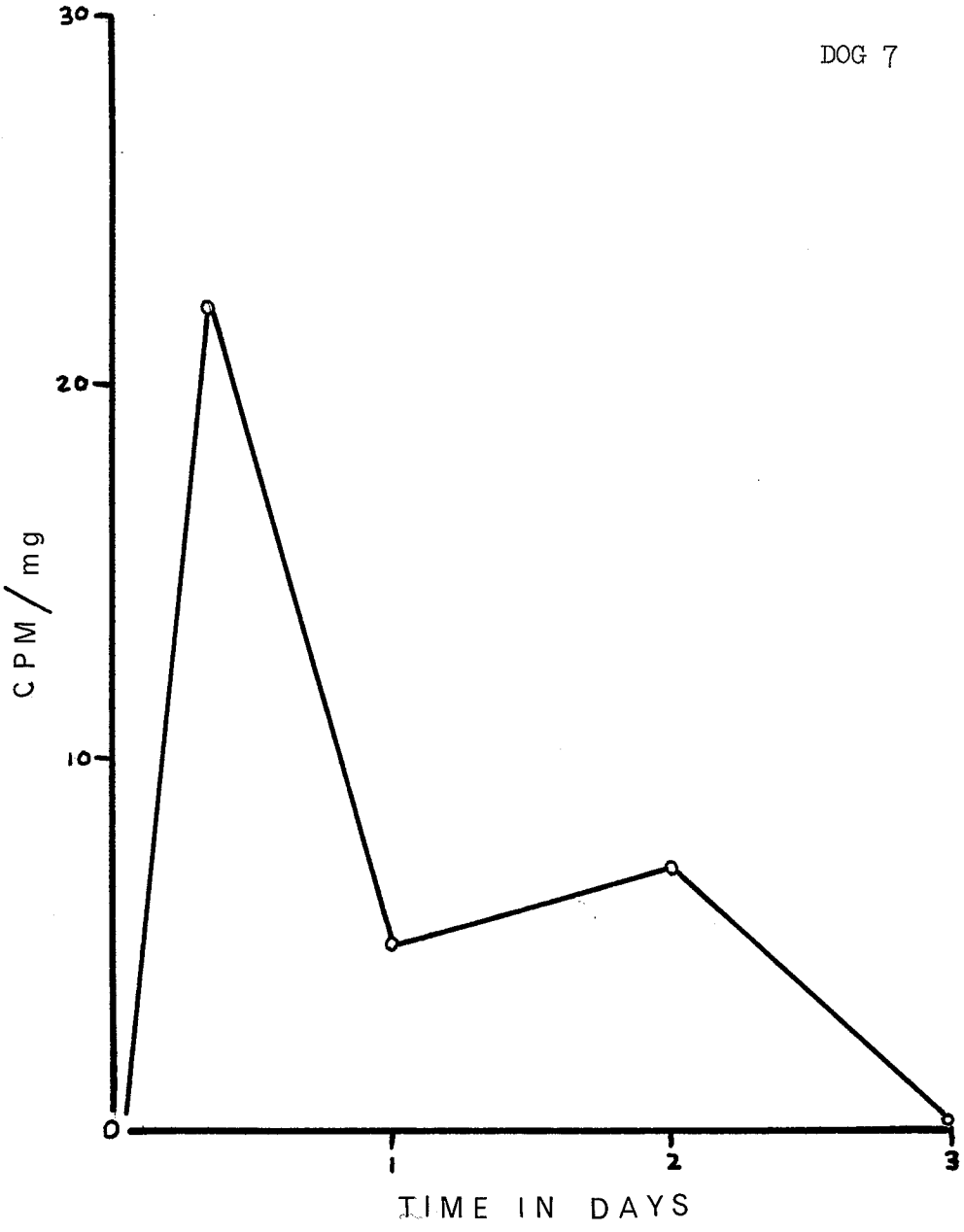

FIGURE 11. The Specific Activity of Bilirubin Prepared from Bile in a Normal Dog Following Intravenous Administration of C^{14} Hemin.

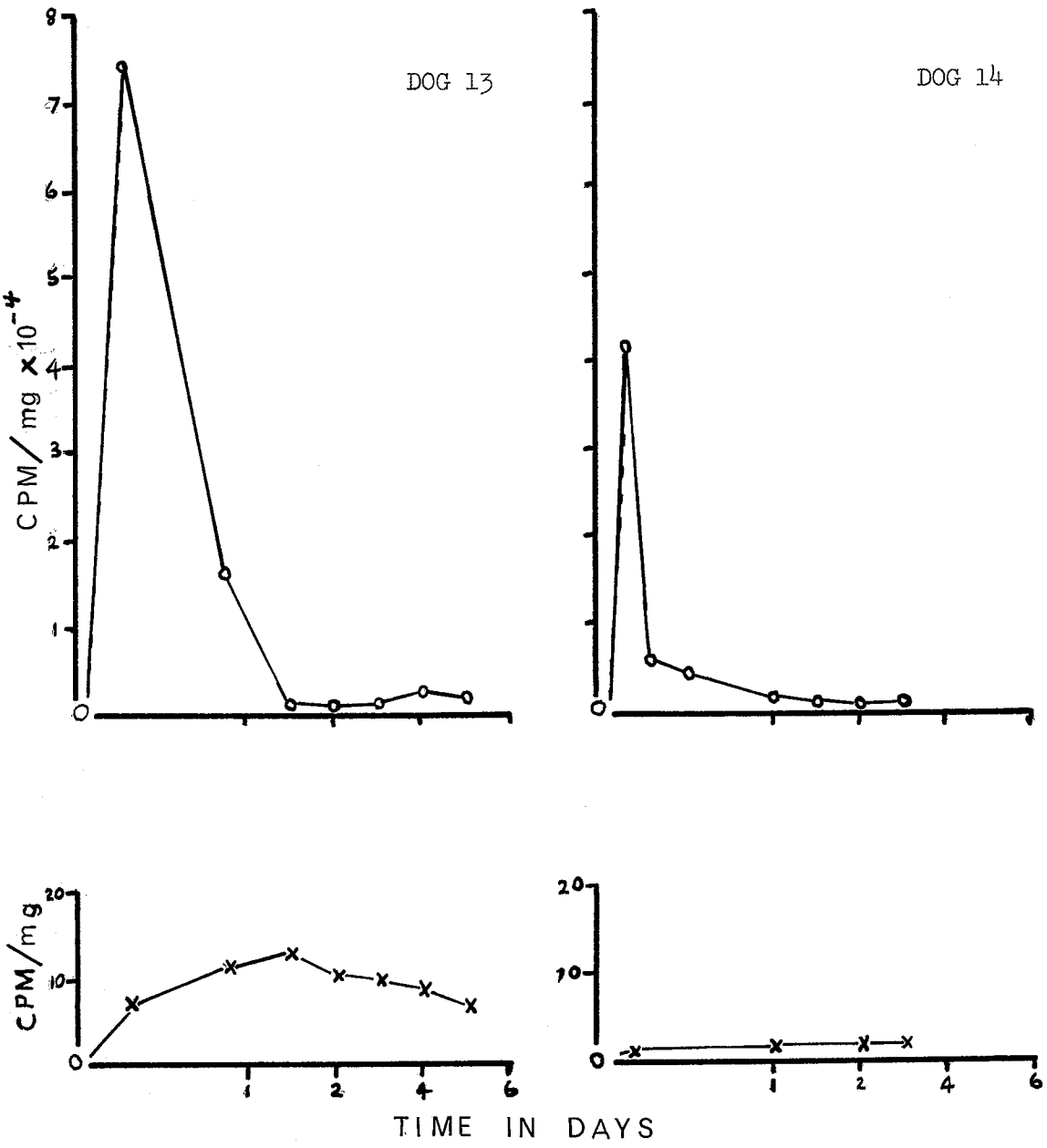

FIGURE 12. The Specific Activity of Bilirubin Prepared from Bile and Hemin Prepared from Peripheral Blood in Two Dogs Given 4 ^{14}C Δ ALA Intravenously at Zero Time. Bilirubin (o-o). Hemin (x-x).

the bilirubin occurred in the first sample of bile collected. In Dog 13, the bilirubin specific activity was higher at 24 hours (when compared to the peak specific activity present within seven hours) than that of Dog 14. Dog 13 incorporated seven counts/min. per mg into circulating heme while Dog 14 incorporated only one count/min. per mg into circulating heme. Dog 13 incorporated 7.7 per cent and Dog 14 incorporated 11.4 per cent of the total injected counts into the early labelled bilirubin as shown in Table VI.

A bone marrow aspiration was performed on Dog 13 seven hours after the injection of 4 ^{14}C Δ ALA and the heme isolated from the buffy coat and red cell layer of the aspirated marrow. The specific activity of the heme isolated from the buffy coat was 7.1 counts/min. per mg, the specific activity of the heme isolated from the marrow red cell layer was 8.8 counts/min. per mg and that of the peripheral blood 6.8 counts/min. per mg. The specific activity of the circulating heme rose to 9.6 counts/min. per mg on day 4. Little increase of the circulating heme occurred after seven hours. Dog 14 incorporated only one count/min. per mg into circulating heme by day 4. In Dogs 13 and 14, 0.537 per cent and 0.226 per cent respectively of the total injected counts appeared in the circulating heme as shown in Table VI. Thus, Δ ALA, which is known to be a poor heme precursor appears rapidly in the bile bilirubin after intravenous administration to normal dogs even though

TABLE VI

Radioactivity in Bilirubin and Hemin in Normal Dogs Given 4 ^{14}C Δ ALA.

Dog	Time After 4 ^{14}C Δ ALA (Hours)	Specific Activity CPM/mg			Total Counts Injected	Percent of Injected Counts in Bilirubin	Percent of Injected Counts in Heme
		Hemin (Blood)	Hemin (Marrow)	Bilirubin			
13	7	6.8	7.1	74,500	1.015×10^6/kg	7.7	0.537
	20	11	-	15,200			
14	5	0.5	-	40,600	3.55×10^5/kg	11.4	0.226
	24	1.0	-	2,160			

circulating heme incorporates relatively little radioactive Δ ALA.

To be sure that the radioactivity was present in the bilirubin and not a contaminant, the bilirubin sample collected at five hours from Dog 14 was chromatographed using a kieselguhr column to separate unconjugated bilirubin as described in the section on methods. The specific activity after alumina chromatography was 40,600 counts/min. per mg and after kieselguhr chromatography the specific activity was 42,500 counts/min. per mg. Thus, no evidence of radioactive contamination was found and the radioactivity was present in unconjugated bilirubin.

Experimentally Induced Porphyria

A state resembling acute porphyria in man can be induced by various agents which include allylisopropylcarbamide (Sedormid), allylisopropyl acetamide, and certain barbiturates (Schmid and Schwartz, 1952). These agents cause a large increase in Δ ALA and porphobilinogen excretion in the urine as well as the excretion of large amounts of coproporphyrin in the bile. In both experimental porphyria and human acute porphyria, the liver is the main metabolic site of synthesis of the porphyrins and their precursors. The bone marrow exhibits no change in this type of porphyria. This then is a means of altering hepatic heme-porphyrin synthesis without change in marrow synthesis of porphyrins.

To determine the effect of a change in hepatic porphyrin metabolism on the excretion of early labelled bilirubin and the relationship in time of synthesis of protoporphyrin to early labelled bilirubin, dogs were intoxicated with Sedormid and the incorporation of 2 ^{14}C glycine and 4 ^{14}C delta aminolevulinic acid into the heme, bilirubin and protoporphyrin was observed. The dogs were fed 50-100 mg/kg per day depending on the drug tolerance of the individual dog. After seven days of Sedormid administration, biliary fistulae were prepared. The degree of Sedormid intoxication was assessed by observing the fluorescence of the bile. In most of the dogs studied, the bile was fluorescent at the time of surgery. If fluorescence was slight, Sedormid administration was continued until the bile was strongly fluorencent. At this time, the animals were given 2 ^{14}C glycine or 4 ^{14}C Δ ALA intravenously and the specific activity of bilirubin and protoporphyrin excreted in the bile, and that of circulating heme was determined.

Dogs 15, 16 and 17 given Δ ALA showed a pattern of incorporation of label into bilirubin and heme very similar to that of normal dogs given Δ ALA. The results are shown in Figure 13. Peak activity of the bilirubin occurred within five hours and dropped rapidly thereafter. The specific activity of circulating heme remained low. The percent of total injected counts incorporated into bilirubin and heme is shown in Table VII. The peak specific activity of protoporphyrin

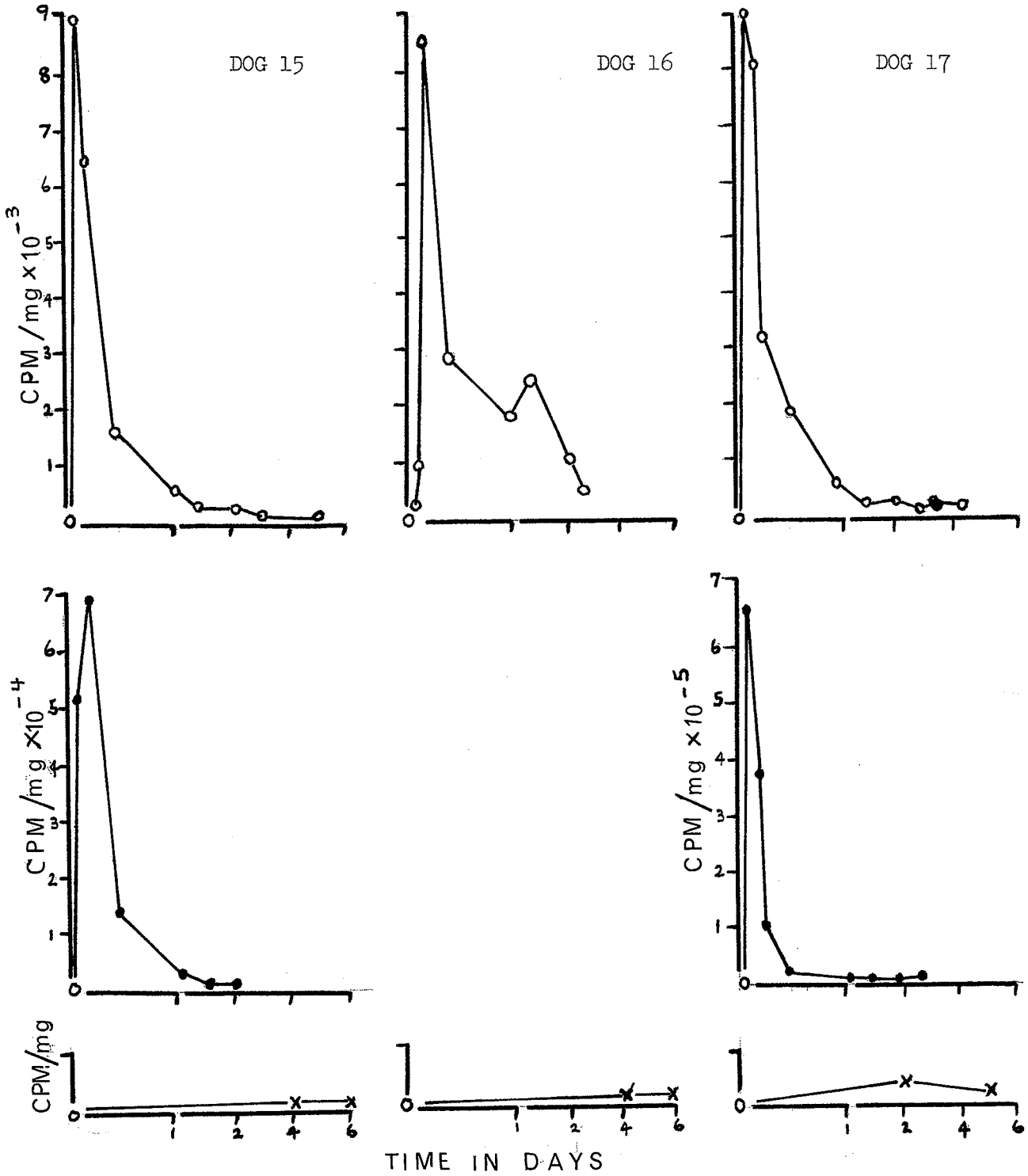

FIGURE 13. The Specific Activity of Bilirubin and Protoporphyrin Prepared from Bile and Hemin Prepared from Peripheral Blood in Three Dogs With Experimentally Induced Hepatic Porphyria Given 4 ^{14}C ALA at Zero Time. Bilirubin (o-o). Protoporphyrin (●-●). Hemin (x-x).

TABLE VII

Radioactivity in Bilirubin, Protoporphyrin, and Hemin in Dogs with Experimentally Induced Hepatic Porphyria After Intravenous Injection of 4 ^{14}C Δ ALA or 2 ^{14}C Glycine

Dog	Tracer	Counts Injected	Percent Total Counts		Maximum Specific Activity (CPM/mg)			Shunt (Per cent)
			Bilirubin	Heme	Time (Hrs)	Bilirubin	Protoporphyrin	
15	4 ^{14}C Δ ALA	1.42×10^5/kg	2.9	0.047	2.5	9,245	69,000	-
16	4 ^{14}C Δ ALA	2.66×10^5/kg	3.6	0.115	4	8,550	-	-
17	4 ^{14}C Δ ALA	3.05×10^5/kg	4.6	0.17	2.5	860	3,750	-
18	2 ^{14}C Glycine	1.3×10^6/kg	.025	0.322	6	110	-	7
19	2 ^{14}C Glycine	2.88×10^6/kg	.089	0.326	3	361	8,740	21.5
20	2 ^{14}C Glycine	4.54×10^5/kg	.635	1.84	6	335	7,750	25

occurred at the same time as that of bilirubin except in Dog 15. In this dog, peak activity of protoporphyrin occurred at 5.5 hours while that of bilirubin occurred at 2.5 hours or sooner.

The pattern of incorporation of 2 ^{14}C glycine into the heme, bilirubin and protoporphyrin or porphyric dogs is shown in Figure 14. In all three dogs, the peak activity of bilirubin occurred within six hours. In Dogs 19 and 20, protoporphyrin was found to have reached peak activity at the same time as bilirubin although the specific activity was over twenty times as high.

The appearance of labelled protoporphyrin at the same time as the appearance of labelled bilirubin indicates that the rate of synthesis of protoporphyrin is at least equal to that of bilirubin. The specific activity of heme was found to be similar to that of normal dogs. The percent of total injected counts appearing in heme and bilirubin is shown in Table VII.

Thus, in porphyric dogs given 2 ^{14}C glycine, the peak specific activity of bilirubin occurs much earlier and is higher than in normal dogs given 2 ^{14}C glycine. The shunt size was also found to be increased in Dogs 19 and 20 as shown in Table VII. The smaller shunt of Dog 18 is open to question as this dog exhibited poor bile secretion and was extremely ill due to Sedormid intolerance. The specific activity of the

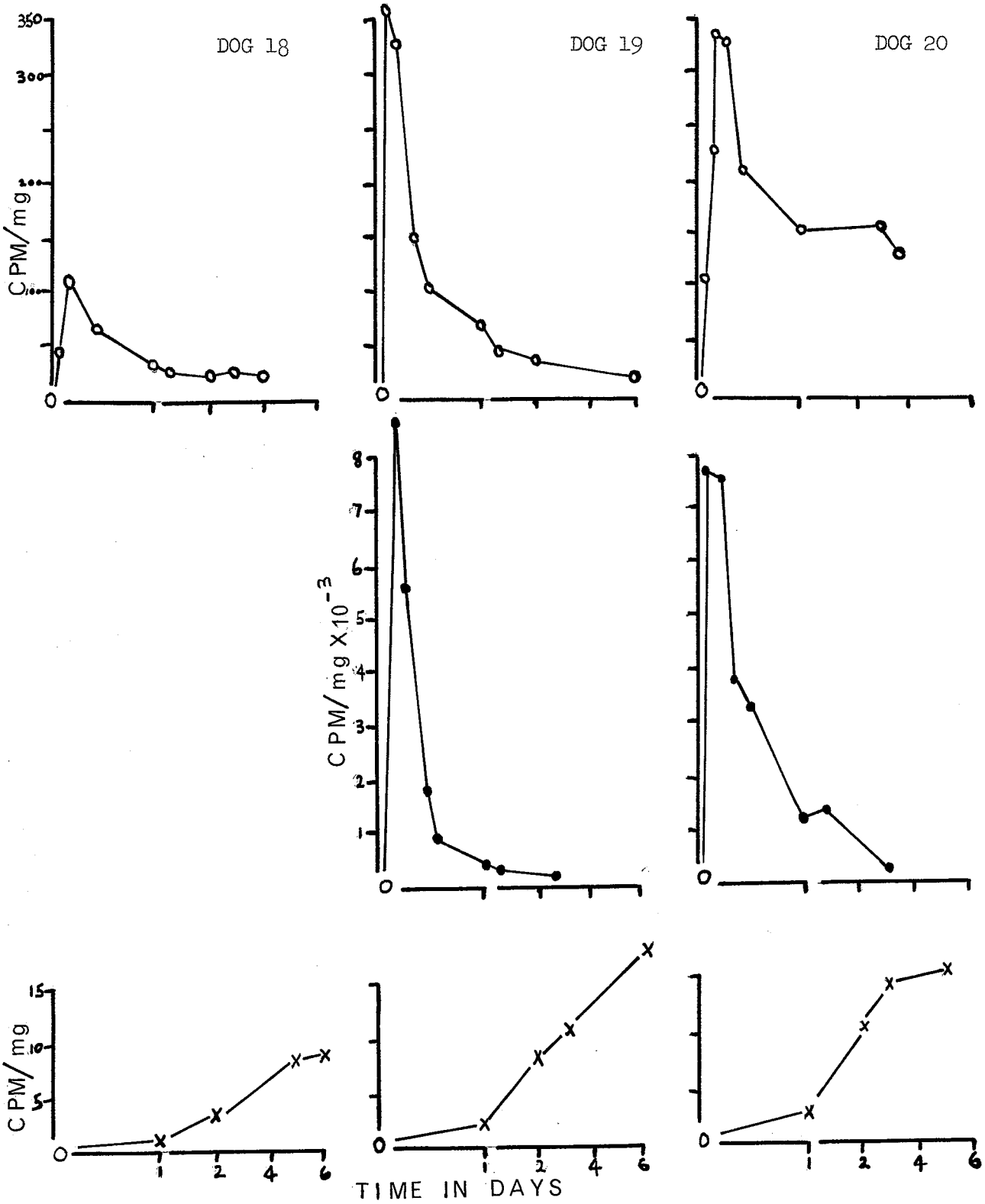

FIGURE 14. The Specific Activity of Bilirubin and Protoporphyrin Prepared from Bile and Hemin Prepared from Peripheral Blood in Three Dogs with Experimentally Induced Hepatic Porphyria Given 2 ^{14}C Glycine Intravenously at Zero Time. Bilirubin (o-o). Protoporphyrin (●-●). Hemin (x-x).

bilirubin beyond twelve hours was comparable to that in normal dogs.

Studies in Man

In all adult subjects 30-40 ml of blood was taken in heparin before and after the injection of 50 uc of 2 ^{14}C glycine or 12.5 uc of 4 ^{14}C Δ ALA. The volume of blood taken depended on the plasma bilirubin level and the hematocrit. In those subjects with a draining T-tube bile collections were obtained before administration of the label, 30 minutes thereafter, and then in two to twelve hour block samples as shown. The glycine and Δ ALA were given intravenously as a single rapid injection in a total volume of 1 to 5 ml of 0.85 per cent saline.

The first subject in which the appearance of early labelled bilirubin in plasma was studied was a woman with untreated pernicious anemia. The clinical findings are given in Table VIII. Bilirubin was isolated from the plasma and stercobilin from block collections of stool. Hemin was isolated from the peripheral blood. The appearance of the ^{14}C label in plasma bilirubin, stercobilin, and hemin are shown in Figure 15.

The first plasma bilirubin determination was made on day 2 at which time the specific activity of the isolated bilirubin was found to be 750 counts/min. per mg which declined to 630 counts/min. per mg on day 3 and rose to peak activity of 1150 counts/min. per mg on day 4.

TABLE VIII

Clinical Data of Subject with Untreated Pernicious Anemia

Sex	Female
Age	72
Weight, kg	52
Hemoglobin, g/100 ml	4.5
Reticulocytes, per cent	3.6
Leukocytes, mm^3	1,500
Bone Marrow	Megaloblastic
Bilirubin, mg/100 ml	
Direct	0.8
Indirect	1.0
Day of B_{12} administration	4
Maximal Response	
Reticulocytes, per cent	-
Hemoglobin, g/100 ml	13.4
Tracer	Glycine 2 ^{14}C

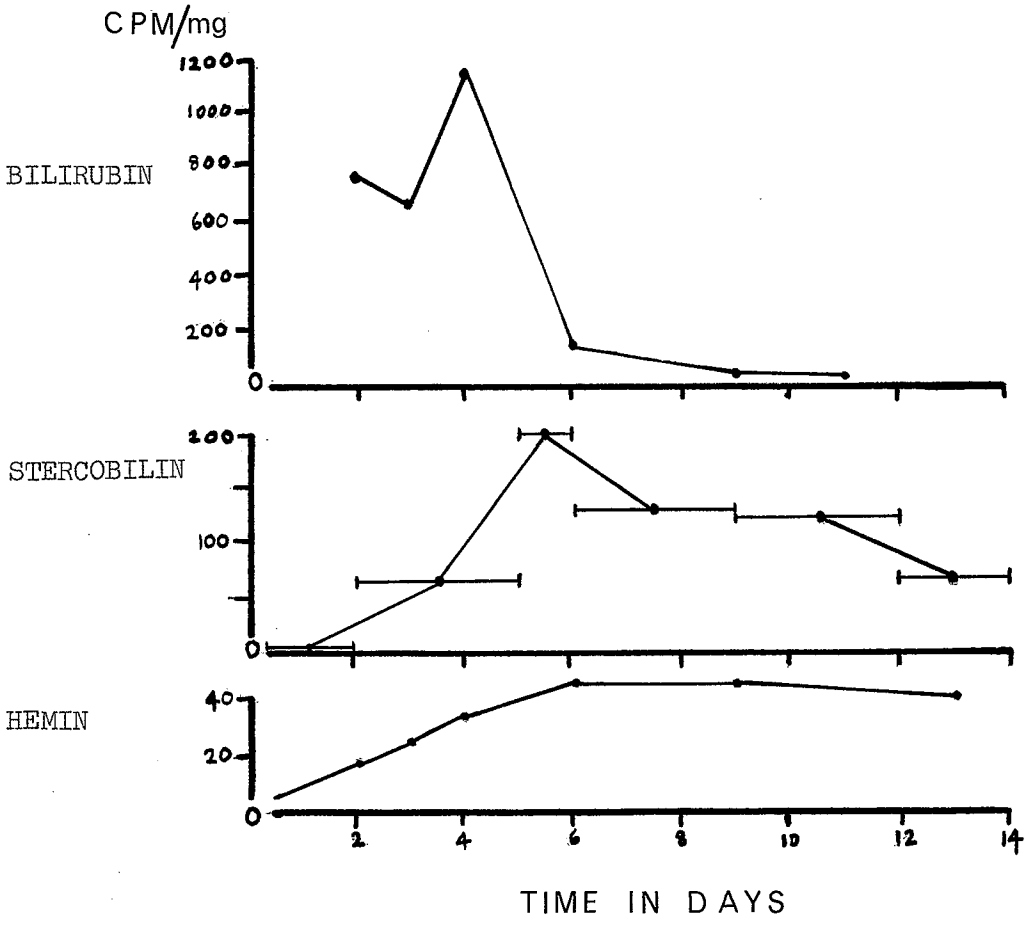

FIGURE 15. The Specific Activity of Bilirubin Isolated from Plasma Hemin Prepared from Peripheral Blood and Stercobilin Isolated from Block Stool Collections in a Patient with Pernicious Anemia Given 50 uc of 2 ^{14}C Glycine at Zero Time.

The specific activity then declined rapidly to 100 counts/min. per mg on day 6. No plasma sample was obtained on day 1 because of the very low hemoglobin concentration of the patient. In the first 24 hours the early labelled bilirubin specific activity reached its peak in bile fistula dogs so that one might expect a high specific activity to be present in plasma bilirubin at this time. It was of great interest that no labelled stercobilin was present in the first two days after the injection of 2 ^{14}C glycine. From day 2 to day 5 the stercobilin had a specific activity of 73 counts/min. per mg and the peak activity was reached on day 5 to day 6 when the specific activity was 200 counts/min. per mg and followed by 24 to 36 hours, the plasma peak of 1150 counts/min. per mg observed on day 4. From day 6 onwards, the stercobilin activity declined slowly to 73 counts/min. per mg on day 16 at which time the experiment was terminated. A total of 1.5 per cent of the injected counts emerged as circulating red cell heme. As there was little decrease in the heme specific activity up to day 17 when the experiment was terminated, there was probably little intravascular hemolysis of young ethrythrocytes which would carry the ^{14}C label. The specific activity of the heme had reached plateau activity by day 6. A very slight decline in activity had occurred by day 17.

As the pattern of plasma bilirubin labelling indicated that more than one component of early labelled bilirubin could be identified

by determining the specific activity of plasma bilirubin in the first five days, further experiments were undertaken using patients with draining T-tubes as subjects. In these experiments it was possible to compare the appearance of labelled bilirubin in plasma and in bile at the same time.

Human Subjects Given 2 ^{14}C Glycine

The appearance of labelled bilirubin in the plasma and in the bile and labelled heme in the circulating red cells of human subjects is shown in Figure 16. Labelled bilirubin was detected in plasma within 90 minutes of the injection of 2 ^{14}C glycine and the specific activity increased to a peak between 12 and 24 hours and then fell to a nadir at 48 to 60 hours and subsequently rose to a second peak on day 3 or day 4. The activity then fell away to day 6 at which time a small amount of radioactivity was still present in the plasma bilirubin of each subject. The specific activity of the bilirubin recovered from the T-tube drainage bile in subjects 2 and 3 was of much lower specific activity than the plasma bilirubin. The parallel changes in bilirubin specific activity in plasma and in bile are seen in Subject 3, but the two peaks are not reflected in the bile in Subject 2.

Labelled heme present in circulating red cells within 24 hours of the administration of 2 ^{14}C glycine began to plateau at day 4 to day 6, the maximum daily increment in the specific activity of

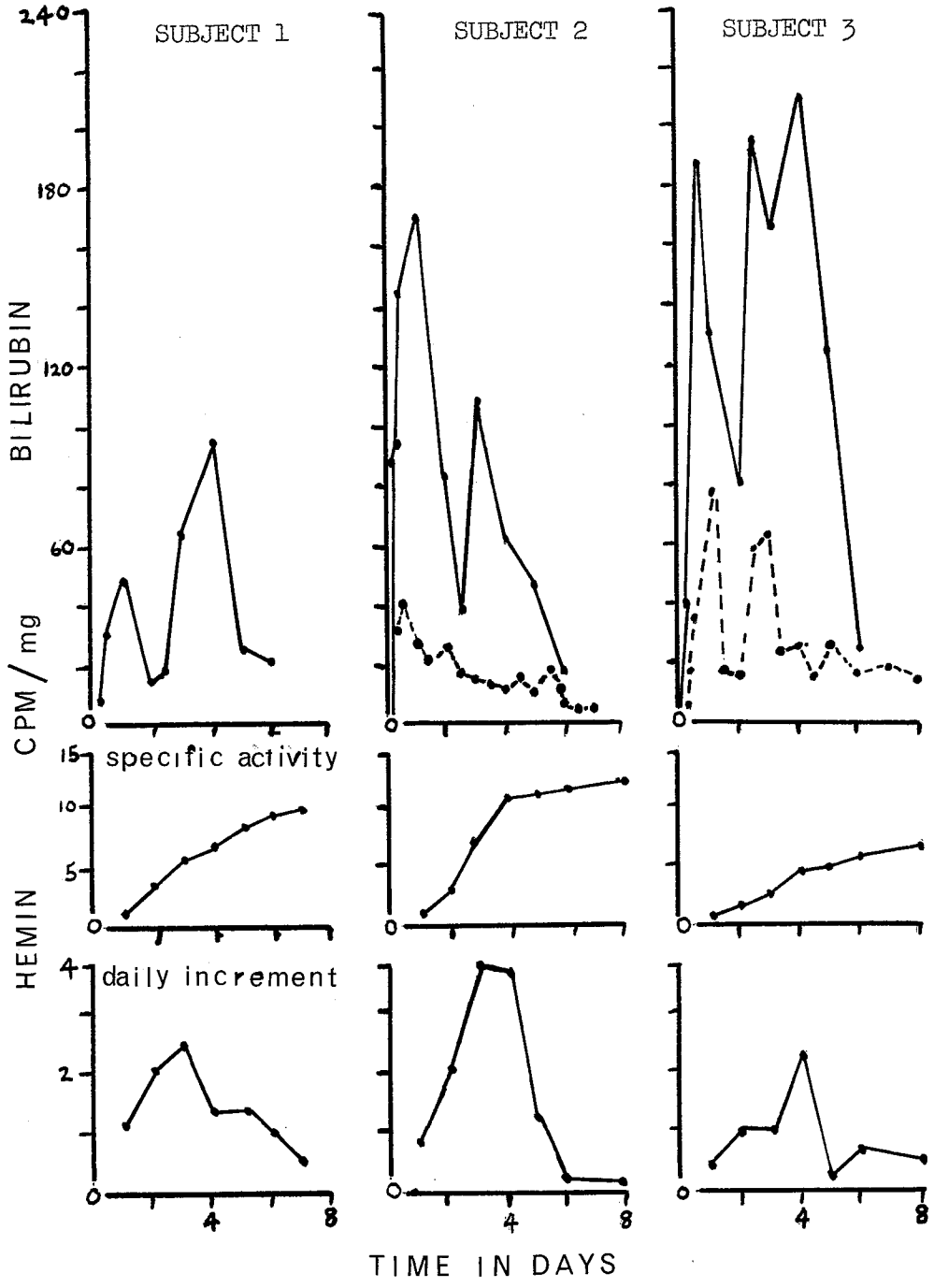

FIGURE 16. The Specific Activity of Bilirubin Isolated from Plasma and Bile and of Hemin Prepared from Peripheral Blood in Three Human Subjects Given 50 uc of 2 ^{14}C Glycine at Zero Time. The Daily Increment in Heme Specific Activity is also shown. Bilirubin in Bile (---). Bilirubin in Plasma (___).

labelled heme occurred on day 3 to 4.

The percentage of injected counts appearing in circulating heme in Subjects 1, 2 and 3 was 2.65 per cent, 2.45 per cent and 1.62 per cent of the total injected counts as shown in Table IX.

Human Subjects Given 4 ^{14}C Δ ALA

The results obtained in subjects 4 and 5 given 4 ^{14}C Δ ALA are shown in Figure 17. In Subject 4, labelled bilirubin was first detected in the plasma 20 minutes following the injection of labelled Δ ALA and peak activity was observed within 90 minutes. In Subject 5, the maximum observed activity was at the time of the first sampling of 1.5 hours and the peak may have occurred prior to this. The specific activity was much higher than that obtained with 2 ^{14}C glycine and the activity fell sharply to low levels at 24 hours. A secondary rise was not observed. Labelled bilirubin in bile was somewhat slower to appear and reached its peak at 6 hours in both subjects. The activity in the bile exceeded that of the plasma from 6 to 72 hours following which the activities were of the same order.

The incorporation of the ^{14}C label into heme was minimal and less than 0.5 counts/min. per mg was present on day 8. The percentage of injected counts appearing in circulating heme in Subject 4 and 5 was 0.28 per cent and 0.12 per cent of the total injected counts as shown in Table IX.

TABLE IX

Per cent of Total Injected Counts Incorporated into Circulating Heme of Human Subjects

Subject	After 2 ^{14}C Glycine		After 4 ^{14}C Δ ALA	
	Per cent	Day	Per cent	Day
1	2.65	7	-	-
2	2.45	8	-	-
3	1.62	8	-	-
4	-	-	0.028	7
5	-	-	0.012	7

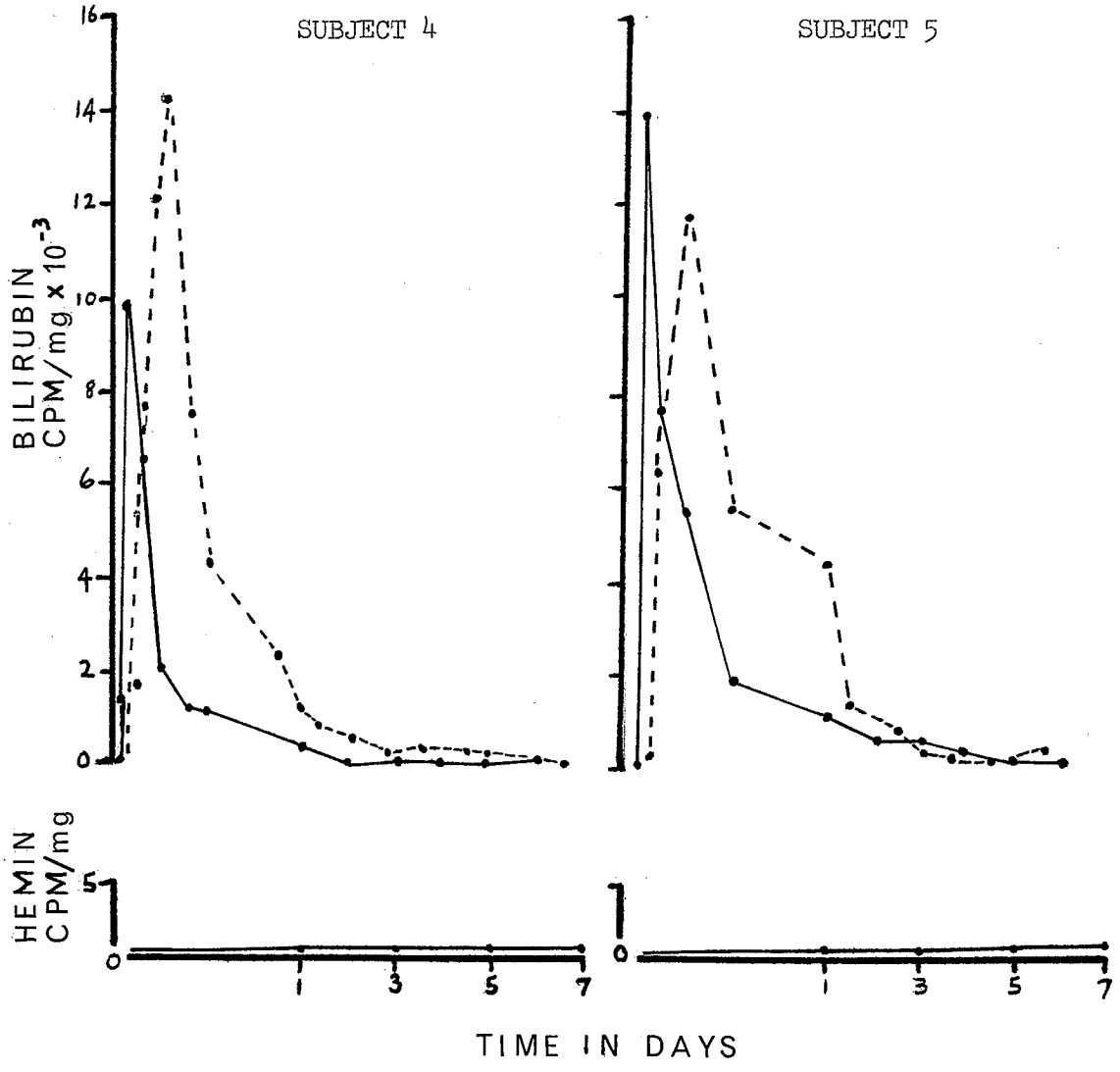

FIGURE 17. The Specific Activity of Bilirubin Isolated from Plasma and Bile, and of Hemin Prepared from Peripheral Blood in Two Human Subjects Given 12.5 uc 4 ^{14}C Δ ALA at Zero Time. Bilirubin in Bile (---). Bilirubin in Plasma (——).

SECTION V

DISCUSSION

Utilizing a bile fistula preparation in dogs it has been possible to determine the time of appearance and the quantity of early labelled bilirubin. The striking finding in all animals was the appearance of labelled bilirubin within 4 to 8 hours of the intravenous injection of 2 ^{14}C glycine and the attainment of maximum specific activity within 24 to 48 hours. Using rats with bile fistulae, Hammaker and Schmid, (1961) found the peak excretion of labelled bile pigment to occur between 6 and 18 hours following administration of 2 ^{14}C glycine. James, (1955) suggested that the rapidity of appearance and the quantity of early labelled bile pigment was against it coming from preformed heme or from the disintegrating red cells of ineffective erythropoiesis. This is also suggested by the work of Berlin, Neuberger and Scott, (1956) who showed that ΔALA may be rapidly converted into stercobilin by a pathway which may not include hemoglobin. The experiments done in this study using 4 ^{14}C ΔALA as the tracer compound showed rapid excretion of labelled bilirubin with little detectable incorporation of label into circulating heme. That hemin may be rapidly converted to bilirubin is seen in one dog given ^{14}C labelled hemin intravenously. Labelled bilirubin was recovered eight

hours after injection. The recovery of the label was poor in that only five per cent of the total injected counts were recovered over a three day period. In a similar study, London, (1950) using N^{15} labelled hematin was able to recover 18 per cent as labelled stercobilin over a nine day period.

The present study demonstrates that in both dogs and man glycine is incorporated into bilirubin within a period of 4 to 8 hours and that Δ ALA is incorporated within 90 minutes. This labelled bilirubin appeared in the normal animals and the human control subject when little or no radioactivity was found in the hemin prepared from circulating red cells on marrow buffy coat and suggests that the heme in these sites may not be an obligatory precursor of the early labelled bile pigment, although a rapidly turning over heme pool is not excluded. This is also suggested by the findings in one dog with marrow aplasia following 700r total body radiation. In this animal no ^{14}C label appeared in the circulating heme although the dog survived for six days after receiving the 2 ^{14}C glycine. Labelled bilirubin was present in the bile within four hours after administration of the labelled glycine. This is further supported by the rapid incorporation of 4 ^{14}C Δ ALA into bilirubin in the absence of significant incorporation of label into circulating heme in the dogs and human subjects studied. If the bone marrow is not the site of production of this early labelled

fraction then it may at least in part be of hepatic origin as suggested by Rimington, (1959).

However, most of the clinical observations of increased production of this early labelled fraction have been in patients with defects in erythropoiesis. This would suggest that the erythropoietic cells may be directly or indirectly related to the synthesis of this fraction. The finding of two peaks of early labelled bilirubin in the plasma of human subjects given 2 ^{14}C glycine and one peak in those given 4 ^{14}C Δ ALA indicate that a portion of the early labelled bile pigment doses indeed originate in the erythropoietic cells. If the early labelled bilirubin is related to erythropoiesis then it may be influenced by changes in the erythropoietic system. Furthermore if the percentage of the precursor pool going through the shunt is an index of marrow efficiency it might be expected to change with the stimulation of depression of erythropoiesis and heme synthesis. The findings in the two bled dogs are suggestive in that the incorporation of the 2 ^{14}C glycine into red cell hemin was greater than in the four normal dogs and in the former group incorporated more of the injected glycine. The specific activity of the excreted bilirubin was high. Some of this increase in specific activity may be spurious as the labelled bilirubin is diluted less by unlabelled catabolic bilirubin derived from destroyed red cells, as the total excretion of bile pigment

derived from senescent red cells decreases following blood loss as shown by Hawkins, Sribhishaj, Robscheit-Robbins, and Whipple, (1931). Gray and Scott, (1959) studied the incorporation of 2 ^{14}C glycine into the early labelled stercobilin before and after hematopoiesis had been stimulated by bleeding in a man with porphyria cutanea tarda. The increased specific activity of the stercobilin formed after bleeding was taken as an indication that this stercobilin fraction was related to the marrow response to bleeding. James, (1955) conducted a similar study in one normal human and also noted an increase in the atoms percentage excess of N^{15} in the stercobilin excreted following bleeding. On calculating the percentage of stercobilin not derived from circulating red cells, he found that because of the relatively increased heme production this had decreased from 20 per cent prior to bleeding to six per cent which would indicate a more efficient system with a smaller relative loss of heme or heme precursors to stercobilin.

In the present study on dogs with biliary fistulae we did not examine the same animal before and after bleeding. The bled dogs, however, did excrete bilirubin of high specific activity, which is in keeping with the findings of James, (1955), and of Gray and Scott, (1959). The percentage of injected 2 ^{14}C glycine finding its way directly into bilirubin was greater than in the normal as was the size of the precursor pool - the active marrow having increased its

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utilization of the 2 ^{14}C glycine pulse. The total quantity of bilirubin passing through the shunt had increased but the percentage of the calculated precursor pool was 3.5 and 5 per cent or of similar order to that in normal dogs.

In the Busulphan treated dogs the excretion of the ^{14}C label in bilirubin was again rapid with little ^{14}C appearing in the heme derived from circulating red cells or marrow. The increased shunt (25 and 37 per cent) in these two dogs results from the low incorporation of ^{14}C into heme as compared with the maintained labelling of bilirubin. The 2 ^{14}C glycine was given to these dogs four days after the administration of Busulphan at a time when the marrow was still cellular. Previous studies done by Israels, Sinclair, Graf and Zipurksy, (1962) had shown a well maintained plasma iron turnover although the incorporation of iron into heme was reduced, suggest a situation resembling "ineffective erythropoiesis".

The dogs that received 700 r total body irradiation with resulting severe marrow hypoplasia incorporated little or no ^{14}C into hemin. Four hours after receiving the 2 ^{14}C glycine, however, the bilirubin was again highly labelled, the relative specific activities being 70 and 170 counts/min per mg. Again the low incorporation into heme and the maintained or smaller depression in the incorporation of label into bilirubin results in a small precursor pool of which 76 to

100 per cent goes directly to bilirubin. In one dog given 500 ϒ total body irradiation the peak bilirubin specific activity was 90 counts/min per mg and occurred at three hours after the injection of 2 ^{14}C glycine. The shunt was found to be 31.6 per cent.

From these findings it is apparent that the early labelled or shunt fraction of bilirubin is present in normal dogs and persists when erythropoiesis is either stimulated by bleeding or depressed by Busulphan or radiation. Thus this channel of bilirubin production remains open under varying conditions of heme synthesis.

As Δ ALA is known to be a poor precursor of mammalian red cell heme in vivo as shown by Scott, (1955), 4 ^{14}C Δ ALA was administered intravenously to two dogs with biliary fistula. The peak specific activity of bilirubin occurred within seven hours and very little activity was present in the circulating or marrow heme at this time or for the duration of the experiment. The specific activity of bilirubin labelled with Δ ALA is much higher than that of bilirubin labelled with glycine and one reason for this difference is that the radioactive glycine is diluted by a large glycine pool while there is a very small and rapidly turning over pool of Δ ALA so that a very large amount of the precursor Δ ALA immediately enters heme and bilirubin. Thus there is rapid excretion of bilirubin even though there is relatively little radioactive Δ ALA incorporated into circulating heme. As the labelled

bilirubin is probably not produced in the bone marrow other sites of production are implicated in the production of the early labelled fraction.

Heme containing enzymes and myoglobin must also be considered as sources of early labelled bile pigment. Quantitatively the most important protein other than hemoglobin is myoglobin. It accounts for approximately five per cent of the total body porphyrin, (Drabin, 1948). On the basis of their observations of the early appearance of N^{15} in stercobilin following the administration of N^{15} glycine, London, West, Shemin and Rittenberg, (1950) concluded that myoglobin could not be the sole source of this pigment fraction although it might contribute to this portion of bile pigment. From studies with radioiron, the rate of myoglobin turnover would seem to be low and out of keeping with the rapidity of appearance of the early labelled bilirubin, (Theorell, Beznak, Bonnichen, Paul and Akenson, 1951). The same authors, from their studies on guinea pigs concluded that cytochrome and catalase had a relatively slow rate of turnover and could not contribute to the early labelled bile pigment. Although the rate of liver catalase was more rapid, its absolute amount argues against it being a significant source of the early labelled pigment. However Price, Sterling, Tarantola, Hartley and Richcigl, (1962) have shown that there is an allylisopropylacetamide-resistant catalase with a more rapid rate of

turnover than the major component of hepatic catalase. Thus rapidly turning over heme-protein may be synthesized and degraded within minutes. Although it forms only a small part of the total heme pool it may contribute to the early labelled bile pigment.

As part of the early labelled bilirubin may be of hepatic origin and have as its precursor either hepatic heme or heme precursors, we next studied animals with chemically induced porphyria to obtain data on the relationship of this bilirubin to biliary porphyrins and the changes which may be produced by Sedormid induced porphyria.

In dogs with experimentally induced porphyria given 4 ^{14}C ΔALA the labelling pattern of early appearing bilirubin was very similar to that shown by normal dogs given labelled ΔALA. In one dog the peak specific activity of protoporphyrin was reached three hours after that of bilirubin. In the other dog studied the labelling patterns of protoporphyrin and bilirubin were very similar. No explanation was readily available for the discrepancy in time of appearance of labelled protoporphyrin. In both dogs the protoporphyrin specific activity was higher than that of bilirubin. This may be because of dilution of the ^{14}C bilirubin by a large pool of unlabelled bilirubin in contrast to the small protoporphyrin pool and its rapid rate of turnover.

In the Sedormid intoxicated animals given 2 ^{14}C glycine the

radioactivity appearing in bilirubin reached peak activity between three and six hours as compared with twelve to twenty-four hours in the normal dog. Peak protoporphyrin activity was also present at this time and exceeded that of bilirubin. This accelerated appearance of the bilirubin labelled from glycine to approximate in time that labelled when 4 ^{14}CΔALA is the precursor is consistant with the findings of Granick and Urata, (1963) that some experimental porphyrias result from enhanced production of delta aminolevulinic acid synthetase activity in liver parenchymal cells. As Sedormid porphyria is primarily a disease of the liver with no demonstrable changes in the bone marrow, (Schmid and Schwartz, 1952), this again suggests the first peak to be of hepatic origin.

As protoporphyrin is synthesized at a rate at least equal to that of the first labelled bilirubin to appear it is probable that this bilirubin fraction arises from hepatic heme or its immediate precursors. The findings of Yamaguchi, Nakajima, and Yamaoka, (1961) on the enzymic degradation of heme proteins in a beef liver system suggests that while hemoglobin is a good, and heme a relatively less efficient precursor of bilirubin, protoporphyrin IX does not yield bile pigment in this in vitro system. In 1963 Nakajima, Takemura, Nakajima and Yamaoka purified the enzyme involved (heme α-methenyl oxidase) from beef and guinea pig liver and showed that while several hemichromes and hemoglobin-haptoglobin were active substrates for the enzyme, alkaline hematin or

protoporphyrin IX were inert as substrates. The enzyme activity was present predominantly in the liver and kidney but was practically absent from the spleen and bone marrow. This is in favor of the precursor substance of the first peak being a heme compound rather than a porphyrin precursor.

Delineation of two distinct peaks of early labelled bilirubin was first observed when plasma bilirubin labelling patterns were studied in human subjects following the administration of 2 ^{14}C glycine. The first ^{14}C plasma bilirubin was detectable at 90 minutes and the activity increased to reach a peak at 12 to 24 hours. The second component reached maximal activity three to five days after the administration of the glycine. This second phase probably began on day one, the initial part of its production being overlapped by the first component.

The second peak corresponded in time to the maximal increment of radioactivity in red cell heme, a time when the red cells that had incorporated three to four days previously were emerging from the marrow.

The observation of Bessis, Breton-Gorius and Thiery, (1961), that some hemoglobin accompanies the nucleus of the cell as it is extruded from the erythroblast led them to suggest this to be the source

of the early labelled stercobilin and it may indeed represent an important source of this second component. This mechanism would be consistant with the coincidence of the second peak with the release of maximally labelled red cells from the marrow.

Another postulated source of early labelled bilirubin is that heme is formed in excess of globin and rapidly excreted as bile pigment. If there is a direct metabolic shunt from heme precursors to bilirubin then following a single pulse of 2 ^{14}C glycine the loss of ^{14}C labelled heme or heme precursors from the heme pool would lower the heme-globin ratio. Thus an increase in the production of early labelled bile pigment and a low heme-globin ratio would be expected to occur together. Low heme-globin ratios have been recorded along with increased production of early labelled stercobilin in thalassemia by Grinstein, Bannerman, Vavra and Moore, (1960). Studies done by Yamamoto, Skanderbeg, Zipursky and Israels (1965) found that the heme-globin ratio in patients with pernicious anemia five days or more after the administration of 2 ^{14}C glycine to be greater than one. These values are higher than those obtained by Nathan and Gardner, (1962) in pernicious anemia but were similar to those of their normal subjects, Nathan and Gardner, (1962) and Nathan, Piomelli and Gardner, (1961). It is unlikely therefore, much of the second bilirubin fraction arises from direct conversion of erythropoietic heme or its precursors within

the marrow.

In bile fistula dogs with total body radiation and resultant marrow aplasia given 2 ^{14}C glycine, the excretion of labelled bilirubin following the first 12 to 24 hour peak is markedly reduced. Thus this second component in bile which is analogous to the second plasma peak falls along with a decrease in the synthesis of circulating red cell heme. This association of a reduced second component and poor incorporation of the label into red cell heme is also seen when Δ ALA is the precursor substance.

In two control subjects receiving 4 ^{14}C Δ ALA 0.28 per cent and 0.12 per cent of the injected counts appeared in circulating heme. This is of the same order as that reported by Berlin, Neuberger and Scott, (1956), (0.24 per cent) and compares with 1.68 per cent to 2.65 per cent for glycine. The absence of a second peak in the subjects receiving Δ ALA who incorporated little label into circulating red cells also is in keeping with the relationship of the second component to red cell heme.

The labelling pattern following 4 ^{14}C Δ ALA showed only a sharp initial peak that was more rapid than glycine, with labelled bilirubin detectable in 20 minutes and reaching higher peak activity within 90 minutes. This difference in time and degree of incorporation

of this precursor into bilirubin may relate to cell permeability, difference in pool size, to the rapid clearance of Δ ALA, and to the fact that Δ ALA enters the heme cycle past the rate limiting step controlled by Δ ALA synthetase. That Δ ALA is a better precursor of the early labelled bilirubin has been shown in normal bile fistula dogs where 7 to 11 per cent of the injected radioactivity of 4 ^{14}C Δ ALA is incorporated into the early labelled bilirubin as compared with 0.07 per cent to 0.15 per cent for 2 ^{14}C glycine.

This difference in the labelling pattern of glycine and Δ ALA is also reflected in their relative labelling of the bilirubin in the plasma and bile. In the subjects given 4 ^{14}C Δ ALA the labelling pattern in plasma and bile are very similar; however, peak specific activity in the bilirubin of the bile followed that in the plasma by four to six hours. At this time the plasma activity had fallen, and the specific activity of the bilirubin in the bile then exceeded that in the plasma over the next 24 hours. Some of the lag in the rise of ^{14}C activity in bile bilirubin as compared with that in the plasma may be due in part to the unlabelled bilirubin occupying the bile ducts and T-tube at the time of injection. If the liver is a primary site of bilirubin synthesis from infused Δ ALA, the bilirubin enters the circulating plasma at a rapid rate and is also rapidly cleared into the bile. The continuing high activity in the bile for a period of 6 to 12 hours

after plasma activity has fallen away suggests hepatic synthesis with direct excretion into the bile.

In contrast to the findings with ΔALA the specific activity of the bilirubin obtained from the bile was much less than that of the plasma in subjects given 2 ^{14}C glycine. The reason for this plasma bile difference is not known but the possibilities include the following:

1) There is a ^{14}C contaminant arising from glycine but not ΔALA that is associated with the isolated plasma bilirubin. If this is so, it is firmly fixed to the plasma bilirubin as the specific activity remains constant through two column systems and crystallization.

2) The bilirubin in the bile is diluted by unlabelled bilirubin arising in the spleen and passing directly to the liver via the splenic vein. The bilirubin content of splenic vein blood is probably greater than that of peripheral blood, and this can be demonstrated in patients with congenital spherocytosis, Dacie, (1960).

3) A part of the plasma bilirubin formed from glycine is excreted as a bile pigment other than bilirubin. This possibility is suggested by the discrepancy between the times of labelling of plasma bilirubin and stool stercobilin. Maximal labelling in the stool does not take place for three to five days with little or no labelling in the first 24 to 48 hours. This is also apparent in other studies done by Israels and

Zipursky, (1962), and Gray, Kulczycka, Nicholson, Magnus and Rimington, (1964). Allowing for gut transit time, the rapid appearance of the label in plasma and bile bilirubin is not mirrored and suggests the possibility that part of the earliest labelled plasma bilirubin is excreted as pigment other than stercobilin. Early labelling of mesobilifuscin in the stool was demonstrated by Gilbertsen, Lowry, Hawkinson and Watson, (1959) and this labelling preceded that of stercobilin. The possibility that mesobilifuscin arises in whole or in part from this bilirubin fraction must be considered.

4) That bilirubin ^{14}C entering the plasma is diluted in the relatively small plasma pool as compared with that bilirubin ^{14}C going into the large hepatic-biliary pool.

As the two main sites of heme and porphyrin synthesis are the erythropoietic cells of the marrow and the hepatic parenchymal cells, this suggests the liver as a possible site of origin of the first peak.

Since the completion of the studies, Robinson, Owen, Flock and Schmid, (1965) have succeeded in producing ^{14}C bilirubin in an isolated rat liver pump perfusion system using Δ ALA as the precursor.

SUMMARY

1) Bile fistula dogs given 2 ^{14}C glycine or 4 ^{14}C ΔALA intravenously have been used to study the time of appearance and magnitude of the 'early labelled' or 'shunt' bilirubin.

2) In all dogs studied, ^{14}C bilirubin appeared in the bile within four to eight hours after the intravenous injection of the 2 ^{14}C glycine. In one human subject studied this preceded the appearance of the label in hemin prepared from both the peripheral blood and the marrow buffy coat.

3) The shunt in normal dogs accounted for 5 to 16 per cent of a proposed common heme-bilirubin precursor pool. In one human subject with a biliary fistula the shunt size was 16 per cent.

4) In two dogs bled to stimulate erythropoiesis the shunt size was 3.5 and 5 per cent of the precursor pool. In two dogs given Busulphan to depress erythropoiesis the shunt size was 25 and 37 per cent. In two dogs given 700 r total body radiation the shunt size was 76 and 100 per cent and in one dog given 500 r total body radiation the shunt size was 31.6 per cent.

5) In bile fistula dogs given 4 ^{14}C ΔALA intravenously early labelled bilirubin reached its peak specific activity within seven hours after the administration of 4 ^{14}C ΔALA. In human subjects given 4 ^{14}C ΔALA

the label was maximal in bile bilirubin within six hours and radio-activity in plasma bilirubin was present within 20 minutes with peak specific activity occurring within 90 minutes. In both dogs and human subjects there was relatively little 4 ^{14}CΔ ALA incorporated into heme.

6) In bile fistula dogs with experimentally induced hepatic porphyrin given 4 ^{14}C ΔALA the labelling pattern of bilirubin resembled that of normal bile fistula dogs given 4 ^{14}C ΔALA. However, the labelling pattern observed when 2 ^{14}C glycine was the precursor was accelerated to resemble in time that of Δ ALA. Peak proloporphyrin radio activity occurred at approximately the same time or before bilirubin peak specific activity with the exception of one dog studied in which peak activity occurred later than that of bilirubin.

7) In human subjects given 2 ^{14}C glycine intravenously, two distinct peaks of bilirubin specific activity were observed, the first occurring between 12 and 24 hours and the second occurring about day three or four. The labelling pattern of plasma bilirubin was reproduced in the bile bilirubin labelling pattern of one subject but two peaks were not found in the other. The maximum daily increment in the specific activity of labelled heme also occurred on day three to four.

8) In human subjects given 4 ^{14}C ΔALA peak specific activity occurred in plasma bilirubin within 90 minutes and no secondary rise was observed. Peak specific activity in bile was reached within six hours. Minimal labelling of heme isolated from peripheral blood was found.

CONCLUSION

Evidence has been presented to indicate that there are at least two distinct fractions of early labelled bilirubin; one of which is erythropoietic in nature and is affected by stimulation or depression of the marrow and the other fraction appears to be extramedullary, persisting in the presence of marrow aplasia and occurring when delta-aminolevulinic acid, a poor heme precursor in mammalian red cells, is the tracer compound.

The most probable site of this extramedullary synthesis is the liver where most porphyrin synthesis occurs outside of the bone marrow. A number of hepatic porphyrin pools may contribute to this first fraction of early labelled bilirubin.

Sources of early labelled bilirubin not involving hematopoiesis or liver porphyrin metabolism are not precluded by this study.

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