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Further Studies on the Nature and Source of the Conjugated Bile Pigments. (28213)

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Bilirubin is formed primarily from degradation of senescent erythrocytes in the reticuloendothelial system(1,2). The manner in which bilirubin is chemically altered and the sites at which it is conjugated for ultimate excretion in the bile have been the topic of much study and controversy. The work of Bollman and Mann(3) on the effect of hepatectomy in dogs showed that, despite absence of the liver, part of the serum bilirubin reacted directly in the Van den Bergh test. It was subsequently demonstrated that water-insoluble bilirubin, prior to its excretion in the bile, is conjugated with glucuronic acid(4) and sulfate(5) forming water-soluble excretory conjugates of bilirubin. The indirect Van den Bergh reaction occurs with free bilirubin whereas a direct reaction is obtained when diazo reagent is added to pigments 1 and 2 obtained by reverse-phase partition chromatography of bile or jaundiced serum. Pigment 1 has been described as bilirubin monoglucuronide whereas pigment 2 is bilirubin diglucuronide and perhaps bilirubin sulfate(6). Although confirmed in rats, the presence of bilirubin sulfate has not been confirmed in dogs or humans(7).

It has been reported(8) that the pigment obtained by reverse-phase partition column chromatography from the plasma of the hepatectomized dog (pigment 1) appeared to have the structure of bilirubin monoglucuronide and was of extrahepatic origin. Exactly where pigment 1 is formed extrahepatically and whether it is formed intrahepatically have not been determined. Nosslin(9) has recently supported the concept that pigment 1 is a complex of one mole of bilirubin diglucuronide (pigment 2) and one mole of unconjugated bilirubin as opposed to the concept of a bilirubin monoglucuronide structure. Pigment 2

TABLE I. Molar Ratio of Glucuronide to Bilirubin in Pigments 2 and 1 as Obtained by Column Chromatography.

Bilirubin		Glucuronide		Molar ratio, glucuronide/ bilirubin
mg/100 ml	10^{-7} moles	mg/100 ml	10^{-7} moles	
Pigment 2, from bile collected by cannulation in 4 rats.				
2.4	419	1.5	773	1.84
2.0	349	1.2	618	1.77
2.8	489	1.6	824	1.69
3.2	559	1.8	927	1.66
				Avg 1.74
Pigment 1, from plasma of hepatectomized dog.				
1.3	227	.48	247	1.08
3.2	559	1.2	618	1.10
2.0	349	.60	308	.88
1.2	210	.48	247	1.18
				Avg 1.06

has not been observed in the hepatectomized dog, indicating that the liver is the sole site of formation of pigment 2 in this species(10). However, it has been reported that the rat kidney also may be capable of forming pigment 2, a species difference that is unexplained(11). Whether the conjugated bilirubin formed by *in vitro* incubations of rat kidney with bilirubin is pigment 1 or pigment 2 has not been determined(12).

In an attempt to study the nature of pigments 1 and 2 and the sites of conjugation, the following experiments were conducted. The results support the concept that pigment 1 is bilirubin monoglucuronide and probably is formed exclusively outside the liver, whereas pigment 2 is formed only within the liver from pigment 1 and unconjugated bilirubin.

Methods. 1. Bile fistulas were prepared in rats by cannulating the common bile ducts with polyethylene catheters. Bile was always collected in the dark and on ice. All experiments were completed as rapidly as possible. 2. The isolated-rat-liver system was modeled after the perfusion apparatus described by Brauer and associates(13), and bile was collected from the cannulated common bile duct of the isolated liver. 3. Total hepatectomy was performed in the dog by the technic of Grindlay and Mann(14). 4. Slices of rat liver were incubated with bilirubin according to the method of Lathe and Walker(15) for study of *in vitro* bilirubin conjugation.

Bile obtained from animals with biliary fistula and from the isolated liver, jaundiced serum from the hepatectomized dog, and the bile pigment produced by liver slices incubated with bilirubin were analyzed for their conjugated bile-pigment content by 3 procedures: the Billing, Cole, and Lathe technic of reverse-phase partition column chromatography(4), the Schachter chemical partition method(16) as modified by McGill and Bollman(17), and the paper chromatographic method as described by Schmid(18).

Bilirubin was estimated by the method of Malloy and Evelyn(19), and glucuronide concentration was determined by the method of Fishman and Green(20). Molar ratios of glucuronide to bilirubin were calculated for pigments 1 and 2.

Serum known to contain bilirubin, pigment 1, and pigment 2 was obtained from 2 jaundiced patients with carcinoma of the pancreas. These sera were subjected to column chromatography; the various pigment bands were separated, redissolved in normal serum and rechromatographed on columns or paper, or submitted to chemical partition, either alone or in combination with other pigments.

Results. Rat-fistula bile. Table I shows the results of simultaneous duplicate analyses for bilirubin and glucuronide in pigment 2 after column chromatography of bile collected *via* the cannulated bile duct of 4 rats. The average calculated molar ratio of glucuronide to bilirubin was 1.74.

The proportion of pigment 1 averaged 14% (range 9-18) while pigment 2 comprised an average of 86% (range 82-91) of the total conjugated pigment in bile of 6 normal rats.

Isolated rat liver. Column chromatographic

analysis of the bile pigment obtained from the cannulated common bile duct of the isolated rat liver yielded only pigment 2. No pigment 1 could be detected in the conjugated pigment by column, chemical partition, or paper chromatography in similar bile samples.

Liver-slice incubation. A pooled sample of the product of 100 liver-slice incubations with bilirubin was analyzed for conjugated pigment. Only pigment 2 was identified by column, paper, and chemical partition chromatography. Pigment 1 was not found even when incubation time was reduced to 60 minutes or 30 minutes instead of the usual 2 hours. The ratio of the glucuronides to bilirubin content of the conjugated bilirubin produced was approximately 1.7.

Hepatectomy. Plasma from the hepatectomized dog always yielded only pigment 1 when tested for conjugated pigment by each of the 3 methods for bile-pigment separation. The calculated average molar ratio of glucuronide to bilirubin in 4 samples of pigment 1 was 1.06 (Table I). Rechromatography of this pigment by the column, paper, and chemical-partition methods likewise revealed only pigment 1.

Jaundiced sera. Column chromatography of jaundiced sera from 2 patients with carcinoma of the pancreas yielded all 3 types of bile pigment. When the bilirubin and pigment 2 from the column were mixed and redissolved in normal serum and rechromatographed using the 3 methods, bilirubin and pigment 2 were recovered and pigment 1 was not formed. Chromatography of pigment 1 alone or in combination with bilirubin or pigment 2 did not appear to change pigments as originally applied to the column.

Comments. If it is accepted that about 15% of the bilirubin is conjugated with sulfate(5), then the molar ratio of glucuronide to bilirubin in pigment 2, found to be about 1.7, may be compatible with the concept of a diglucuronide structure of pigment 2.

Pigment 1 was not found in the bile obtained from the isolated liver or as a product of the *in vitro* liver-slice formation of bilirubin glucuronide. These findings, plus the fact that pigment 1 appeared in the plasma of the hepatectomized dog, suggest that this pigment is formed solely outside the liver. The observation that pigment 1 was not formed upon incubation of bilirubin with liver slices in the synthesis of bilirubin diglucuronide, suggests that the formation of pigment 1 is not a necessary step in conversion of bilirubin to its diglucuronide. Weinbren and Billing(21), having infused bilirubin in amounts sufficient to saturate the capacity of the rat liver to excrete the pigment, noted that, in addition to bilirubin, pigment 1 but not pigment 2 accumulated in the plasma. This serves as further evidence for the extrahepatic formation of pigment 1. Hoffman and associates(11), however, have observed that intravenous injection of bilirubin or hemoglobin to rats led to relative increase in biliary excretion of pigment 1 which they interpreted as exidence that pigment 1 may be formed within as well as outside the liver. It may be that extrahepatic pigment 1 may gain access to the bile through bypassing the conjugating enzymes of the microsomal fraction of the hepatic cells(22).

The exact site or sites of formation of extrahepatic pigment 1 are not known. Although the kidney, the gastrointestinal mucosa, and to a lesser extent the brain are capable of conjugating bilirubin to a direct-reacting pigment(12), it has not been demonstrated that this pigment is a glucuronide or that it corresponds to pigment 1. Inasmuch as the liver contains a significant proportion of the reticuloendothelial system and pigment 1 is not found in the bile of the isolated liver, the reticuloendothelial system seems an unlikely site for formation of pigment 1.

The strongest evidence against the hypothesis that pigment 1 is a complex of pigment 2 and bilirubin rather than a bilirubin monoglucuronide structure alone, is the presence of pigment 1 without pigment 2 in the plasma of the hepatectomized dog. If pigment 1 were a complex, then some pigment 2 should have been identified in the hepatectomized animal. Pigment 2 is formed in the hepatectomized rat, but if both the liver and kidneys are removed from the rat, only free bilirubin and pigment 1 are found in the blood. That the molar ratio of glucuronide to bilirubin in pigment 1 is approximately 1 is, of course, compatible with either structure. We were unable to bring about the formation of pigment

1 by the combination of bilirubin and pigment 2 from columns. This is contrary to the report of Nosslin(9) that, under similar circumstances, a "small but nevertheless distinct" band with a mobility of pigment 1 could be detected.

Summary and conclusions. Rat-fistula bile, isolated rat liver, liver slices incubated with bilirubin, and the hepatectomized dog were utilized to study the nature and sources of conjugated bile pigment. The technics of reverse-phase column chromatography, chemical-partition chromatography, and paper chromatography were employed and molar ratios were calculated. Probably pigment 1 is formed solely in extrahepatic sites. The evidence favors a bilirubin monoglucuronide structure for pigment 1 rather than a complex of bilirubin and pigment 2. In the dog, pigment 2 probably is formed only within the liver. It is suggested that bilirubin diglucuronide may be formed intrahepatically from bilirubin directly, as well as from part of the extrahepatic pigment 1.

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Renal Blood Flow in Acute Renal Failure Measured by Renal Arterial Infusion of Indocyanine Green. (28214)

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Measurements of renal blood flow in oliguric patients with acute renal failure are limited. Early reports using the para-aminohippurate (PAH) clearance and extraction technique(1,2), have been criticized on technical grounds for the very low extraction of PAH by the oliguric kidney. Clearances cannot be measured by this technique if anuria is present, and the extremely low figures for

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