fluence. In these mice the units of TSH/ml of blood times 40 gives an approximate measure of total TSH in units in the body. Thyroxine treatment caused a parallel lowering of the level of TSH in blood and tumor.

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Identification of Extrahepatic Bilirubin Monoglucuronide and Its Conversion to Pigment 2 by Isolated Liver. (26362)

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Bollman and Mann(1) noted that as the amount of serum bilirubin increased after hepatectomy in dogs, the van den Bergh reaction changed from indirect to direct, and bile pigment began to appear in the urine. This indicated that the liver was not the only organ capable of forming bilirubin or converting it to direct bilirubin. Later, work by Billing and Lathe(2), Schmid(3), Talafant(4) and Schachter(5) indicated that conjugation of bilirubin prior to its excretion involves solubilization and accounts for the nature of the reaction of the pigments in the van den Bergh test. The indirect reaction occurs with the water-insoluble free bilirubin, whereas the direct reaction comprises pigment 1 and pigment 2, the water soluble conjugates of bilirubin. Pigments 1 and 2, as obtained by reversed phase partition chromatography, have been described as bilirubin monoglucuronide and diglucuronide, respectively. However, other nonglucuronide conjugates of bilirubin also may be found in pigment 1 and 2 fractions. Bollman(6) observed that the direct pigment in the plasma of the hepatectomized dog has the characteristics of pigment 1 with respect to chromatographic mobility and diazotization reaction. Whether this pigment is a glucuronide or some other conjugate of bilirubin had not been established(7). The present report determines this conjugated pigment of extrahepatic origin to be bilirubin monoglucuronide.

Methods and procedures. Total hepatectomy was performed as previously described by Grindlay and Mann(8), the dog being maintained postoperatively by a saline-glucose solution administered by a constant injection apparatus. Blood was collected for analysis of the plasma pigments at 42 hours after complete removal of the liver.

The proteins were precipitated from the plasma using ethyl alcohol for 30 minutes. After centrifugation, the supernatant was evaporated *in vacuo* and aliquots were dissolved in 0.5 ml of the mobile phase of the solvent system used in the chromatographic separation. It was noted that, as found by Hoffman and associates(9), when ammonium sulfate was used in addition to ethanol for precipitation of the proteins from the plasma of the liverless dog, the pigment appeared to remain bound to the ammonium sulfate crys-

TABLE I. Reactions of Pigment 1 (Isolated by Columns).

Procedure	Glucuronide determination	Direct bilirubin determination			
		NaOH then diazotized	H_2O then diazotized	Diazotized then NaOH	Diazotized then H_2O
mg/100 ml	.48	0	1.3	.564	1.2
10^{-7} moles	247	—	219	—	253
Molar ratio of glucuronide to pigment	—	—	1.13	—	.98

tals and it was not possible to redissolve the pigment sufficiently for satisfactory chromatography. With use of ethyl alcohol only, there was no difficulty in bringing the pigment to solution.

Siliconized kieselguhr was prepared according to the method of Howard and Martin(10). Reversed-phase partition chromatography of the plasma pigments was performed by the method used by Cole and associates(11) and the solvent system used was the butanol-phosphate buffer pH 6 system for separation of the 3 bile pigments.

Three simultaneous columns, with 1 ml of plasma from the hepatectomized dog for each determination, were studied under the following conditions:

The pigment band was washed from the kieselguhr with 5.5 ml of distilled water and separated by filtration. Five milliliters of this substance was utilized for quantitation of glucuronides by the naphthoresorcinol reaction by the method of Fishman and Green (12). Determinations were done in duplicate.

The pigment band was washed from the kieselguhr with 4.5 ml of distilled water and separated by filtration. Two equal aliquots (2 ml each) were added to an equal amount of tenth-normal sodium hydroxide and distilled water respectively. After 30 minutes, diazotized sulfanilic acid was added to both in amounts for determination of the "30-minute direct" van den Bergh reaction, correcting calculations to conform to the modification of Malloy and Evelyn(13).

The pigment band was washed from the kieselguhr with 5 ml of diazotized sulfanilic acid and separated by filtration. Two equal aliquots (2 ml each) were added to an equal amount of tenth-normal sodium hydroxide and distilled water respectively for 30 minutes. The diazotized pigment in each was then estimated with the Beckman spectrophotometer.

A total of 35 ml of plasma from the 42-hour hepatectomized dog was chromatographed on a large kieselguhr column and the single mobile band of pigment was eluted from the column, with the mobile phase of the butanol phosphate buffer pH 6 solvent system. This pigment was dried *in vacuo* at 30°C and reconstituted with rat plasma. The material was then injected into the perfused isolated rat liver system after a control study had been done on the bile pigments. This apparatus was modeled after that described by Brauer and co-workers(14). The bile and plasma were analyzed for the change in amount and type of pigment at 4 hourly intervals after injection of the pigment.

Results. The bilirubin of the hepatectomized dog was 8.5 mg total and 5.0 mg direct for each 100 ml of plasma. The results of the studies of the 3 simultaneous columns prepared from the plasma of the hepatectomized dog are shown in Table I. When the pigment was first treated with sodium hydroxide and then diazotized, all of the pigment was found to be labile to the alkali treatment. However, when the pigment was first diazotized and then treated with alkali, about half (47%) was found to be alkali stable. The molar ratio of glucuronide to pigment (expressed as bilirubin) was approximately 1. Internal controls were inherent in the system.

Table II illustrates the increase in bile pigment and specifically of pigment 2 as measured in the bile collected *via* the cannulated bile duct. This increment occurred after the pigment 1 obtained from the plasma

TABLE II. Biliary Excretion (Perfused Liver) after Pigment 1.

Hourly interval	Bile vol, ml	Direct pigment in bile, mg/100 ml	Pigment 2 in bile, mg/100 ml
Preinj. control	.7	7.6	4.2
1st	.7	15.9	6.1
2nd	.52	19.4	6.9
3rd	.5	19.0	5.5
4th	.5	7.3	3.9

of the hepatectomized dog had been injected into the perfused preparation. Control values were reached again 4 hours after the injection. In control experiments without injection of pigment 1 into the perfusion apparatus, excretion of pigment during a similar 4-hour period remained essentially the same as during the first hour of experiment. After losses of pigment during isolation of pigment 1, it was estimated that 0.18 mg of pigment 1 was injected into the perfusion system. All pigment was removed from the perfused blood and no pigment 1 was found in the bile. Over a 3-hour period 95% of the injected pigment 1 was recovered as pigment 2 in the bile as determined by the increment (0.17 mg) in the direct van den Bergh reaction and chromatography of the bile. The figures given for pigment 2 recovered by column chromatography indicate the loss of this pigment during the procedure. Were any appreciable amounts of pigment 1 present, however, they would have been detected by the columns.

Comment. Both the 42-hour survival and the high levels of plasma bilirubin of the hepatectomized animal afforded the opportunity to carry out these studies. This level of total plasma bilirubin is equivalent to that found after obstruction of the common bile duct for a period of similar duration.

Isselbacher and McCarthy(15) showed that about 15% of the conjugated azopigment in bile is an alkali-stable sulfate conjugate. Work in progress in this laboratory indicates that the sulfate conjugate migrates with the pigment 2 band, whereas no alkali-stable sulfate component is found in pigment 1. Pigment 1 can be accounted for by the monoglucuronide structure. Thus, in this study, the lability of the pigment from the plasma of the hepatectomized dog is compatible with a monoglucuronide structure.

Overbeek and associates(16), in studying the kinetics of the van den Bergh reaction, concluded that after bilirubin is split at the methene bridge, both of the dipyrrol compounds couple with the diazonium salt. Thus, when the pigment from the plasma of the hepatectomized dog is diazotized prior to alkali treatment, only that half of the molecule esterified with the glucuronic acid is labile to the sodium hydroxide. In this situation, alkali lability of 50% speaks for the monoglucuronide structure.

The indications of a monoglucuronide structure that have been mentioned were confirmed when a molecular ratio of 1:1 for glucuronic acid to bilirubin was found in the pigment.

Summary and conclusions. The pigment obtained by reversed-phase partition chromatography from the plasma of the hepatectomized dog has been shown to be bilirubin monoglucuronide (pigment 1). In the experiments performed, pigment 1 was of extrahepatic origin. The isolated perfused rat liver converts bilirubin monoglucuronide to bilirubin diglucuronide and excretes the diconjugate in the bile.

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Parabiotic Anemia-Polycythemia.* (26363)

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Surgical parabiosis of genetically diverse animals frequently results in severe and persistent anemia of one partner and polycythemia of the other(1,2,3). This may be observed as early as the third day after junction. In the absence of genetic diversity this is rare, and, if present, only mild and of short duration. Anemia-polycythemia (AP) has been most commonly observed in instances of unilateral genetic diversity, *i.e.*, after parabiosis of a mouse of one inbred strain with its F_1 hybrid(4). AP has been interpreted generally as due to an immune response of the inbred mouse against the unrelated parent component of the hybrid. In this combination the hybrid becomes anemic and the inbred mouse polycythemic, a development probably brought about by a shift of red cell mass from the hybrid into the inbred mouse. If the AP were the result of an immune response to incompatible tissue, it could be used as an assay of this response. However, this assay should only be of aid in unilateral immune responses. Bilateral immune responses, both aiming at AP, should tend to cancel each other.

The data presented here were obtained from mice of 2 unrelated strains, parabiosed to teach other and to their common F_1 hybrids. They suggest that at least 2 separate factors operate to bring about AP, only one of them being immunologic in nature.

Material and Methods. Experimental animals were inbred mice from our colonies, of the C57BL/6 and BALB/c strains, and their reciprocal hybrids. Partners were of the same sex, with approximately equal numbers of male and female pairs. The technique of parabiosis has been described(3). Blood for microhematocrit determination was obtained from blood of the tail vein. The mice were bled on the 6th, 10th, 13th, and 17th days after parabiosis.

Observations. Parabiosis of *BALB/c mice with (C57BL/6 x BALB/c)F_1* hybrids resulted in AP on the 6th day in all pairs, invariably "favoring" (= creating polycythemia in) the BALB/c partner (Table 1). It increased in severity until the end of the 3rd week when death had occurred in most instances.

Parabiosis of *C57BL/6 mice with (C57BL/6 x BALB/c)F_1* hybrids did not result in similar AP on the 6th day. Instead, 18 of 25 pairs showed a moderate AP favoring the *hybrid* partner (mean: 15.7 S.D. $\pm$ 8.4), while the remaining 7 pairs showed mild AP favoring the C57BL/6 (mean: 9.4, S.D. $\pm$ 8.1). However, the trend reversed itself on successive bleeding days; results became uniform, all pairs showing AP favoring the C57BL/6 side, but significantly less severe than in the BALB/c-hybrid combination ($p<0.02$).

Parabiosis of *BALB/c with C57BL/6* mice

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