Increased Bilirubin Production from Sources Other Than Circulating Erythrocytes in Mutant Southdown Sheep[1] (35232)

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Studies on bilirubin-^{14}C turnover (1) in mutant Southdown sheep with congenital hyperbilirubinemia have indicated the existence of a hepatic uptake defect for unconjugated bilirubin (UCB) resembling that in Gilbert's syndrome in man (2, 3). "Early-labeled bilirubin," which originates from sources other than the turnover of hemoglobin of circulating erythrocytes, may account for 10–20% of the total bilirubin production under normal conditions in man (4). At least two early-labeled peaks of plasma bilirubin radioactivity have been observed in man (5, 6) following the injection of glycine-2-^{14}C, one related to erythropoiesis and the other derived from the rapid turnover of hepatic hemoproteins including some of the microsomal cytochromes. The overproduction of "early-labeled bilirubin" from either source can account for increased bilirubin production and hyperbilirubinemia.

Since erythrocyte volume was significantly less on the average in three mutants ($p<0.05$) while bilirubin production in individual mutants was either similar to or more than that in normal sheep (1), the present study was undertaken to determine what percentage of bilirubin production could be accounted for either from the turnover of hemoglobin of circulating erythrocytes or from other sources.

Materials and Methods. The total bilirubin-production rate was determined in 3 normal and 3 mutant mature Southdown sheep by an indirect method (1), in which the plasma UCB-^{14}C disappearance curve was used to calculate intercompartmental rate constants, pool sizes, and irreversible removal (IR) of UCB. Since steady state conditions were present in both normal and mutant sheep, UCB-IR was considered equal to the UCB production rate. The endogenous conjugated bilirubin (CB) excretion in bile measured directly by bile duct cannulation in normal and mutant sheep varied from 70 to 92% of the indirect UCB-IR calculations. Approximately 78% of bilirubin constantly infused at a submaximal rate is excreted into bile in normal sheep (7). These findings support the use of UCB-IR in the indirect method to estimate the rate of total bilirubin production.

Bilirubin produced from the turnover of hemoglobin in circulating senescent erythrocytes was calculated from the mean erythrocyte life span of each sheep and the erythrocytic cell mass determined from the microhematocrit and plasma volume. Since the mean corpuscular hemoglobin concentrations in both mutant and normal ovine erythrocytes were on the average 33.5% and each gram of hemoglobin is capable of generating 35 gm of bilirubin, the turnover of each ml of erythrocytes would result in the production of 11.7 mg of bilirubin. Erythrocyte life span was determined by the *in vivo* labeling of erythrocytes with ^{75}SE-selenomethionine according to the method of Penner (8). Plasma volume was measured using ^{131}I-labeled albumin.[3] The rate of bilirubin production from sources other than the circulating erythrocytes was calculated for each sheep from the difference between total bilirubin produc-

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tion rate (UCB-IR) and that originating from erythrocyte turnover.

Glycine-2-^{14}C was injected intravenously into 2 normal (13.1 and 13.6 μCi/kg) and 2 mutant (11.0 and 13.2 μCi/kg) sheep to determine if 2 major components of plasma "early-labeled bilirubin" could be detected as previously observed in man (5, 6). Plasma bilirubin was separated into conjugated and unconjugated fractions according to a modification of the technique of Weber and Schalm (1). The unconjugated bilirubin fraction was washed twice with two volumes of distilled water. It was then absorbed on a small column of neutral aluminum oxide for column chromatography. The bilirubin was next washed with 10 ml of chloroform, eluted from the column with 3 ml of 1% acetic acid in chloroform, and subsequently counted for radioactivity by previously described methods (1). Radioactive glucose, glycine, and methionine added to the plasma prior to the separation of CB and UCB were found to be excluded from the final bilirubin-containing eluate. The specific activity of UCB remained constant even after its precipitation from eluate. The specific activity of UCB was calculated from the radioactivity of the eluate and its bilirubin concentration, determined by comparing its optical density at 450 mμ with that of standard solutions of bilirubin in eluting fluid.

Plasma and erythrocyte iron turnover was measured in two normal and one mutant sheep according to the method of Huff *et al.* (9); to determine if ineffective erythropoiesis could be present in the mutant. Plasma iron concentrations and the unsaturated iron-binding capacities were determined by the method of Williams and Conrad (10).

Results and Discussion. Bilirubin production rates from circulating erythrocytes and other sources in 3 normal and 3 mutant sheep are presented in Table I. Mean erythrocyte life spans of normal and mutant sheep did not differ significantly and were within the normal range previously reported (11). The erythrocytic cell mass, however, was significantly lower ($p<0.05$) on average in mutants (17.63 $\pm$ 2.2 ml/kg) than in normal sheep (23.7 $\pm$ 1.9 ml/kg). Blood volumes were similar due to a compensatory increase in plasma volume of mutants (41.1 $\pm$ 3.0 ml/kg) compared to normal sheep (32.0 $\pm$ 0.7 ml/kg).

The production rates of bilirubin in mutants as calculated from the bilirubin-^{14}C transfer rates appear on average as a group statistically similar to normal sheep (Table I); however, the two mutants with the lowest circulating red blood cell masses exhibited apparent elevated rates of production. In normal sheep, bilirubin produced from sources other than turnover of circulating erythrocyt-

TABLE I. Bilirubin Production in Normal and Mutant Southdown Sheep.

Animal no.	Mean erythrocyte life span (days)	Circulating erythrocyte turnover (ml/day/kg)	Bilirubin production: Total (mg/day/kg)	From circulating erythrocytes (mg/day/kg)	% of total	From other sources (mg/day/kg)	% of total
Normal							
1	72	0.30	4.52	3.52	78	1.00	22
2	114	0.19	2.92	2.23	76	0.69	24
3	128	0.22	3.54	2.58	73	0.96	27
	105 $\pm$ 17	0.24 $\pm$ 0.03	3.66 $\pm$ 0.47	2.78 $\pm$ 0.38	76 $\pm$ 2	0.88 $\pm$ 0.1	22 $\pm$ 2
Mutant							
5	123	0.16	7.52	1.88	25	5.64	75
6	128	0.11	5.69	1.29	23	4.50	77
7	136	0.13	2.86	1.52	55	1.34	47
	129 $\pm$ 4	0.1 $\pm$ 0.02[a]	5.36 $\pm$ 1.4	1.56 $\pm$ 0.17[a]	34 $\pm$ 10[a]	3.83 $\pm$ 1.3[a]	66 $\pm$ 10[a]

[a] Significantly different from normal by one-tailed t test.

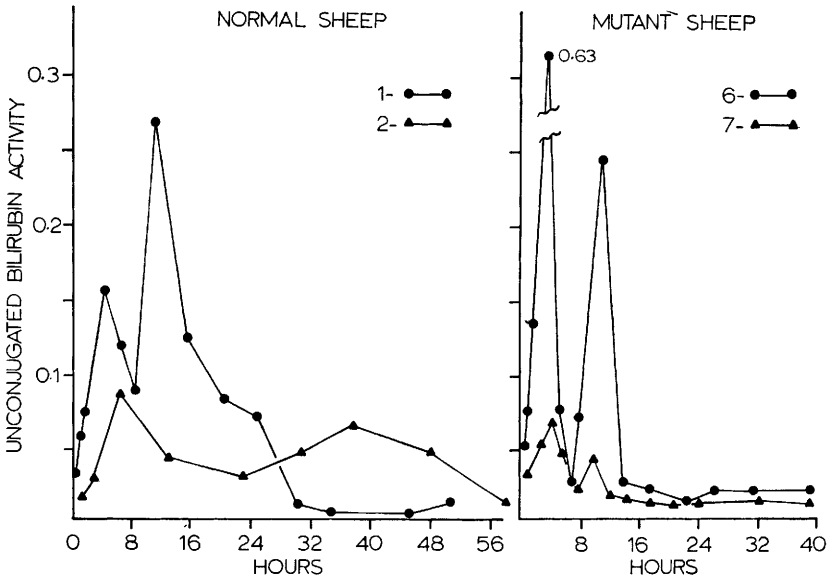

FIG. 1. Percentage of injected ^{14}C per milligram of unconjugated bilirubin recovered in plasma unconjugated bilirubin fraction following intravenous injection of glycine-2-^{14}C into 2 normal and 2 mutant Southdown sheep.

ic hemoglobin averaged 22% of the total production; a similar value has been reported for normal man (4). In mutant sheep, 66% (av) of the bilirubin originated from sources other than from circulating erythrocyte turnover and represented a 4-fold absolute increase above normal for bilirubin production from such sources ($p<0.05$).

Following the intravenous injection of glycine-2-^{14}C into normal and mutant sheep, at least two separate "early-labeled" peaks were observed in the plasma UCB specific activity curves (Fig. 1). In both the normal and mutant sheep, the first "early labeled" fraction peaked at 4–8 hr while the second fraction peaked 10–36 hr after injection, indicating a similarity to observations reported for man (5, 6). The first fraction has been postulated to be due to the turnover of tissue "easily split" or microsomal hemes while the second fraction may result from erythroid precursors in bone marrow. Data for plasma iron concentration and turnover, unsaturated iron-binding capacity, and erythrocyte iron turnover in one mutant and two normal sheep are presented in Table II. Ferrokinetic data from the mutant and normal sheep were within normal limits (12, 13), suggesting that "ineffective erythropoiesis" most likely was not present in the mutant. That was confirmed when hypoplastic bone marrow was observed in two mutants at necropsy.

TABLE II. Ferrokinetics in Normal and Mutant Southdown Sheep.

Animal no.	Plasma iron conc (μg/100 ml)	Unsaturated iron-binding capacity (μg/100 ml of plasma)	Iron turnover (μg/day/kg)	
			Plasma	Erythrocyte
Normal				
2	147	428	520	260
4	161	405	530	270
Mutant				
6	153	414	520	220

There is substantial and growing evidence that jaundice in many liver disorders may partly result from overproduction of bilirubin from nonhemoglobin sources within the liver (14). Using data presented by Barrett *et al.* (3) on a patient with Gillert's syndrome and no evidence of hemolysis, we calculated that 35% of bilirubin produced was from sources other than from turnover of circulating erythrocytes. Overproduction of bilirubin by the liver both in Gilbert's syndrome in man and in congenital hyperbilirubinemia in Southdown sheep should be considered and investigated further.

Summary. Bilirubin production from circulating erythrocytes and from other sources was studied in normal and mutant Southdown sheep with congenital hyperbilirubinemia. In mutant sheep, 66% on average of the total bilirubin produced originated from sources other than turnover of hemoglobin of circulating erythrocytes. This represented a 4-fold absolute increase above the bilirubin production rate from such other sources in normal sheep. Two separate "early-labeled" fractions were observed in both normal and mutant sheep following the injection of glycine-2-^{14}C. Since mutant sheep exhibited no signs of ineffective erythropoiesis as evidenced by hypoplastic bone marrow and normal plasma and erythrocyte iron turnover, the hepatic overproduction of bilirubin should be considered and further investigated.

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