Bilirubin-^{14}C Turnover Studies in Normal and Mutant Southdown Sheep with Congenital Hyperbilirubinemia[1] (34603)

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Mutant Southdown sheep exhibit a marked delay in the plasma clearance of organic anions such as unconjugated bilirubin (UCB), sulfobromophthalein, sodium cholate, and indocyanine green (1). A delay in the plasma clearance of various injected anions, the absence of any recycling of BSP conjugates from the liver into plasma, and the existence of an unconjugated hyperbilirubinemia primarily have been interpreted as evidence that the hepatic uptake of certain organic anions may be defective in Southdown mutants (1). Impaired hepatic uptake of bilirubin has been demonstrated by Billing *et al.* (2) in Gilberts' syndrome in man.

Barrett *et al.* (3) administered tracer quantities of bilirubin-^{14}C to patients and normal volunteers to determine the plasma bilirubin-^{14}C disappearance rates. They proposed a two-compartment model for the transport of UCB and calculated intercompartmental rate constants, bilirubin production rates, and pool sizes from plasma bilirubin-^{14}C disappearance curves.

Kinetic data on bilirubin transport in sheep is lacking. In the present study, bilirubin-^{14}C was injected as a tracer to estimate intercompartmental transfer rates and pool sizes of UCB in normal and mutant Southdown sheep.

Materials and Methods. Five mutant and six normal Southdown sheep were housed indoors for 2 months prior to the experimental trials. This prevented the photosensitization which occurs in mutants due to their inability to adequately excrete phylloerythin into the bile.

Bilirubin-^{14}C was prepared essentially as recommended by Barrett *et al.* (4), except that approximately 300 μCi of Δ-aminolevulinic acid-4-^{14}C (19-22 mCi/m*M*) were injected into a dog in three divided doses at 3 to 4-hr intervals following bile duct cannulation. Purification on aluminum oxide columns was omitted and the pooled chloroform extracts were washed twice with distilled water following a phosphate buffer wash. Chloroform was evaporated at 80° under a nitrogen stream. Specific activities of purified bilirubin ranged from 2–6 μCi/mg and its molar extinction coefficient at 450 mμ in a chloroform solution was more than 57,000.

Immediately prior to intravenous injection, 2–7 μCi of recrystallized bilirubin were dissolved in 5–10 ml of 0.05 *N* NaOH under subdued light, and an equal volume of sheep plasma or 5% bovine albumin was added. Isotopic bilirubin solutions were injected into sheep via polyethylene tubing inserted into the left jugular vein. Injected bilirubin equalled 4 to 13% of the total calculated bilirubin pool. To study the effect of hyperbilirubinemia on bilirubin turnover kinetics, two normal sheep were continuously infused with nonradioactive bilirubin solutions at a rate of 4 μg/kg/min. Bilirubin-^{14}C was injected into the infused sheep 1 hr after plasma UCB levels had stabilized at a level similar to that of the mutants. Infusion of nonradioactive bilirubin was continued during the 6-hr trials. Four-ml-heparinized blood samples were withdrawn at specific intervals from the right jugular vein for 6 hr to determine

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the disappearance rate of plasma UBC-^{14}C radioactivity.

Plasma bilirubin was separated into unconjugated and conjugated fractions by the technique of Weber and Schalm (5). One ml of plasma and 2.3 ml of chloroform were added to 6.5 ml of the lactic acid-ethylacetate (8:5) mixture, shaken for 0.5 min and centrifuged at 1500*g* for 3 min to separate the unconjugated and conjugated bilirubin fractions. Three ml of the unconjugated fraction were transferred to a counting vial and evaporated at 80° to remove the volatile solvent. Solubilizer (BBS-3[3], 0.5 ml) and counting solution (0.4% PPO and 0.01% POPOP in toluene, 10 ml) were added to the vials after evaporation. Radioactivity was counted in a liquid scintillation counter according to the method of Bruno and Christian (6), which allows correction for quenching by two-channel counting. The efficiency of the separation technique was measured using bilirubin-^{14}C and varied directly with concentration of UCB at concentrations below 5 μg/ml. Appropriate corrections were made for both the concentration and radioactivity of plasma UCB.

The concentrations of plasma bilirubin were determined by the techniques of Weber and Schalm (5) and Malloy and Evelyn (7). Concentrations of plasma bilirubin determined by those two methods agreed reasonably well. Specific activity of plasma UCB was determined from its concentration and the counted radioactivity, and expressed as disintegration per min (D.P.M.) per μg of plasma UCB.

A model for bilirubin turnover and the equations derived by Richards *et al.* (8) and Barrett *et al.* (3) for the calculation of transfer rates and sizes of the mixing pool (MP) and storage pool (SP) are presented in Fig. 1. The validity of such a mathematical model to calculate turnover and transfer rates between compartments has been demonstrated (9). The model assumes that the labeled bilirubin rapidly mixes in the MP (primarily plasma) and is in constant equilibrium with SP (primarily hepatic cell mass). UCB is irreversibly removed from the SP by conjugation and rapid excretion into the bile. At steady state, the rate of production of UCB is postulated to be equal to the rate of its irreversible removal.

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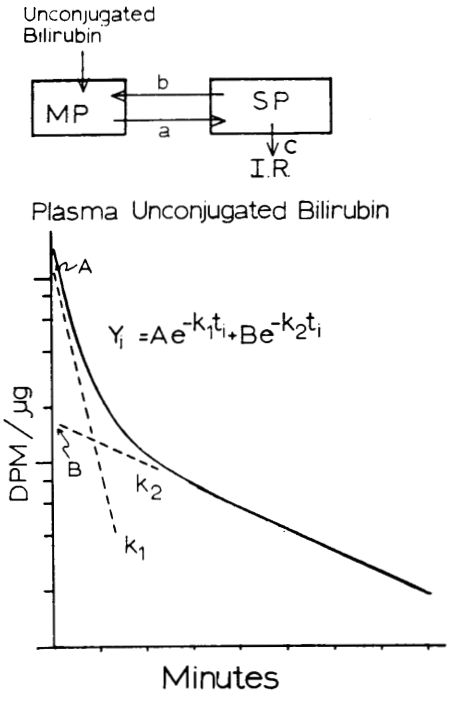

FIG. 1. Model for bilirubin turnover: A and B are the intercepts, and k_1 and k_2 are the slopes of the two components of the disappearance curve; *MP* and *SP* are the mixing and storage pools; *a*, *b*, and *c* are the fractional transfer constants. I.R. is the irreversible removal. $a = \frac{Ak_1 + Bk_2}{A+B}$; $b = (k_1 + k_2) - (a + c)$; $c = \frac{k_1 k_2}{a}$; $MP = \frac{injected\ dose}{A+B}$; $SP = \frac{a}{b+c} \times MP$.

Results and Discussion. Data pertaining to the disappearance of bilirubin-^{14}C in five mutants and six normal sheep are presented in Table I. Best-fit curves representing two exponentials were drawn following analysis on a digital computer using the SAAM program of Berman and Weiss (10). A third slower exponential process was not apparent; this was additionally confirmed in one trial in which the plasma radioactivity was followed for 14 hr. Transfer rates, fractional transfers,

TABLE I. Data Pertaining to the Disappearance of Plasma Unconjugated Bilirubin-^{14}C in Normal and Mutant Southdown Sheep.

Animal (no.)	Body wt (kg)	Radioactivity injected (dpm[a] × 10^8)	Extrapolated radioactivity of plasma unconjugated bilirubin at zero times (dpm × $10^3/\mu g$)		Half-time (min)	
			1st component	2nd component	1st component	2nd component
Normal						
1	23	2760	1.612	0.139	10.5	71.1
2	34	8930	7.220	0.331	12.0	126
3	24	7105	5.083	0.334	12.2	133
4	25	6906	4.667	0.203	6.9	150
Normal (infused)						
5	31	7447	1.795	0.116	5.5	140
6	38	6458	0.588	0.097	14.8	132
Mutant						
7	17	5494	1.226	0.178	14.5	126
8	30	12,196	1.421	0.831	17.1	145
9	36	4440	0.393	0.250	11.4	90.5
10	45	5824	0.236	0.220	11.2	119
11	43	7235	0.614	0.365	10.8	212

[a] dpm = disintegration per minute.

and pool sizes of UCB are presented in Table II.

The significantly reduced fractional transfer of UCB from the MP to the SP in mutant sheep with hyperbilirubinemia may indicate a defect in the membrane transfer mechanism. Fractional transfer of UCB from the SP back to the MP in mutants was nearly 5 times greater than in normal sheep. Approximately 63% of the UCB transferred from the MP to the SP was transported back to the MP in mutants, compared with only 33% in normal sheep. This difference occurred even though the UCB SP's were of similar magnitude in both normal and mutant sheep. The net hepatic uptake of UCB per minute in mutants was on average only 1.4 of the MP as compared with 4.4% in normal sheep. The relative decrease in net uptake resulted in a MP enlarged to such a proportion that the amount of UCB removed from the MP at this reduced fractional uptake equalled the production of UCB. Thus, a steady state is maintained with hyperbilirubinemia in the mutant sheep.

Fractional UCB transfer from the MP to the SP (Table II) and MP/SP ratios were not significantly altered in two normal sheep during the constant infusion of bilirubin to simulate a comparable hyperbilirubinemia as observed in mutant sheep. Fractional transfer from the SP to the MP in infused sheep was nearly twice that of normal controls and suggested that a lesser proportion of UCB remains within hepatic cells in loading experiments.

The total amount of UCB irreversibly removed (I.R.) from the SP was proportional to the total UCB pools in both mutant and normal sheep. Therefore, no major conjugation or excretory defect would likely be present as a rate limiting step in the mutant sheep. The possibility of other routes of UCB degradation or sites of excretion have not been investigated as yet. Bilirubin UDP-glucuronyl transferase activities in liver were found to be within the normal range in a previous study on one mutant Southdown sheep (1). Black and Billing (11) recently reported that low glucuronyl transferase levels are present in Gilberts' syndrome in man if a new enzyme method is employed. Preliminary studies using this new enzyme method (11) on biopsy specimens from two

TABLE II. Transfer Rates, Fractional Transfer, and Pool Sizes of Unconjugated Bilirubin in Normal and Mutant Southdown Sheep.

	Units	Normal sheep (4)	Mutant sheep (5)	Normal sheep infused with bilirubin (2)
		$\bar{X} \pm$ SE	$\bar{X} \pm$ SE	$\bar{X} \pm$ SE
Unconjugated plasma bilirubin	μg/ml	0.8 ± 0.07	4.0 ± 0.5[b]	3.5 ± 0.5[b]
Total plasma bilirubin	μg/ml	1.0 ± 0.08	7.4 ± 0.2[b]	4.1 ± 0.6[b]
Mixing pool (M.P.)	μg/kg	55.8 ± 6.9	217 ± 22[b]	170 ± 53
Storage pool (S.P.)	μg/kg	398 ± 90	403 ±110	971 ±137
M.P./S.P.	%	15.5 ± 2.9	63.9 ± 11[b]	18.7 ± 8.5
Transfer rates from:				
1. M.P. to S.P.	μg/kg/min	3.77 ± 0.79	8.1 ± 0.99[a]	11.49 ± 2.1[a]
2. S.P. to M.P.	μk/kg/min	1.32 ± 0.43	4.99 ± 0.67[a]	6.06 ± 0.86[a]
3. S.P. to I.R.[c]	μg/kg/min	2.45 ± 0.42	3.11 ± 1.6	5.43 ± 0.11[a]
Fractional transfer from:				
1. M.P. to S.P.	min^{-1}	0.0666 ± 0.01	0.0372 ± 0.0028[a]	0.0794 ± 0.038
2. S.P. to M.P.	min^{-1}	0.00312 ± 0.0003	0.0153 ± 0.003[a]	0.00616 ± 0.009
3. S.P. to I.R.[c]	min^{-1}	0.00662 ± 0.0012	0.00795 ± 0.0013	0.00555 ± 0.00001

[a] or [b] = Probability less than 5% or 1%, respectively, as compared with normal group.
[c] I.R. = irreversible removal.

normal and three mutant sheep showed no significant differences in hepatic bilirubin UDP-glucuronyl transferase activity.

The hepatic uptake defect for bilirubin described in the mutant Southdown sheep closely resembles that reported in Gilberts' syndrome in man (2, 3). A large percentage of the total UCB is also present in the MP in Gilberts' syndrome in man as in mutant sheep. Unlike the mutant sheep, both the UCB SP and the UCB MP are greater in Gilberts' syndrome than in normal man. From data reported in a study by Barrett *et al.* (3), it can be calculated that 69 and 33% of the UCB transferred to the SP from the MP is transferred back to the MP in Gilberts' syndrome and normal man, respectively. In comparison, 63 and 33% on average are transferred back in mutant and normal sheep, respectively. Net hepatic uptake per minute of UCB was likewise calculated to be 0.42 and 1.77% of the MP in Gilberts' syndrome and normal man (3), compared with 1.4 and 4.4% in mutant and normal sheep, respectively.

Billing *et al.* (2) in an earlier study on Gilberts' syndrome, reported that the fractional transfer rates of UCB from plasma to liver and liver to plasma were respectively one-half and twice that of normal man. They proposed that a hepatic cell membrane defect and a decreased UCB holding capacity of the hepatic cells could account for such phenomena. Unlike mutant Southdown sheep, they (2) reported that certain patients with Gilberts' syndrome exhibited a lower fractional irreversible removal of UCB and suggested a possible reduction in bilirubin conjugation.

Since a marked hapatic uptake defect for BSP has been observed in mutant Southdown sheep, in contrast to apparently no defect in Gilberts' syndrome in man, a common uptake mechanism for bilirubin and BSP seems unlikely. This is further supported by recent *in vitro* studies (12) in which bilirubin did not compete with BSP for binding sites on hepatic cell membranes isolated from rats.

Summary. Bilirubin-^{14}C turnover was studied in normal and mutant Southdown sheep with congenital hyperbilirubinemia. Intercompartmental rate constants, pool sizes,

and transfer rates of unconjugated bilirubin (UCB) were calculated from the plasma bilirubin-^{14}C disappearance curve. The net hepatic uptake per minute of UCB was on the average 1.4% of the mixing pool in mutants as compared with 4.4% in normal sheep. The hepatic uptake defect for UCB in mutant Southdown sheep closely resembles that previously reported in Gilberts' syndrome in man.

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