Hepatic Uptake of Organic Anions Affects the Plasma Bilirubin Level in Subjects With Gilbert's Syndrome Mutations in *UGT1A1*

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Although in Gilbert's syndrome (GS), bilirubin glucuronidation is impaired due to an extra TA in the TATA box of the promoter of the gene for bilirubin UDP-glucuronosyltransferase 1 (*UGT1A1*), many GS homozygotes lack unconjugated hyperbilirubinemia. Accordingly, an additional defect in bilirubin transport might be required for phenotypic expression. Plasma bilirubin and the early fractional hepatic uptake rate (BSP K_1) of a low dose of tetrabromosulfophthalein (0.59 μmol/kg) were determined in (1) 15 unrelated patients with unconjugated hyperbilirubinemia plus 12 random controls; (2) 4 unrelated GS probands and 15 of their first-degree relatives; (3) 7 unrelated patients with hemolysis due to β-Thalassemia minor. Subjects were classified by DNA sequencing of the promoter region of both *UGT1A1* alleles. In group 1, GS homozygotes showed a highly significant negative linear correlation between plasma bilirubin levels and BSP K_1. BSP K_1 values overlapped considerably between GS and normal subjects, whereas, in group 2, they were clustered within, and sharply segregated among, families. Patients with hemolysis, despite elevated plasma bilirubin levels, had mean BSP K_1 values similar to the normal subjects. Within each GS subgroup with defined *UGT1A1* mutations, the plasma bilirubin level is in part determined by the organic anion uptake rate, assessed by early plasma disappearance of low-dose BSP. The lower BSP uptake in GS is not secondary to the hyperbilirubinemia, but probably caused by (an) independent, genetically determined defect(s) in hepatic transport mechanism(s), shared by BSP and bilirubin, that are likely necessary for phenotypic expression of GS. (HEPATOLOGY 2001;33:627-632.)

Gilbert's Syndrome (GS) is an inherited form of mild, chronic, unconjugated hyperbilirubinemia,[1-3] which is associated with an extra TA in the promoter region of both alleles for bilirubin UDP-glucuronosyltransferase 1 (*UGT1A1*).[4-8] The resultant 65% decrease in transcription of the $(TA)_7TAA$ mutant alleles explains the impaired conjugation of bilirubin found in all GS patients.[9,10]

The abnormal $(TA)_7TAA$ allele has a reported prevalence of 34% to 40% in white adults[4,5] and Sephardic Jewish neonates.[11] Thus, 12% to 16% should be and are homozygous for this abnormality,[4-8] yet only 3% to 9% of such populations, or less than half the expected proportion of homozygotes, have an increased level of unconjugated bilirubin (UCB) in plasma.[7,8,12-14] This suggests that additional steps in bilirubin metabolism and/or transport must be impaired for hyperbilirubinemia to be manifested in $(TA)_7TAA$ homozygotes with reduced expression of UGT1A1.

Thus far, both increased bilirubin production (from hemolysis,[1-3,11,15,16] which may not be overt[16-18]) and/or decreased hepatic uptake of UCB have been documented in many presumed GS subjects. Some also exhibit decreased plasma clearance of tetrabromosulfophthalein (BSP)[20-22] and other organic anions,[20,23,24] including several therapeutic agents,[24-26] which appear to share hepatic uptake mechanisms with UCB.[27] With BSP, impaired uptake may be unmasked in GS patients if the administered dose is reduced to 0.59 μmol/kg body weight (one tenth of the usual dose).[22]

To assess if decreased hepatic uptake must coexist with decreased conjugation of bilirubin for the phenotypic expression (hyperbilirubinemia) of *UGT1A1* mutations in GS subjects without overt hemolysis, we determined the presence of the extra TA (GS allele) and the plasma disappearance rate of the low dose of BSP in 2 subgroups: unrelated subjects with mildly elevated or normal plasma bilirubin levels and 4 unrelated, jaundiced GS probands and their first-degree relatives. A group of patients with hemolytic anemia were also tested to determine if hyperbilirubinemia *per se* would impair the removal of the low dose of BSP. The results strongly suggest that the rate of hepatic uptake of a low dose of BSP, and thus, presumably, of bilirubin, is a key determinant of the presence and severity of hyperbilirubinemia in patients with impaired bilirubin glucuronidation related to the abnormal GS allele of *UGT1A1*.

Abbreviations: GS, Gilbert's syndrome; UGT1A1, bilirubin-UDP glucuronosyltransferase 1; UCB, unconjugated bilirubin; BSP, tetrabromosulfophthalein; CN, Crigler-Najjar II syndrome; TB, total plasma bilirubin; BSP K_1, fractional initial plasma disappearance rate of BSP (min^{-1}); 7&7, GS homozygotes with TA_7TAA polymorphism in the promoter region of both alleles of the *UGT1A1* gene; 6&6, normal subjects with the normal TA_6TAA pattern in both alleles; 6&7, GS heterozygote with TA_7TAA polymorphism in only one allele; 6CN&7, compound heterozygotes, with a structural Crigler-Najjar mutation in the coding region of one allele of *UGT1A1* and the TA_7TAA polymorphism in the other allele; OATP, organic anion transporting polypeptide.

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Received June 5, 2000; accepted December 4, 2000.

Supported by the Ministries of University Technology and Scientific Research (Cofin 98), the Ministry of Health (ICS060.1/RF98.67), and the Liver Study Foundation, Trieste, Italy; and the Gastroenterology Foundation, Academic Medical Center, University of Amsterdam, The Netherlands.

Presented in part at the annual meeting of the AASLD, Dallas, TX, Nov. 1999. Abstract in HEPATOLOGY 1999;30:501A.

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0270-9139/01/3303-0019$35.00/0

doi:10.1053/jhep.2001.22499

PATIENTS AND METHODS

The protocols were approved by the Human Subjects Committees of the University of Naples and University of Trieste, according to the ethical guidelines of the 1975 Declaration of Helsinki; each subject gave informed consent in writing. Three groups were studied (Table 1):

Group 1 consisted of 15 hyperbilirubinemic subjects (presumed GS), 12 men and 3 women, ages 22 to 35 years, and 12 randomly selected controls (9 men and 3 women, ages 22 to 37 years) with normal bilirubin levels.

Group 2 consisted of 19 subjects, in 4 families, ages 18 to 57 years, including 4 mildly jaundiced GS probands (3 men and 1 woman). Except for an additional patient in family D, who had Crigler-Najjar II (CN) syndrome, none of the other 15 first-degree relatives (4 men and 11 women) had hyperbilirubinemia.

Group 3 consisted of 4 men and 3 women, ages 25 to 41 years, with mild chronic hyperbilirubinemia due to β-Thalassemia minor.

In groups 1 and 2, the clinical diagnosis of GS was based on the standard criteria of mild chronic, unconjugated hyperbilirubinemia, normal liver function tests, and no overt signs of hemolysis (normal erythrocyte and reticulocyte counts, erythrocyte osmotic fragility, and hemoglobin electrophoresis).[1,27] None of the subjects had a history of hepatic or hematologic disorders, excessive alcohol intake, or chronic use of medications or narcotics, and none received any drug during the 2 weeks before investigation. The CN patient in family D was not considered further because he required chronic therapy with high-dose phenobarbital, which decreases plasma bilirubin levels by increasing hepatic clearance of UCB.[28]

In each subject, after an overnight fast, serum levels of total bilirubin (TB) and direct-reacting bilirubin were determined by diazo methods[29,30] at least 3 times within 6 months prior to the BSP study; the upper normal limit for TB (mean + 2 SD) was 20 μmol/L. The early fractional plasma disappearance rate (K_1) of BSP (E. Merck, Darmstadt, Germany) at the low dose of 0.59 μmol/kg body weight was determined after an overnight fast, as described previously.[22] Following bolus injection of the dose into one forearm vein over 15 seconds, venous samples were taken from the opposite forearm every minute for 10 minutes, and plasma BSP concentration was determined spectrophotometrically after alkalinization. BSP K_1 (min^{-1}) was calculated, by least-squares fit, as the slope of the logarithm of the BSP concentration versus time, using only BSP concentrations from 3 to 10 minutes (after the dye had equilibrated in the volume of distribution).

Sequence analysis of the *UGT1A1* gene was performed on genomic DNA, isolated from blood lymphocytes of the subjects; a segment of 359 bp, encompassing nucleotides −227 to +132, was amplified and sequenced as reported previously.[4] In family D, the entire coding region was analyzed also.[4] Each patient was then classified genetically as 7&7, a GS homozygote with the TA_7TAA polymorphism in the promoter region of both alleles of the *UGT1A1* gene; 6&6, a

TABLE 1. Characteristics of Groups 1 to 3

Subgroups (TATA Alleles)	n	Sex (M/F)	Age (yrs)	BSP K_1 (1/min)	TB (μmol/L)
Group 1					
GS Homozygotes (7&7)	12	10/2	26.8 ± 3.4	0.191 ± 0.036[a,b]	35.0 ± 7.1[c,d]
GS Heterozygotes (6&7)	6	4/2	28.5 ± 5.4	0.256 ± 0.050	17.8 ± 8.2
Normal (6&6)	9	7/2	28.3 ± 4.2	0.249 ± 0.047	12.7 ± 2.8
All subjects	27	21/6	27.7 ± 4.1	0.225 ± 0.052	23.7 ± 12.0
Group 2*					
GS Homozygotes (7&7)	7	3/4	38.6 ± 15.3[e]	0.241 ± 0.027[f]	30.1 ± 14.9
GS Compound Heterozygotes (6CN&7)	5	1/4	37.2 ± 14.0[f]	0.267 ± 0.027[g]	22.2 ± 9.4
Combined (7&7) + (6CN&7)	12	4/8	38.0 ± 14.1[g]	0.252 ± 0.029[h]	26.8 ± 13.0
GS Heterozygotes (6&7)	6	3/3	26.3 ± 10.9	0.217 ± 0.002	18.9 ± 0.4
All subjects	19	8/11[i]	34.5 ± 13.7[j]	0.239 ± 0.029	23.9 ± 10.9
Group 3					
β-Thalassemia Minor	7	4/3	31.7 ± 5.5	0.250 ± 0.054	26.1 ± 2.0[k]
Groups 1 + 2					
GS Homozygotes (7&7)	19	13/6	31.1 ± 10.9	0.209 ± 0.041	33.2 ± 10.5[k,l]
GS Heterozygotes (6&7)	12	7/5	27.4 ± 8.3	0.236 ± 0.039	18.3 ± 5.6[m]
Normal (6&6)	10	7/3	29.7 ± 5.8	0.237 ± 0.059	13.4 ± 3.2[n]
Combined (7&7) + (6CN&7)	24	14/10	32.4 ± 11.6	0.221 ± 0.045	30.9 ± 11.1[k,o]
Combined (6&7) + (6&6)	22	14/8	28.5 ± 7.2	0.241 ± 0.042	16.1 ± 5.2[n]
All subjects	46	29/17	30.5 ± 9.8	0.231 ± 0.044	23.8 ± 11.5

NOTE. Values are mean ± SD. Comparisons are within corresponding group(s), unless specified otherwise (*e,g,h,i,j*).

[a] $P < .01$ vs. normals (6&6).
[b] $P < .05$ vs. GS heterozygotes (6&7).
[c] $P < 5E\text{-}08$ vs. normals (6&6).
[d] $P < .002$ vs. GS heterozygotes (6&7).
[e] $P < .05$ vs. GS homozygotes (7&7) from group 1.
[f] $P < .02$ vs. GS heterozygotes (6&7).
[g] $P < .005$ vs. GS homozygotes (7&7) from group 1.
[h] $P < .0002$ vs. GS homozygotes (7&7) from group 1.
[i] $P < .02$ vs. all subjects from group 1.
[j] $P < .05$ vs. all subjects from group 1.
[k] $P < 5E\text{-}05$ vs. normals (6&6), groups 1 and 2.
[l] $P < 5E\text{-}05$ vs. GS heterozygotes (6&7).
[m] $P < .05$ vs. normals (6&6).
[n] $P < 1E\text{-}05$ vs. combined (7&7) + (6CN&7).
[o] $P < 5E\text{-}04$ vs. GS heterozygotes (6&7).
*The one normal (6&6) subject is not shown.

normal subject with the TA_6TAA pattern in both alleles; 6&7, a GS heterozygote with TA_7TAA polymorphism in only one allele; or 6CN&7, a compound heterozygote with a structural Crigler-Najjar mutation in the coding region of one allele of *UGT1A1* and the TA_7TAA polymorphism in the other allele.

Values are reported as mean ± SD. Comparisons of continuous variables between groups or subgroups were analyzed by the 2-tailed *t* test, assuming unequal variances, or by the z test. Gender distributions were analyzed by the χ^2 test. Linear regression (*r*) was performed by the least-squares method. *P* values less than .05 were considered significant.

RESULTS

Study 1—Unrelated Subjects (Table 1). Of the 15 hyperbilirubinemic patients (presumed GS), 12 were GS homozygotes (7&7) and 3 were heterozygotes (6&7). Three other GS heterozygotes were found among the 12 controls with normal TB levels; the remaining 9 had normal TATA boxes (6&6). There were no significant differences in age or sex distribution among the 3 genetic subgroups. Mean plasma TB levels among the GS homozygotes (7&7) were almost 3 times the levels in the normal subjects (6&6) ($P < 5E\text{-}08$).

The mean BSP K_1 value of the 7&7 subjects was 23% lower than the value in the 6&6 subjects ($P < .01$), but there was considerable overlap between the 2 subgroups (5 of 9 normal subjects had BSP K_1 values as low as the GS subjects). Of the 6 GS heterozygotes (6&7), the 3 with the lowest BSP K_1 values had elevated TB levels, whereas the 3 with higher BSP K_1 values had normal TB levels. A highly significant negative linear correlation ($P < .0005$) was observed between bilirubin levels and BSP K_1 values in the GS homozygotes (7&7) (Fig. 1).

Study 2—Families (Table 1 and Fig. 2). Three of the clinically jaundiced probands were homozygous (7&7) for the $(TA)_7TAA$ abnormality. The fourth (subject D1) was a compound heterozygote, with a structural CN mutation at codon 806 on one allele and the $(TA)_7TAA$ abnormality on the other allele (6CN&7); all but one of his relatives were likewise compound heterozygotes.

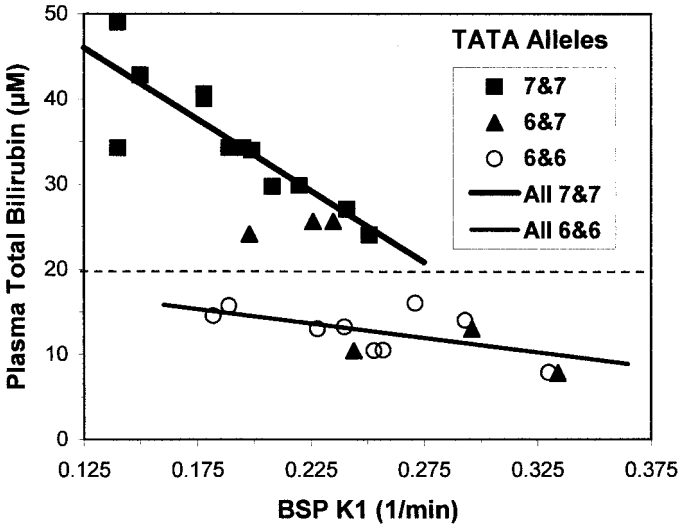

FIG. 1. Plasma total bilirubin concentrations versus early fractional uptake rates (BSP K_1) of low-dose BSP (0.59 μmol/kg) in unrelated subjects from study 1. The *solid lines* represent linear regressions for the 12 GS homozygotes (7&7, *upper heavy line*, TB = 67 − 168 × BSP K_1, $r = .86$, $P < .0005$) and the 9 normal subjects (6&6, *lower light line*, TB = 21 − 34 × BSP K_1, $r = .59$, $P = .09$). GS homozygotes (7&7), *squares*; GS heterozygotes (6&7), *triangles*; genetic normals (6&6), *circles*. The *horizontal dashed line* represents the upper normal limit of plasma total bilirubin concentration (mean + 2 SD).

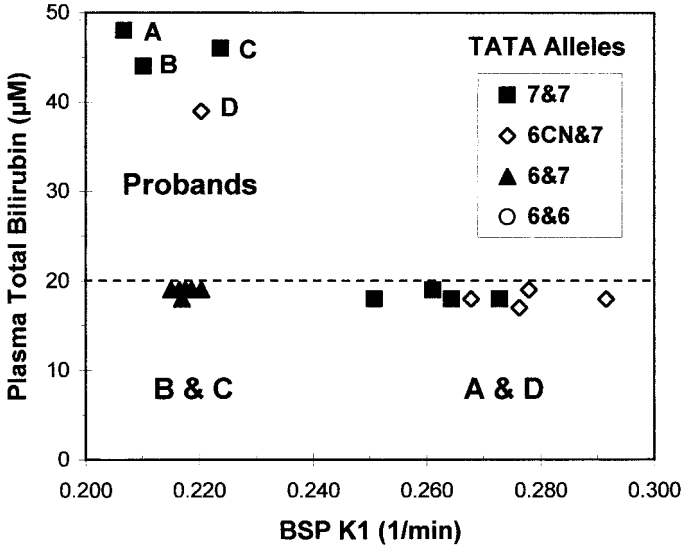

FIG. 2. Plasma total bilirubin concentrations and BSP early fractional uptake rates (BSP K_1) in subjects from family study 2. The capital letters identify the 4 families; the subject with Crigler-Najjar II syndrome (family D) is not shown. The *dashed line* and *symbols* for individual subjects are as in Fig. 1, except for the addition of compound heterozygotes (6CN&7), *diamonds*. Note the clustering and narrower range of BSP K_1 values on the x axis compared with unrelated subjects in Fig. 1.

In the 4 probands, mean TB levels ranged from 39 to 48 μmol/L and BSP K_1 values were all below 0.23 min^{-1}. Families A and D included 4 additional homozygotes (7&7) and 4 other compound heterozygotes (6CN&7), all of whom had normal TB levels, but higher BSP K_1 values. By contrast, all relatives in families B and C, like the probands, had BSP K_1 values below 0.23 min^{-1}, yet none was homozygous (7&7) and none had elevated plasma TB concentrations. The mean BSP K_1 values for relatives from families A and D (0.268 ± 0.013 min^{-1}) did not overlap with and were significantly higher than ($P < .00001$) the BSP K_1 values of relatives from families B and C (0.217 ± 0.002 min^{-1}) and of the 4 jaundiced probands (0.215 ± 0.008 min^{-1}).

Combined Studies 1 and 2 (Table 1). The data points for the 4 jaundiced subjects from group 2 lay above, but were still within the 95% confidence limits of, the regression line for the GS homozygotes in group 1. There were also no significant differences between groups 1 and 2 in the TB levels for any given genetic subgroup. This permitted pooling of the subjects from the two studies. In the combined subgroups, TB levels were significantly higher in subjects with two abnormal alleles (7&7 or 6CN&7) as compared with both GS heterozygotes (6&7) or normal subjects (6&6) and among GS heterozygotes compared with normal subjects (Table 1). By contrast, the BSP K_1 values did not differ significantly among the 3 genetic subgroups, and showed considerable overlap.

Each genetic subgroup showed a significant negative linear correlation between the plasma TB levels and the BSP K_1 values. The slope was steepest (TB = 74 − 193 × BSP K_1) and the correlation most significant ($r = .75$, $P < .0005$) for the 19 GS homozygotes (7&7). The slopes and the significance of the correlations were progressively lower for the 12 heterozygotes (6&7, TB = 43 − 105 × BSP K_1, $r = .73$, $P < .01$) and the 10

normal subjects (6&6, TB = 23 − 41 × BSP K_1, $r = .75$, $P < .02$), though the correlation coefficients remained similarly high for each subgroup.

Within the 7&7 subgroups and the groups as a whole, the plasma TB levels were significantly higher ($P < .001$) and the BSP K_1 values significantly lower ($P < .01$) in men compared with women, as expected.[31] Although there were significantly more men in group 1 than 2 ($P < .02$), there were no significant differences in gender frequency among the combined genetic subgroups and the data points for both sexes all fell along the same regression lines.

Study 3—Subjects With Hemolysis (Table 1 and Fig. 3). The gender and age distributions of the Thalassemic patients were not significantly different from any other group. The BSP K_1 values for the 7 Thalassemic subjects did not differ significantly from those for the normal subjects from studies I and II, even though their plasma bilirubin levels averaged almost twice those of the normals ($P < 5E-05$). On the plot of TB versus BSP K_1 values, all data points for the Thalassemic patients lay above the regression line and data points for the normal subjects (Fig. 3).

DISCUSSION

In GS homozygotes, the 65% to 80% decrease in hepatic glucuronidation of bilirubin[9,10] is related to an extra TA in the TATA box of the promoter region of the *UGT1A1* gene.[4-6] This promoter abnormality causes a decrease of 67% to 82% in the transcription and expression of this gene,[4] the only isoform that is functionally significant for bilirubin glucuronidation in humans.[32] Screening of large groups of presumably healthy controls has shown that 12% to 16% are homozygous for the $(TA)_7TAA$ abnormality and should thus have reduced expression of UGT1A1, yet less than half of these have the elevated plasma bilirubin levels characteristic of GS.[4-6] This suggested that, in hyperbilirubinemic GS subjects, a second abnormality in bilirubin metabolism and/or transport coexists with deficient glucuronidation.

Kinetic modeling of the plasma disappearance of intravenously injected bilirubin[15,19] had indicated defects in hepatic uptake as well as conjugation of UCB in many GS patients. Impaired hepatic clearance of BSP, indocyanine green, tolbutamide, nicotinic acid, and Rifamycin SV, other organic anions that share uptake mechanisms for UCB, have also been reported in some GS patients.[20-26] In particular, when low doses of BSP were administered to subjects with GS, a significant decrease in apparent affinity for the hepatic transport mechanism(s) was observed, together with inhibition by Rifamycin SV.[23] Collectively, these data suggested that the function of the transport system(s) for organic anion uptake was impaired in GS.

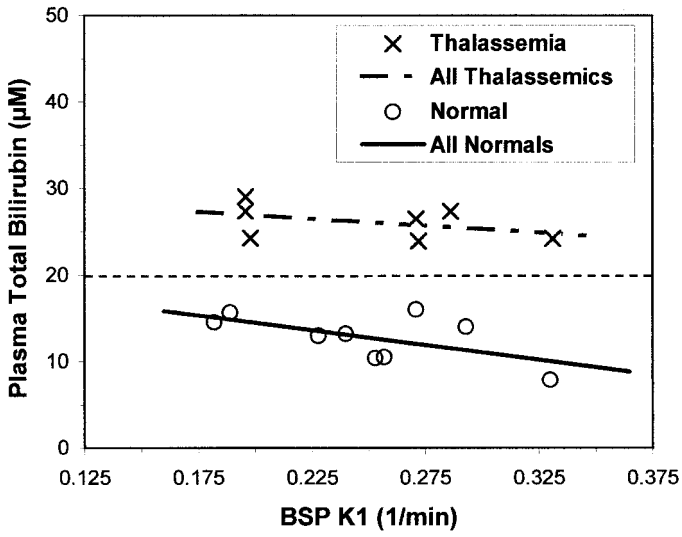

FIG. 3. Plasma total bilirubin concentrations and BSP early fractional uptake rates (BSP K_1) in subjects with β-Thalassemia from study 3, compared with normal subjects. The *dashed line* and *symbols* and *solid regression line* for normal subjects are as in Fig. 1. Xs represent the Thalassemic subjects.

The present study expands this conclusion by showing that impaired organic anion uptake may be required for the phenotypic manifestation of GS (hyperbilirubinemia) in subjects with genetically defined polymorphisms in the TATA box of the *UGT1A1* gene, but without overt hemolysis. Plasma TB levels showed strong negative linear correlations with BSP K_1 values in GS homozygotes (7&7) plus compound heterozygotes (6CN&7), in GS heterozygotes (6&7) and in normals (6&6). Plasma bilirubin levels were normal if the BSP K_1 value was at least 0.255 min^{-1}, even in subjects with two mutant *UGT1A1* alleles (7&7 or 6CN&7). Much lower BSP K_1 values engendered little or no elevation of bilirubin levels in subjects with no more than one abnormal allele (6&7 or 6&6). The degree of hyperbilirubinemia thus seemed to be determined by both the impairment in conjugation, caused by abnormal *UGT1A1* genes and by the slower rate of organic anion uptake reflected by a lower BSP K_1 value.

Potential Limitations of the Studies. Direct proof of decreased UGT1A1 activities was not obtained, because it is unethical to perform liver biopsies strictly for research purposes on controls or GS subjects. Data published previously has shown that: (1) the $(TA)_7$ mutation in the *UGT1A1 (BUGT1)* promoter decreases expression of a reporter gene to a relatively circumscribed low range of values (33% to 18% of normal),[4] comparable with the decreases in bilirubin conjugating activity reported in liver biopsy specimens from clinically diagnosed GS patients[9,10] and (2) the expression of the other bilirubin conjugating enzyme, UGT1A4 (BUGT2) is not compensatorily increased, even in the complete absence of functional UGT1A1.[32] In Fig. 1, the excellent fits of the regression lines with the data indicate that the variances in rates of bilirubin conjugation must have been similarly small in our subjects in whom exposure to inducing agents had been excluded.[1,4,9,10] We feel, therefore, that the bilirubin conjugating activity in our subgroups of subjects may be assumed to be representative of that reported for each *UGT1A1* genotype.

The modest variance in the relationships of bilirubin levels to BSP uptake within each genetic subgroup also suggests that there were relatively small variations in bilirubin production rates.[2,3,11,15,16] Although hemolysis was not assessed directly by ^{51}Cr-erythrocyte survival,[16,17] or CO production rate,[28,33] none of our subjects evidenced overt hemolysis on standard hematologic tests. In addition, none (except the CN patient) were exposed to xenobiotics that decrease plasma bilirubin levels by inducing the activity of UGT1A1 or organic anion uptake.[28,34]

An important issue is whether the reduced organic anion uptake, or the retained bilirubin, is the primary phenomenon producing their correlations. Theoretically, retained UCB might competitively inhibit uptake of the low dose of BSP.[27] This is unlikely, however, because BSP K_1 values in the same

low range as the clinically jaundiced GS patients were seen in more than half the heterozygous and normal patients with little or no elevation in plasma bilirubin levels. This result extends the findings of prior, more extensive, kinetic studies, which showed that neither clearance or fractional plasma removal of the standard dose of BSP,[21] nor fractional uptake or clearance of tracer radiolabeled bilirubin,[15,21] was affected by hemolytic hyperbilirubinemia in either GS subjects or controls. Several other anionic drugs likewise exhibit normal pharmacokinetics in Thalassemic patients with hemolytic hyperbilirubinemia.[24-26] Collectively, this evidence mitigates against incriminating the modestly elevated plasma bilirubin levels as the cause of the lower BSP K_1 values found in GS subjects.[22]

The significant difference ($P < .02$) in gender distribution between groups 1 and 2 (Table 1) must be considered, because, as with our subjects, gender affects serum bilirubin levels, primarily because of differences in hepatic uptake of organic anions.[31] There were, however, no significant differences in gender distribution among the 3 genetic subgroups in group 1 or combined groups 1 + 2, or of the Thalassemic patients (group 3) versus groups 1 and/or 2. Thus, there is no gender bias in Figs. 1 or 3. Since, for both group 1 and combined groups 1 and 2, the data points for both sexes all fell along the same regression lines, it appears that the relationships between plasma bilirubin levels and organic anion uptake incorporated the effects of gender.

Significant differences in age were found between subjects in groups 1 and 2, but not among genetic subgroups or for group 3 (Table 1). Our own data with the low-dose of BSP, however, reveals no effect of age on BSP K_1 values in our subjects (Fig. 4). Thus, the age differences could not influence our findings regarding the negative correlation between TB levels and BSP K_1 values in groups 1 and 2. Our plots (not shown) of raw data from published studies at higher BSP doses, both in controls and GS subjects, with or without hemolysis, likewise revealed no alteration in various indices of BSP uptake or storage up to age 60 years.[21,35-40] Fractional uptake and clearance of bilirubin are also reportedly unaltered up to age 60, both with tracer doses of radiolabeled bilirubin[15,21] and high doses (2 mg/kg intravenously) of unlabeled bilirubin.[19]

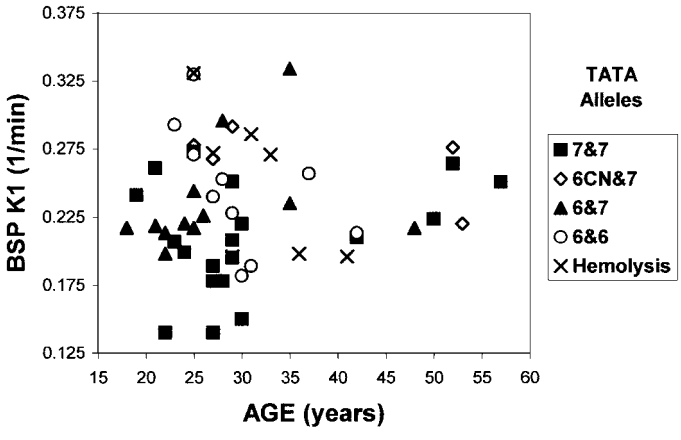

FIG. 4. Early fractional uptake rates (BSP K_1) after intravenous administration of a low dose (0.59 μmol/kg) of BSP at different ages. All subjects studied are shown. Those from groups 1 and 2 are categorized according to the TATA box polymorphisms in *UGT1A1*; the hemolytic patients are the subjects with Thalassemia from group 3. There are no significant changes in BSP K_1 values with age over the range from 17 to 57 years ($r = .11$, $P > .45$).

Implications of Results. From the above considerations, the most reasonable interpretation of our findings is that the impaired organic anion uptake is an important determinant of the hyperbilirubinemia in GS. This concept is supported by the report that administration of phenobarbital to GS subjects decreased plasma bilirubin levels by over 50%, due to a marked increase in UCB clearance, without any change in the activity of hepatic bilirubin-UDPGA transferase.[28] Our findings suggest further that these variances in uptake rates are also inherited, mediated by (a) high-affinity, low capacity basolateral transporter(s) of UCB and BSP,[22-24] encoded by gene(s) that segregate independently of the GS mutation. In support of this are the strong negative linear correlations between plasma bilirubin levels and BSP K_1 values within each genetic subgroup, whether or not the GS mutation is present; and the clustering of BSP K_1 values within families and sharp segregation among families in group 2, contrasting with the marked overlap of BSP K_1 values among genetic subgroups of the unrelated subjects in group 1. This overlap, observed previously but overlooked,[20-22] suggests that mutations of the putative transporter(s) mediating hepatic uptake of bilirubin and BSP may have a high prevalence, even among subjects with normal bilirubin levels. Among our 12 unrelated subjects in group 1 who had normal bilirubin levels, 6 had BSP K_1 values below 0.255 min^{-1}, the highest value found in the 15 subjects with hyperbilirubinemia. This estimated prevalence of 0.50 is remarkably similar to the estimate of 0.44, obtained by dividing the reported median prevalence of GS (0.06)[12-14] by the mean prevalence (0.137) of the double $(TA)_7TAA$ mutation in 6 series[4-8] (plus Bosma PJ, unpublished data). These tentative estimates imply that impaired organic anion uptake, due to presumed genetic abnormalities in basolateral transporter(s), with high affinity for BSP and UCB, may be nearly as common as the extra TA mutation in the promoter region of *UGT1A1*.

Direct proof of this proposal and determination of the lower limit of normal for BSP K_1 values must await studies of the expression of basolateral UCB transporters in tissues from GS subjects and controls. At present, however, none of the cloned organic anion transporting polypeptides (OATPs), including the newly-cloned OATP8,[41] have been reported to transport unconjugated bilirubin. (After acceptance of this work, a paper in press[42] has appeared online with evidence that OATP2 (SLC21A6) may be involved in the heaptic uptake of both BSP and UCB.) Indeed, no transport of highly radiolabeled UCB has been detected in *Xenopus laevis* oocytes expressing individual OATPs after injection with the corresponding messenger RNA,[43] although each was shown to specifically transport bile salts by a saturative mechanism.

In summary, in the absence of an overt increase in bilirubin production or inducing xenobiotics, decreased hepatic uptake is likely necessary for the expression of hyperbilirubinemia when conjugation is not severely impaired, as in GS. The decrease in UCB conjugation, caused by abnormal alleles for *UGT1A1*, is apparently the primary determinant of whether unconjugated hyperbilirubinemia can be present in GS. Within each *UGT1A1* subgroup, the presence and degree of jaundice is apparently determined by the rate of organic anion (*e.g.*, UCB uptake), and only the very small proportion of GS homozygotes with the lowest uptake rates will be visibly jaun-

diced. The severity of the impairment in hepatic uptake of UCB and other organic anions, reflected in the plasma bilirubin level, may have additional clinical implications, because impaired clearance of a number of drugs (*e.g.,* tolbutamide and Rifamycin), independent of defects in conjugation, have been reported in GS patients.[24-26]

Acknowledgment: The authors thank Dr. Sum P. Lee, Seattle, WA, for his helpful suggestions with the manuscript.

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