Sources of bile pigment overproduction in Gilbert's syndrome: studies with non-radioactive bilirubin kinetics and with $\delta$-[3,5-$^3$H]aminolaevulinic acid and [2-$^{14}$C]glycine

M. L. Zeneroli, V. Piaggi, C. Cremonini, C. Gozzi and E. Ventura

Istituto di Semeiotica Medica, University of Modena, Italy

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Summary

1. The kinetics of non-radioactive bilirubin were investigated in 100 patients with chronic non-haemolytic unconjugated hyperbilirubinemia and in nine normal volunteers.

2. The patients with Gilbert's syndrome were divided on the basis of a two-compartment model into two distinct groups: group 1 (74 patients) with decreased hepatic uptake and conjugation of bilirubin and a bilirubin turnover rate within the normal range, and group 2 (26 patients) having normal uptake, impaired conjugation and increased bilirubin turnover rate.

3. On the basis of these findings, studies were made of early pigment production in four patients of group 1, in six patients of group 2 and in four of the normal volunteers, with $[^{14}$C]glycine and $\delta$-[3H]aminolaevulinic acid.

4. After receiving injections of labelled haem precursors, the four patients in group 1 had a normal rate of incorporation of both tracers, whereas abnormalities in incorporation were found in group 2. Four patients showed an increase of the first component of the early peak of $[^{14}$C]bilirubin as well as of the early peak of $[^{2}$H]bilirubin, suggesting an increased turnover of hepatic haem. Two patients showed an increased incorporation of $[^{14}$C]glycine characterized by the fusion of the two early peaks, whereas the incorporation of the $\delta$-[3H]aminolaevulinic acid was normal, indicating the presence of a primary shunt hyperbilirubinemia.

5. The results confirm that Gilbert's syndrome is a heterogeneous condition with respect to bilirubin turnover rate. The population of Gilbert's syndrome with increased bilirubin turnover rate comprises not only patients with an increased haem erythroid turnover; but also patients with increased metabolism of non-erythroid haem protein in the liver.

Key words: $\delta$-[3,5-$^3$H]aminolaevulinic acid, Gilbert's syndrome, [2-$^{14}$C]glycine, non-radioactive bilirubin.

Abbreviations: BRT, bilirubin turnover rate; CBR, hepatic bilirubin clearance.

Introduction

Gilbert's syndrome is a familial disorder of bilirubin metabolism involving one or more steps in the handling of bilirubin by the liver [1]. Studies have attributed the hyperbilirubinemia variously to a defect in hepatic uptake and conjugation [2, 3] and to a defect of UDP-glucuronyltransferase activity [4–6]; a decreased proportion of bilirubin diglucuronide in the bile has been described as a consequence of the impaired bilirubin metabolism [7, 8]. The syndrome has also been associated with defects inducing a rise in turnover of erythrocyte haem, such as mild haemolysis escaping clinical detection [9–11].

Although abnormalities in bilirubin metabolism in Gilbert's syndrome have been extensively investigated, relatively little attention has been
paid to the hepatic turnover of haemoproteins in these patients.

It has been established [12–16] that, after injection of radiolabelled haem precursors (glycine and \( \delta \)-aminolaevulinic acid), maximum labelling of bilirubin occurs at approximately 120 days, consequent upon destruction of senescent erythrocytes. A fraction (approximately 10–26%) [12, 16] of labelled pigment appears in the blood within a few days of injection, and is referred to as early labelled bilirubin. Early glycine incorporation has two components: the first is thought to be largely of hepatic origin, the second is a by-product of erythropoiesis [14]. \( \delta \)-Aminolaevulinic acid, which penetrates poorly into erythrocyte haem, is the preferential precursor of non-erythroid sources of bile pigment [15].

In this paper we report studies on the kinetics of bilirubin in a large group of patients with Gilbert’s syndrome carried out in order to investigate the proportion of patients having an increased bilirubin turnover. In some of these patients we have examined the haem sources of early labelled bilirubin formation, particularly the initial non-erythropoietic phase, by studying the early labelled peaks of bilirubin formation after injection of \([14C]glycine\) and \(\delta-[3H]amino-laevulinic\) acid. The two tracers were administered simultaneously in order to determine the relationship between the two sources of the early labelled peaks.

**Materials and methods**

**Subjects**

Non-radioactive bilirubin kinetics were studied in 100 patients with Gilbert’s syndrome (77 males and 23 females, mean age 24 ± 8 years) and nine normal volunteers (six males and three females, mean age 22 ± 5 years). All patients were admitted to hospital with a history of periodic scleral icterus due to unconjugated hyperbilirubinaemia (higher than 2 mg/dl). Other liver function tests were normal, and overt haemolysis was excluded by routine tests (haemoglobin, packed cell volume, erythrocyte morphology, reticulocyte count, erythrocyte osmotic fragility, haemoglobin electrophoresis, glucose 6-phosphate dehydrogenase assay and Coomb’s antiglobulin test).

Ten of the patients with Gilbert’s syndrome and four of the normal volunteers, randomly selected from the above-mentioned subjects, were submitted to further investigation in order to estimate the production of the early labelled peak of bilirubin from \([14C]glycine\) and \(\delta-[3H]amino-laevulinic\) acid. This study was performed with the fully informed consent of each subject.

**Protocol**

**Non-radioactive bilirubin kinetics.** Studies were begun at 09.00 hours after an overnight fast in patients who had been medication free at least 1 month. Crystalline bilirubin (Merek, provided by Boehringer, S.P.A., Milano, Italy), 2 mg/kg body wt., dissolved in sodium carbonate solution (0.1 mol/l) [2] was injected intravenously, and venous blood samples were obtained from the opposite arm every 5 min during the first half-hour and at 40, 50, 60, 90, 150, 180 and 240 min after the injection. A preinjection blood sample was collected in order to evaluate the baseline bilirubin levels. Samples were analysed for total and direct bilirubin by the Malloy & Evelyn method [17].

The plasma bilirubin clearance data were processed by use of the Simulation Analysis and Modeling (SAAM) program [18] and were subsequently analysed according to the two-compartment model [2, 3, 19].

**Early labelled peaks.** The early labelled peaks of bile-pigment formation were investigated by simultaneous intravenous injections of \(\delta-[3H]\)aminolaevulinic acid (25 \(\mu\)Ci, specific radioactivity 1.3 Ci/mmol) and \([14C]glycine\) (25 \(\mu\)Ci, specific radioactivity 54 mCi/mmol). The whole-body radiation dosage was 6–6 mrad/\(\mu\)Ci for \(^{14}\)C and 0–8 mrad/\(\mu\)Ci for \(^3\)H [20]. \([14C]Glycine\) was purchased in aqueous solution from The Radiochemical Centre, Amersham, Bucks., U.K., and \(\delta-[3H]\)aminolaevulinic acid hydrochloride was obtained from NEN Chemicals (Dreieichenhain, West Germany).

Venous samples were collected after 1, 6 and 12 h and 1, 2, 3, 4, 5 and 6 days after the injection. Unconjugated labelled bilirubin was separated by chromatography in all samples with chloroform as eluent [15]. A thin-layer chromatographic analysis, performed on a total of six samples obtained from six different studied subjects, using propan-1-ol/butan-1-ol/acetic acid/water (30:40:1:29, by vol.) as solvent mixture and methanolic diazo reagent (1:1, v/v) as chromogen biliary pigment [21] revealed that the radioactivity recovered from the chloroform fraction migrated as authentic unconjugated bilirubin. \(^3\)H and \(^{14}\)C radioactivity counts were recorded twice (for 5 min each time) for each sample by a three-channel liquid scintillation counter (Packard Tri-Carb). All samples were re-counted after the addition of \(^{14}\)C-labelled internal standard, and further counted after
addition of $^3$H-labelled internal standard to enable appropriate corrections for quenching. The mean 5 min background count provided 204 counts (SD 12) for $^{14}$C and 127 counts (SD 16) for $^3$H, and the minimum counts per sample for $^{14}$C and for $^3$H were respectively 11 and 20 times higher than background. Because of the low yield of bilirubin from plasma in normal subjects, the radioactivity was expressed as d.p.m./ml of plasma and not as specific radioactivity as described before in the method [15], in order to avoid possible underestimation.

**Results**

**Non-radioactive bilirubin kinetics**

Plasma bilirubin clearance curves were resolved into two exponential functions, and the discriminating variable between controls and Gilbert's syndrome was the $R_{240}$, as previously defined [22, 23]. The three transfer rate constants per min between compartment (blood and liver) were calculated according to the two-compartment model [2, 3, 19]. $K_{11}$ (min$^{-1}$) represents bilirubin transferred from blood to liver, $K_{12}$ (min$^{-1}$) is the reflux rate from liver to blood and $K_{22}$ (min$^{-1}$) corresponds to bilirubin conjugation and excretion into bile. CBR (ml min$^{-1}$ kg$^{-1}$) is the volume of plasma cleared from bilirubin in 1 min and BRT (mg day$^{-1}$ kg$^{-1}$) represents the daily turnover of bilirubin, slightly underestimating the daily production rate of bilirubin.

In normal volunteers the mean values for model-dependent and -independent parameters (Figs. 1 and 2) were: $K_{11}$ 0.0251 min$^{-1}$ (SD 0.0059), $K_{12}$ 0.0086 min$^{-1}$ (SD 0.0059), $K_{22}$ 0.0142 min$^{-1}$ (SD 0.0079), BRT 4.09 mg day$^{-1}$ kg$^{-1}$ (SD 1.66; range 0.86–6.04 mg day$^{-1}$ kg$^{-1}$), CBR 1.06 ml min$^{-1}$ kg$^{-1}$ (SD 0.37), $R_{240}$ 5% (SD 3), and the mean values of baseline plasma unconjugated bilirubin were 0.32 mg/dl (SD 0.16).

When model-dependent parameters were obtained in Gilbert's-syndrome patients (Fig. 1), the analysis of the data revealed two different populations with respect to bilirubin turnover rate (BRT): one, represented by 74 patients of both sexes, having a BRT in the range of normality (mean ± SD: 3.34 ± 1.43 mg day$^{-1}$ kg$^{-1}$; range: 0.95–5.95 mg day$^{-1}$ kg$^{-1}$), and one, represented by 26 patients of both sexes, having a mean BRT 9.71 mg day$^{-1}$ kg$^{-1}$ (SD 3.32; range 6.21–18.86

![Fig. 1. Mean values ± SD of model-independent parameters calculated from the plasma clearance curves of non-radioactive unconjugated bilirubin in control subjects (C), and in Gilbert's-syndrome patients group 1 (G I) and group 2 (G II). BRT, Bilirubin turnover rate; CBR, hepatic bilirubin clearance; $R_{240}$, percentage retention of exogenous bilirubin at 4 h. Mean and SD of baseline plasma unconjugated bilirubin are shown in the right panel. Variance analysis between G I and G II is represented by *P < 0.01 and **P < 0.005.](image-url)
mg day⁻¹ kg⁻¹). On the basis of BRT values, patients with Gilbert's syndrome were divided in two groups: group 1 having normal BRT and group 2 having increased BRT, higher than 6 mg day⁻¹ kg⁻¹. Patients in group 2 had higher baseline levels of unconjugated bilirubin than those in group 1 (mean ± SD: 2.11 ± 0.84 vs 1.08 ± 0.50 mg/dl; P < 0.005). Interestingly, they had a higher clearance rate (CBR) (mean ± SD: 2.33 ± 0.11 vs 2.23 ± 0.11 ml min⁻¹ kg⁻¹; P < 0.01) and lower K₀₂ (conjugation) (mean ± SD 24 ± 7 vs 31 ± 9%; P < 0.005) in comparison with group 1. Model-dependent parameters (Fig. 2) in group 1 were characterized by decreasing uptake (K₁₂) (P < 0.005) and conjugation (K₀₂) (P < 0.01) in comparison with controls, and group 2 had normal hepatic uptake by the liver and conjugation was decreased. Both groups had increased reflux (K₁₁) in comparison with control subjects and, in particular, the reflux rate of Gilbert's-syndrome patients in group 2 was significantly higher than that of group 1 (P < 0.005). However, when the reflux was calculated as percentage of bilirubin entering the hepatic pool and returning unaltered to plasma, by the equation [100 × K₁₁/(K₀₂ + K₁₁)] [10], the values in the two Gilbert's-syndrome groups were not statistically different (mean ± SD: controls 35 ± 12%; Gilbert's-syndrome patients group 1 68 ± 17%; Gilbert's-syndrome patients group 2 67 ± 16%). This apparent discrepancy indicates that the differences of K₁₁ values between groups were simply related to the different rates of uptake and conjugation in these groups.

Representative plasma bilirubin clearances in the two groups, shown in Fig. 3, illustrate the greater clearances in group 2 compared with group 1.

Table 1 shows the parameters of the plasma non-radioactive bilirubin kinetics and the baseline unconjugated bilirubin levels of normal volunteers (four) and of Gilbert's patients (ten) selected at random for early pigment production studies.

Early labelled peaks

One hour after the injection of the tracers, both normal subjects (four) and patients with Gilbert's syndrome of group 1 (four) had a sharp peak of [¹⁴C]bilirubin, reaching the maximum value at 24h, followed by a brief decline and then a
Sources of bilirubin in Gilbert's syndrome

Table 1: Kinetic characteristics of non-radioactive bilirubin and baseline unconjugated bilirubin levels of patients selected for early labelled bilirubin studies

<table>
<thead>
<tr>
<th>Subject no.</th>
<th>Sex</th>
<th>K₁₁ (min⁻¹)</th>
<th>K₁₂ (min⁻¹)</th>
<th>Reflux ratio (%)</th>
<th>K₂₁ (min⁻¹)</th>
<th>K₂₂ (min⁻¹)</th>
<th>BRT (mg/day⁻¹kg⁻¹)</th>
<th>CBR (ml/min⁻¹kg⁻¹)</th>
<th>R₁40 (%)</th>
<th>Plasma unconjugated bilirubin (mg/dl)</th>
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<td>0.0039</td>
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<td>2</td>
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<td>7.6</td>
<td>0.30</td>
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</table>

* Primary shunt hyperbilirubinaemia.

Fig. 4. Plasma curves of [14C]bilirubin (a) and of [3H]bilirubin (b) in normal subjects (hatched areas) and in Gilbert's syndrome (group 1) (continuous line). Values are reported as means ± SD.

Progressive rise that reached the maximum value at day 4 (Fig. 4). The peak of [3H]bilirubin from δ-[3H]aminolaevulinic acid appeared earlier in the blood, reaching the maximum value after 12 h, and then fell rapidly, declining to a baseline level by day 3 in both normal subjects and patients with Gilbert's syndrome group 1 (Fig. 4). The amount of δ-[3H]aminolaevulinic acid incorporation into early labelled bilirubin was much higher than that observed with [14C]-glycine, as previously described [24]. In four of the six patients, classified on the basis of BRT as belonging to group 2 (Fig. 5), the first component of the early peak of [14C]bilirubin appeared earlier than that in controls, reaching values 39% higher (P < 0.001), whereas the second peak was similar in amplitude but appeared earlier, i.e. on day 3. The early peak of [3H]bilirubin (Fig. 5) was respectively 28% and 26% higher than in controls and in group 1 (P < 0.001). The other two patients of group 2 had a normal [3H]-bilirubin peak, but they were different with respect to [14C]-glycine incorporation. The [14C]-bilirubin early peak was characterized by a single curve having a single high peak (maximum at day 3) (Fig. 5).

In order to establish whether the described rise of plasma labelled bilirubin in Gilbert's syndrome...
patients was proportional to the reflux rate of the non-radioactive bilirubin and specifically related to this phenomenon, both the $K_{12}$ values and the percentage ratio of reflux in Gilbert’s-syndrome patients (ten) were tested by linear regression analysis against the maximum value of $[{}^3H]$bilirubin (12 h), and no significant correlations were demonstrable ($K_{12}: r = 0.2446$; percentage ratio of reflux: $R = 0.2290$).

Discussion

The data derived from our extensive study on non-radioactive bilirubin kinetics confirm the existence of two different populations in Gilbert’s syndrome with respect to the daily turnover of bilirubin (BRT) [10, 22, 23]. Although the syndrome is generally characterized by a normal rate of bilirubin production, in some cases, assuming that BRT only slightly underestimates the total bilirubin production [25], the defect is associated with an increased daily production of the pigment. According to previous reports [22, 23], it is noteworthy that group 2 patients with increased BRT had a conjugation defect ($K_{02}$) with normal hepatic bilirubin uptake ($K_{21}$), whereas patients in group 1 showed a defect in both uptake and conjugation. Moreover, group 2 had higher values for hepatic bilirubin clearance (CBR) and significantly lower 4 h retention ($R_{240}$) in comparison with group 1. In spite of this, the defect in group 2 was accompanied by higher baseline levels of unconjugated bilirubin in the blood. From the analysis of dependent and independent variables it seems likely that these patients (group 2) had a simpler defect in bilirubin metabolism in respect of conjugation, but complicated by superimposed factors inducing higher levels of bilirubin in the blood.

Bilirubin overproduction in Gilbert’s syndrome has been attributed to a mild compensated haemolytic state, as documented by erythrocyte survival studies or by an association with spherocytosis [9, 10].

When the early peaks of radiolabelled bile pigments were studied in patients with Gilbert’s syndrome, it was found that patients in group 1 had normal incorporation of the labelled precursors, and patients of group 2 had increased incorporation. Within limitations imposed by the small number of studies, the linear regression analysis seems to exclude any correlation between the reflux rate of non-radioactive bilirubin and an increased proportion of incorporation of the tracers. On the other hand, the observation that Gilbert’s-syndrome patients group 2 had an increased incorporation of tracers, involving in some cases one tracer and in some others both of them, adds support to these changes being related to other factors. Indeed, in group 2 two cases showed increased $[{}^{14}C]$glycine incorporation and normal $[{}^3H]$amino-laevulinic acid incorporation. The profile of the specific $[{}^{14}C]$bilirubin radioactivity was characterized by an enlargement and heightening of the second peak, reaching a fusion with the first component. The high $[{}^{14}C]$bilirubin peak, in association with a normal $[{}^3H]$bilirubin peak is attributable to an increased direct metabolic shunt from haem precursors to bilirubin in bone marrow, and is characteristic of the pattern found in ineffective erythropoiesis of the primary shunt hyperbilirubinaemia type [15].

In contrast, in the other four patients of group 2, the increase in the first component of $[{}^{14}C]$bilirubin followed by the normality of the second peak, together with an increase in $[{}^3H]$amino-laevulinic acid incorporation, is compatible with an increased turnover of haem enzymes in the liver. This would be in keeping with the increase in catalase and hypertrophy of the hepatocyte smooth endoplasmic reticulum described in some cases of Gilbert’s syndrome [26, 27].

Leaving aside the two cases of Gilbert’s syndrome associated with primary shunt hyperbilirubinaemia, we can offer only a tentative explanation for the alterations of the early pigment production in the four patients in group 2.

The increased incorporation of $[{}^3H]$amino-laevulinic acid into the early labelled peak of bilirubin [13, 28] together with the ultrastructural and biochemical changes in the hepatocyte previously described [26, 27] are similar to those seen in liver after enzyme induction by phenobarbital or by ethanol. However, the four patients studied were receiving no medication and were not consuming ethanol. It is thus possible that the increase in activities of haem enzyme in these cases of Gilbert’s syndrome may represent an adaptive phenomenon to expedite the conversion of lipid-soluble compounds into more water-soluble forms. The phenomenon may represent an unsuccessful attempt to eliminate lipofuscin pigment that sometimes accumulates in the liver in Gilbert’s syndrome [29] and/or an attempt to provide some additional enzymes for bilirubin conjugation.

The present study therefore provides biochemical confirmation of the previously reported abnormalities in bilirubin production in Gilbert’s syndrome, but underlines the presence of a sub-population with increased synthesis of haem enzymes in the liver.
Acknowledgment

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