Definition of a conjugation dysfunction in Gilbert’s syndrome: studies of the handling of bilirubin loads and of the pattern of bilirubin conjugates secreted in bile

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Summary

1. Intravenous doses of bilirubin (3-4 μmol/kg) were given to normal subjects and patients with Gilbert’s syndrome. Both groups displayed an identical initial disappearance of a substantial proportion of the bilirubin but, late in time, the Gilbert’s patients exhibited reduced clearance with a sustained elevation of the plasma bilirubin and no reflux into the plasma space of conjugated bilirubin. Increasing the dose in normal subjects (by factors of 3 and 6) failed to reproduce the response found in the Gilbert’s patients.

2. In the bile-containing duodenal aspirates of Gilbert’s patients the average proportion of bilirubin found as bilirubin diglucuronide was 68% (normal 88%) and of bilirubin monoglucuronide, 23% (normal 7%). Both differences were significant at the $P < 0.001$ level. In the Gilbert’s patients restriction of caloric intake to 1569 kJ/day for 2 days characteristically raised the serum bilirubin with no modification of the biliary pigment pattern; phenobarbital (180 mg/day for 2 weeks) decreased the plasma bilirubin to the normal range with, concomitantly, a reversion of the biliary pigment pattern towards normal.

3. We conclude that there is no hepatic uptake defect in Gilbert’s syndrome but that there is decreased activity in the conjugation process underlying the addition of the second glucuronic acid moiety to bilirubin, to form bilirubin diglucuronide.

Key words: bile, bilirubin, Gilbert’s syndrome, hyperbilirubinaemia.

Introduction

Chronic low-grade primarily unconjugated hyperbilirubinaemia, originally described by Gilbert & Lereboullet (1901), is of clinical significance only because it may confuse otherwise easily discernible diagnostic problems. However, from the point of view of bilirubin metabolism, it is of some importance. A study of the changes in bilirubin metabolism in these subjects may not only provide insight into the factors underlying Gilbert’s syndrome but also allow dissection of the steps concerned with the processing of bilirubin in the normal individual. Gilbert’s syndrome has been postulated to arise from a defect of kinetic uptake or conjugation, or both. We have used plasma disappearance curves in normal volunteer subjects and patients with Gilbert’s syndrome to study bilirubin handling after single injections, looking particularly for evidence of a rapid initial removal of a major fraction of the load. Increasing bilirubin doses were given to the normal subjects, to see if we could reproduce the delayed clearance, which is characteristic of patients with Gilbert’s syndrome.
We have obtained bile-containing duodenal aspirates from normal subjects and patients with Gilbert's syndrome, and determined the nature and pattern of the bilirubin conjugates secreted in the bile by these subjects. In the Gilbert's patients the effects on the pattern of conjugates of fasting (which characteristically raises the serum bilirubin further) and also of phenobarbital administration (which decreases the plasma bilirubin to normal) were determined.

Methods and materials

We studied 21 normal healthy subjects (males, age range 20–60 years) and 19 patients with Gilbert's syndrome (males except for one, age range 16–58 years). The patients with Gilbert's syndrome were otherwise healthy subjects, who were found on routine screening to have unconjugated hyperbilirubinaemia. Overt haemolysis was excluded by a normal haemoglobin concentration, a reticulocyte count below 2% of erythrocytes count, and a normal serum haptoglobin concentration. They had no clinical evidence of liver disease and serum alkaline phosphatase, glutamate oxaloacetate transaminase and protein concentrations were all normal.

Single-injection studies

The single-injection studies were carried out in eight normal subjects and three patients with Gilbert's syndrome. A bilirubin dose of 3.4 \( \mu \text{mol/kg} \) (2 mg/kg) was given to each subject in both groups. The study was repeated in all normal subjects with 10-2 \( \mu \text{mol/kg} \) and, in five of the eight normal subjects, with 20-4 \( \mu \text{mol/kg} \). The studies were at least 3 days apart. Subjects fasted overnight, a control blood sample was drawn, the bilirubin (British Drug Houses Ltd) was given over 30 s, and blood samples were obtained from an indwelling arterial needle at intervals of 2 min, over the first 10 min, 5 min, over the next 50 min and 30 min, over the next 90 min. At the time of the study, the plasma volume was measured with labelled albumin. Expected increments in plasma bilirubin concentration at the time of injection were calculated by dividing the dose given by the plasma volume. With this initial value, the arterial plasma disappearance curve provides a direct measure of the proportion of the dose which has disappeared between injection and the time of the first sample. Chromatography of the bilirubin showed it to contain solely the \( \text{IXa} \) form (McDonagh & Assissi, 1971). It was dissolved in sterile sodium carbonate solution (0.1 mol/l) and injected soon after preparation (Billing, Williams & Richards, 1964). Care was taken to shield it from light.

Serum samples were assayed by separating bilirubin and its conjugates in a two-phase extraction (Weber & Schalm, 1962; Berk, Howe, Bloomer & Berlin, 1969), each moiety being measured as its diazo derivative. The conjugates in serum were not further characterized. Plasma disappearance curves were expressed both in terms of bilirubin concentration (Fig. 1), and also as a fraction of the initial increment in unconjugated bilirubin predicted from the plasma volume measurement (Fig. 2).

Studies of bile-containing duodenal aspirates

In a second group of 16 patients with Gilbert's syndrome and 13 normal subjects, fasted overnight, bile-containing duodenal aspirates were obtained through a Dreiling tube after intravenous cholecystokinin (Boots, 1 unit/kg). Additionally, to ascertain that the Gilbert's patients had a characteristic response to fasting, their caloric intake was restricted to a glucose drink (523 kJ), three times daily, for 2 days. Their serum bilirubin then increased on average from 28 \( \mu \text{mol/l} \) (SD 8) to 58 \( \mu \text{mol/l} \) (SD 19), as expected (Owens & Sherlock, 1973). Four of these patients again were intubated after this 2 days of caloric restriction. Seven of the patients with Gilbert's disease were given phenobarbital, 180 mg/day for 2 weeks, and then intubated once again. Their average serum bilirubin fell to 14 \( \mu \text{mol/l} \) (SD 2), a normal value, as expected (Black & Sherlock, 1970).

The protocol of each of the foregoing studies was approved by the Ethics Committee of the institution and the informed consent of each subject was obtained. Liver biopsy was thought inappropriate.

Biochemical analysis of the bile-containing fluid

The aspirated fluid was protected from the light, thoroughly mixed, frozen and stored at \(-4^\circ\text{C}\). At a later time, the bile pigments in the sample were separated and quantified (Gordon, Chan, Samodai & Goresky, 1977). Previously, in studies of gallbladder bile, phospholipids were removed by precipitation with acetone saturated with MgCl\(_2\) (Gordon et al., 1977). Here this step was omitted.
We diluted a portion (1·0 ml) of the duodenal juice fivefold with water and the bile pigments were quantitatively extracted with chloroform containing tetraheptyl ammonium chloride (10 \( \mu \text{mol/l} \)). The extract was taken to dryness under vacuum, the precipitate redissolved in a small volume of chloroform, and the bile pigments were separated by thin-layer chromatography (Gordon et al., 1977).

Four distinct yellow bands were usually detected in the extracts of the duodenal juice. Each yellow bilirubin tetrapyrrrole band was scraped from the thin-layer plate and extracted from the gel in a known volume of methanol/water (8:2, v/v). The pigment present was quantified by determining the absorbance of a portion at 454 nm. The chemical identity of each yellow band was established by formation of its diazo derivatives (Gordon, Goresky, Chan & Perlin, 1976). This was considered necessary because we did not know whether any abnormal conjugates might be present in the patients with Gilbert's syndrome and because we wished to be sure that no artifacts had been introduced by omission of the precipitation step. The azopigments formed were separated by thin-layer chromatography, identified and quantified (Gordon, Dadoun, Goresky, Chan & Perlin, 1974; Gordon et al., 1976), and from this identification and quantification (in terms of molar ratios), the identity of the parent tetrapyrrrole was deduced (Gordon et al., 1977).

**Results**

Results are given as mean value \( \pm 1 \) sd. Comparisons are made by paired \( t \)-test.

**Single-injection studies**

In the normal subjects an initial major proportion (36 \( \pm 13 \)% of the bilirubin dose (3·4 \( \mu \text{mol/kg} \)) was removed within 2 min and the remainder then disappeared over the next 3 h (Fig. 1 and Fig. 2). In Fig. 2 the increment in unconjugated bilirubin above base line has been expressed as a fraction of the expected initial increment (as derived from the plasma volume measurements) and the resultant values are plotted on a logarithmic scale against time. On analysis of these curves a minimum of three exponential components can be obtained (details are available on request to authors).

The initial serum bilirubin concentrations in the Gilbert's patients vary somewhat and the level attained on injection of the bilirubin dose (3·4 \( \mu \text{mol/kg} \)) varies correspondingly (Fig. 3a). The dose–response curve in these patients shows an initial sharp fall in plasma concentration with, after this, a delay in plasma disappearance. When the data are replotted as a fraction of the predicted initial increment in concentration, and are compared with the range found in normal subjects (Fig. 3b), the initial sharp fall is seen to lie within the normal range, whereas the late protracted elevation of the serum bilirubin lies far above the normal range.

To see if this response in the Gilbert's subjects resulted from a rate limitation, related to the concentration of serum bilirubin rather than from a specific characteristic of that group of patients, larger doses of 10·3 and 20·5 \( \mu \text{mol/kg} \) (three and six times the original dose) were also given to the normal subjects. At the intermediate dose 45 \( \pm 13 \)% of the injected dose was removed by 2 min; at the higher dose, 49 \( \pm 13 \)%.

The increase in the initial proportional removal rate with dose occurred presumably because of the increase in the bilirubin/albumin molar ratio with dose. Thereafter the removal proceeded more or less in the manner seen with the smallest dose, and was relatively complete within 3 h. The response seen in the Gilbert's patients, with the prolonged elevation of bilirubin, was not reproduced. In both the normal subjects and the Gilbert's patients there was only a minor increase in the conjugated bilirubin in the plasma space.

**Studies of bile-containing duodenal aspirates**

The distribution of bilirubin tetrapyrrroles in the bile-containing duodenal aspirates from normal subjects, presented in Table 1, confirms the values previously reported by Gordon et al. (1977). In the diluted bile samples, omission of the phospholipid precipitation step has had no effect on the analysis. In the patients with Gilbert's syndrome the pattern is changed. The proportional content of bilirubin diglucuronide is decreased from an average of 88% to 68% of the total, and that of bilirubin mono-glucuronide increased from 7% to 23% of the total. No significant difference is observed in the proportion of the mixed conjugate or of unconjugated bilirubin.

In the patients with Gilbert's syndrome, partial fasting with intake of only 1674 kJ/day resulted in no significant change in the pattern of distribution of the tetrapyrrroles. After partial fasting the average values for the distribution were: bilirubin...
Fig. 1. Average plasma disappearance curves for bilirubin after its intravenous injection at the doses indicated in normal subjects. ●, Total bilirubin; ○, conjugated bilirubin. Bars are used to indicate standard deviations about each point. In the lower two panels, the standard deviations for conjugated bilirubin lie within the limits of the symbols.

Discussion

Bilirubin single-injection plasma disappearance curves

The characteristic feature of the plasma disappearance curve in the patients with Gilbert’s syndrome is the slow terminal phase after the initial distribution is complete. This phenomenon was previously described by Billing et al. (1964) and
Bilirubin loads and Gilbert's syndrome

indicates that, in comparison with normal subjects, these patients have a limited clearance of unconjugated bilirubin from the liver–plasma–body system. The delayed terminal clearance of unconjugated bilirubin in the patients with Gilbert's syndrome could therefore be due to a saturation phenomenon related to the pre-existing larger load, at the time the 3.4 μmol/kg dose is introduced into the circulation. However, our studies in normal subjects, in which a larger dose was given, did not result in the delayed terminal clearance found in the Gilbert's patients. This phenomenon is therefore not a function of the load but is related to the underlying defect in these patients.

Use of the calculated initial increment based on the plasma volume uncovered a second characteristic in both normal subjects and patients with Gilbert's syndrome: an initial very rapid clearance, so that by 2 min 36–49% of the injected dose had been cleared from the circulation. The observed increase in initial clearance with increase in the bilirubin/albumin molar ratio (that is, with dose) corresponds to past observations that increase in the bilirubin/albumin molar ratio accelerates the early

Fig. 2. Semilogarithmic plots of the average normalized bilirubin disappearance curves for the three doses of bilirubin indicated. For each curve the initial point is, of course, unity. Bars are used to indicate standard deviation about each point.
Fig. 3. (a) Plasma disappearance curves for bilirubin in three patients with Gilbert's syndrome, after an intravenous 3.4 μmol/kg dose: ●, total bilirubin concentrations (both before and after the intravenous load); ○, mean values for conjugated bilirubin. (b) Semilogarithmic plots of the normalized bilirubin disappearance curves in the three patients with Gilbert's syndrome (●). For comparison, the average data from the normal subjects (○), together with a range corresponding to ±1 SD, are also displayed. Δ, Increment.

![Graph showing plasma disappearance curves for bilirubin](image)

### Table 1. Distribution of bile pigments in biliary aspirates from normal control subjects and patients with Gilbert's syndrome

<table>
<thead>
<tr>
<th>Bilirubin tetapyrrole</th>
<th>Relative content (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bilirubin diglucuronide</td>
<td>87.6 ± 4.3</td>
</tr>
<tr>
<td>Bilirubin monoglucoside</td>
<td>2.2 ± 3.7</td>
</tr>
<tr>
<td>Bilirubin monoglucuronide diester</td>
<td>6.8 ± 4.7</td>
</tr>
<tr>
<td>Bilirubin monoglucuronide</td>
<td>6.8 ± 4.7</td>
</tr>
<tr>
<td>Bilirubin</td>
<td>4.4 ± 3.5</td>
</tr>
</tbody>
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Mean values ± SD are shown. * Significance (t-test): P < 0.001.

Rapid uptake of bilirubin by the liver (Barnhart & Clarenberg, 1973; Berstein, Ben Ezzer, Gartner & Arias, 1966; Goresky, 1977). If one assumes average values for the plasma volume, analysis of the data collected by Billing et al. (1964) indicates that the average proportion of the bilirubin load removed from the plasma in the earliest phase (the component neglected in their analysis) was 39%. Studies with radioactive bilirubin have shown a similar phenomenon. In early studies, the average
**Table 2. Effects of phenobarbital therapy on the distribution of bile pigments in bile of patients with Gilbert's syndrome**

Mean values ± SD are shown. * Significance (paired t-test): P < 0.05.

<table>
<thead>
<tr>
<th>Bilirubin tetrapyrrrole</th>
<th>Relative content (%)</th>
<th>Before treatment (n = 7)</th>
<th>After phenobarbital (n = 7)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bilirubin diglucuronide</td>
<td>61.4 ± 10.0</td>
<td>76.6 ± 10.3*</td>
<td></td>
</tr>
<tr>
<td>Bilirubin monoglucoside</td>
<td>4.0 ± 4.5</td>
<td>1.9 ± 1.3</td>
<td></td>
</tr>
<tr>
<td>monoglucuronide diester</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bilirubin monoglucuronide</td>
<td>29.8 ± 9.8</td>
<td>14.6 ± 11.0*</td>
<td></td>
</tr>
<tr>
<td>Bilirubin monoglucuronide</td>
<td>5.1 ± 2.0</td>
<td>5.0 ± 4.4</td>
<td></td>
</tr>
</tbody>
</table>

Proportion of the bilirubin reported to undergo initial rapid removal was 27% (Barrett, Berk, Menken & Berlin, 1968); in later studies the proportion has been smaller (Berk, Howe, Bloomer & Berlin, 1969; Berk, Bloomer, Howe & Berlin, 1970). This phenomenon has previously been neglected during kinetic analysis. Single-passage studies of hepatic uptake of labelled bilirubin suggest that initial hepatic uptake is very rapid (Goresky, 1975; Paumgartner & Reichen, 1976), so that it must represent a substantial proportion of the initial disappearance phenomenon, and studies in normal and Gunn rats in which the liver was removed and assayed for uptake of labelled bilirubin after intravenous injection document that over 40% of the labelled bilirubin is in the liver at 4 min (Bernstein et al., 1966). These experimental studies indicate that it is inappropriate to neglect the very first part of the disappearance curve (for either a bilirubin load or trace amounts of labelled bilirubin) and that the initial disappearance (the proportion of the injected bilirubin removed within 2 min) must represent, in large part, hepatic uptake. The initial parts of the single-injection plasma disappearance curves in our study are virtually identical for both normal subjects and patients with Gilbert’s syndrome. This identity implies that no evidence can be adduced in these studies for a defect in the mechanism of hepatic uptake of bilirubin in the Gilbert’s patients. The changed pattern of removal in these patients appears consequent to a decreased rate of removal of unconjugated bilirubin from a rapidly equilibrating hepatic pool.

**Studies of the bile-containing duodenal aspirates**

Studies of the bile-containing duodenal aspirates show that, in patients with Gilbert’s disease, there is a characteristic change in the bilirubin tetrapyrrrole pattern, the proportion of bilirubin diglucuronide falling from a normal value of 88% to 68%, and that of bilirubin monoglucuronide increasing from 7% to 23%. This difference in the pattern is so characteristic that, ultimately, its chromatographic definition may become the easiest and, probably, best method for characterizing this disorder clinically. The difference between this pattern and that in normal subjects must result from a dysfunction or deficiency in the enzymatic mechanism underlying bilirubin conjugation in these patients. Indeed, studies of digitonin-activated liver biopsy specimens from patients with Gilbert’s syndrome (Black & Billing, 1969; Felsher, Craig & Carpio, 1973; Black, Fevery, Parker, Jacobson, Billing & Carson, 1974) have demonstrated low activities of bilirubin uridine diphosphate (UDP)-glucuronyl transferase (EC 2.4.1.17).

In a child with Crigler–Najjar syndrome type II (moderately severe unconjugated hyperbilirubinaemia), which is characteristically ameliorated by phenobarbitone (Arias, Gartner, Cohen, Ben Ezzer & Levi, 1969) and in which the enzyme deficiency is more extreme, the deviation of the bilirubin conjugates from the normal pattern in bile was found to be even larger: only 10% of the pigment was bilirubin diglucuronide, and 90% was bilirubin monoglucuronide (Gordon, Shaffer & Sass-Kortsak, 1976).

The failure of fasting to affect the biliary bilirubin conjugate pattern in the patients with Gilbert’s syndrome suggests that the increase in plasma bilirubin with fasting results from a quantitative rather than a qualitative change. The plasma disappearance data suggest that there is a rate limitation on the excretion of bilirubin and it is possible that the biliary transport maximum in this syndrome may be at or approximately equivalent to the daily rates of bilirubin production. The excretion of bilirubin in bile both at maximal excretory
rates (Goresky, Haddad, Kluger, Nadeau & Bach, 1974) and at ordinary rates of bilirubin excretion (Shull, Wagner, Trotman & Soloway, 1977) varies with the rate of bile salt secretion. The relative lack of enterohepatic recirculation of bile salts during fasting would therefore be expected to reduce the rate of plasma clearance of bilirubin and thus raise the plasma concentration. Two other factors, changes in enzymatic activity and in the hepatic production of bilirubin, could also play a role. In the rat, 3 days of starvation results in a 20% decrement in total hepatic bilirubin UDP-glucuronyl transferase activity (Duvaldestin, Mahu & Berthelot, 1975). Such a decrement in the Gilbert's patients would reduce excretion. Increase in the bilirubin produced from the cytochromes in the liver, which preempts the excretory pathway (Kirshenbaum, Shames & Schmid, 1976), could also reduce the plasma clearance.

In the present group of Gilbert's patients, phenobarbitone treatment resulted in reversion of the biliary pigment pattern in five of seven patients towards normal, and in all seven the plasma concentration decreased into the normal range. Black et al. (1974) reported that in three of five patients treated with phenobarbitone, the hepatic bilirubin UDP-glucuronyl transferase activity, assayed on liver biopsies, increased into the normal range but it did not in the other two. These results suggest that the reduction in serum bilirubin concentrations after phenobarbitone therapy may have been mediated both by changes in enzyme activity and by an increase in liver size.

Jansen, Chowdhury, Fischberg & Arias (1977) have recently provided evidence that the enzymatic basis for glucuronidation of bilirubin is a two-step process, bilirubin monoglucuronide being synthesized at a microsomal site and the second glucuronide being added to form bilirubin diglucuronide at a plasma membrane site. In light of this, the results of tissue assay procedures will need reappraisal. The inference arising from the patterns of bile pigment conjugates excreted in the bile is that the dysfunction in Gilbert's syndrome is at the second step; the data of Gordon, Shaffer & Sass-Kortsak (1976) indicate that the Crigler–Najjar syndrome type II is a more severe variant of this, in which the dysfunction and the divergence of the conjugation products from the normal are more marked. The change in the pattern of bilirubin conjugates in bile with phenobarbitone therapy would appear to indicate that the fundamental genetic basis for the dysfunction in Gilbert's syndrome is a disorder at the level of a regulatory gene.

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