EFFECT OF PHENOBARBITONE ON PLASMA [\(^{14}\)C]BILIRUBIN CLEARANCE IN PATIENTS WITH UNCONJUGATED HYPERBILIRUBINAEMIA

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SUMMARY

1. The clearance of a tracer dose of \(^{14}\)C-bilirubin from the plasma was studied in patients with Gilbert's syndrome, congenital non-haemolytic jaundice, haemolytic jaundice and in normal subjects. Clearance was significantly impaired in the patients with Gilbert's syndrome and in those with congenital non-haemolytic jaundice when compared with the normal subjects, and was normal in the patient with haemolytic jaundice.

2. Treatment for 2 weeks with phenobarbitone (180 mg/day) lowered the plasma bilirubin concentration and improved all indices of plasma clearance of the isotope in patients with Gilbert's syndrome, so that they became indistinguishable from those of normal subjects. The improvement in plasma \(^{14}\)C-bilirubin clearance in these patients was associated with modest increases in hepatic bilirubin glucuronyl transferase in some subjects.

3. Phenobarbitone treatment improved plasma bilirubin concentrations and plasma \(^{14}\)C-bilirubin clearance in patients with congenital non-haemolytic jaundice, so that they resembled those seen in patients with untreated Gilbert's syndrome. Despite this improvement hepatic bilirubin glucuronyl transferase activity remained undetectable.

4. These results are compatible with the hypothesis that Gilbert's syndrome is a manifestation of a relative deficiency of hepatic bilirubin glucuronyl transferase, and differs from congenital non-haemolytic jaundice only in severity.

Key words: unconjugated hyperbilirubinaemia, Gilbert's syndrome, congenital non-haemolytic jaundice, phenobarbitone, \(^{14}\)C-bilirubin clearance.

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Isotopically labelled bilirubin has provided a valuable means of studying bilirubin metabolism in man and experimental animals, particularly in situations where jaundice is the result of increased plasma unconjugated bilirubin. Delayed rates of clearance of the isotope from the plasma have been reported in patients with congenital non-haemolytic jaundice (Schmid & Hammaker, 1963; Crigler & Gold, 1969) and in the Gunn rat (Schmid & Hammaker, 1963), due, it is assumed, to a deficiency of the enzyme, bilirubin uridine diphosphate glucuronyl transferase (E.C.2.1.4.17) (bilirubin glucuronyl transferase).

Barrett, Berk, Menken & Berlin (1968) and Berk, Bloomer, Howe & Berlin (1970) have performed similar investigations in patients with the milder type of unconjugated hyperbilirubinaemia of Gilbert's syndrome. Their studies, which confirmed earlier observations by Billing, Williams & Richards (1964) with 'cold' bilirubin, demonstrated that such patients have a decreased ability to clear $[^{14}\text{C}]$bilirubin from the plasma when compared with normal controls. Multicompartmental analysis of the plasma disappearance curves was interpreted as indicating defects in both the hepatic uptake and conjugation of bilirubin. So far, no collateral evidence has been presented to support the hypothesis of a primary defect in uptake of bilirubin. It has, however, been shown that bilirubin glucuronyl transferase is significantly decreased in patients with Gilbert's syndrome (Black & Billing, 1969), suggesting that this may be the primary defect in the abnormality of plasma $[^{14}\text{C}]$bilirubin clearance.

In order to clarify the relationship between enzyme deficiency and abnormal handling of isotopic bilirubin in patients with Gilbert's syndrome, plasma $[^{14}\text{C}]$bilirubin clearance studies were performed before and after 2 weeks treatment with phenobarbitone. This drug has been shown to enhance bilirubin glucuronyl transferase activity in experimental animals (Catz & Yaffe, 1968) and in man (Black, Perrett & Carter, 1973) and to decrease plasma bilirubin concentrations in patients with Gilbert's syndrome (Black & Sherlock, 1970). In addition, the effect of phenobarbitone administration on $[^{14}\text{C}]$bilirubin clearance was studied in four normal subjects, a patient with haemolytic jaundice and four children with congenital non-haemolytic jaundice (referred to by Arias, Gartner, Cohen, Ben Ezzer & Levi, 1969, as Type II, Crigler-Najjar syndrome). The effect of glutethimide, another drug which is able to increase hepatic microsomal enzymes (Kato & Chiesara, 1962), was also investigated in three patients. Part of this study has appeared in abstract form (Black, Fever, Parker, Jacobson & Billing, 1971).

**MATERIALS AND METHODS**

**Patients** (Table 1)

Twelve patients with Gilbert's syndrome were studied (Group I), nine of whom had plasma $[^{14}\text{C}]$bilirubin clearance studies done while receiving no medication, and eleven of whom had studies carried out after 2 weeks therapy with phenobarbitone (180 mg/day in divided doses). One patient (no. 1) was unable to attend for study while off all treatment, and in two others (nos. 10 and 11) technical problems prevented satisfactory pretreatment data from being obtained. A further patient (no. 12), the mother of patients 15 and 16, experienced marked dizziness while taking phenobarbitone and the medication was discontinued. In all, eight patients had satisfactory studies performed both before and after 2 weeks therapy with phenobarbitone. Two patients (nos. 1 and 3) had additional studies after receiving glutethimide (500 mg *nocte* for several months, and 500 mg *nocte* for 2 weeks respectively), no phenobarbitone or other medication having been taken for at least 2 months before the studies. The full con-
sent of all patients was obtained after a detailed explanation of the investigation being carried out was given.

**TABLE 1.** Effect of 2 weeks phenobarbitone (PB) therapy on plasma bilirubin concentration and bilirubin glucuronyl transferase activity of liver. The plasma bilirubin concentration is the mean value on the day of the test. The normal range of bilirubin glucuronyl transferase activity is 540–1660 μg g⁻¹ h⁻¹ (Black & Billing, 1969). Patient 12 was the mother of normal subjects 20 and 21. N.D. means not detectable.

<table>
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<tr>
<th>Group</th>
<th>No.</th>
<th>Age (years)</th>
<th>Sex</th>
<th>Plasma bilirubin concentration (mg/100 ml)</th>
<th>Hepatic bilirubin glucuronyl transferase activity (μg g⁻¹ h⁻¹)</th>
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</table>

* Treatment discontinued.

The ages of the patients ranged between 16 and 57 years, the majority being male and less than 25 years old. Diagnosis was based on documentation of a persistently raised plasma unconjugated bilirubin level in the absence of overt haemolysis or coexisting hepatic or systemic disease; all who underwent liver biopsy showed normal histology on light microscopy. In eight patients assays of hepatic bilirubin glucuronyl transferase activity had been carried out as an aid to diagnosis using the digitonin activation method of Black, Billing & Heirwegh (1970); two patients had this investigation carried out only after the 2 week course of phenobarbitone was completed, and a further three patients had enzyme assays both before and after therapy. The results of these studies have been published previously (Black & Sherlock, 1970).
Two children (nos. 13 and 15) with congenital non-haemolytic jaundice (Crigler–Najjar syndrome, Type II; Arias et al., 1969) were also studied, and an affected younger sibling of each child underwent part of the investigation. These four patients comprise Group II. Patient 13 was studied before and after a 2 week course of phenobarbitone (120 mg/day), while patient 15 was studied before and after a 4 week course of the medication (120 mg/day), and again 9 months later, the phenobarbitone being continued throughout. Hepatic bilirubin glucuronyl transferase activity was assayed in each of the four patients before treatment, and in patients 15 and 16 this investigation was repeated 4 weeks after initiation of phenobarbitone therapy. Full parental consent and co-operation was given for all aspects of these studies. (The decision to proceed with repeat studies including liver biopsy in these children was based upon the desirability of obtaining as complete information as possible before commencing them on treatment which was likely to be life-long. Although it was not customary at the time these studies were done to refer such matters to the Ethical Committee of the hospital, the decision to perform follow-up investigations, including a second liver biopsy, was made after consultation with senior members of the department and detailed discussions with the children's relatives.)

One patient with chronic haemolytic jaundice (Group III) was studied on three occasions; the first study was performed while he was on no drug therapy, and the second and third studies were carried out after courses (with several months in between) of phenobarbitone (180 mg/day for 2 weeks) and glutethimide (500 mg nocte for 2 weeks). The nature of the haemolytic condition was obscure; Hb (haemoglobin concentration) was 12.5 g/100 ml; a peripheral blood film showed anisocytosis and polychromasia, and 51Cr-labelled erythrocyte studies showed an erythrocyte half-life of 17 days. This was associated with marked overproduction of bilirubin, since on two separate occasions faecal urobilinogen measurements gave results in excess of 2000 mg/day (normal range, 150–250 mg/day). Assay of hepatic bilirubin glucuronyl transferase activity in this patient gave a normal result.

The normal control group (Group IV) comprised one male patient without hyperbilirubinaemia or liver disease who was undergoing investigation for possible endocrine disease (none was demonstrated), and three normal volunteers of comparable age to the patients with Gilbert's syndrome.

Chemical methods and clearance studies

**Plasma bilirubin determination.** Plasma bilirubin was determined by the method of Michaëllsson, Nosslin & Sjölin (1965); results are expressed as total bilirubin since in normal subjects and in patients with unconjugated hyperbilirubinaemia the amount of conjugated bilirubin present is minimal and cannot be determined with accuracy or precision. The upper limit of normal was taken to be 0.8 mg/100 ml (Powell, Hemingway, Billing & Sherlock, 1967).

**Assay of hepatic bilirubin glucuronyl transferase activity.** Bilirubin glucuronyl transferase activity (expressed as μg of bilirubin conjugated g of liver⁻¹ h⁻¹) of 10% (w/v) liver homogenates in sucrose (250 mmol/l) with EDTA (1 mmol/l), pH 7.4, was determined with digitonin activation (Black et al., 1970).

**Preparation of [¹⁴C]bilirubin.** [¹⁴C]Bilirubin was prepared biosynthetically using the method of Barrett, Mullins & Berlin (1966) by injecting δ-aminolaevulinic acid (usually 0.6 mCi) dissolved in phosphate buffer (0.05 mol/l; pH 6.0), intravenously into a dog with a patent bile fistula. The δ-aminolaevulinic acid (specific radioactivity 113 μCi/mg) was obtained from The Radiochemical Centre, Amersham, Bucks.
Isotopically labelled bilirubin was extracted from bile by the method of Ostrow, Hammaker & Schmid (1961), recrystallized twice to constant specific radioactivity, and then divided into aliquots each containing approximately $5 \times 10^6$ d.p.m. and less than 1 mg of bilirubin, and stored at $-20^\circ$C protected from the light. After 2 months storage the specimens were recrystallized before use.

Before commencing each study the tracer dose of $[^{14}C]$bilirubin (approx. 1·5 μCi for the adults and 1·0 μCi for the children) was dissolved in 0·5 ml of NaOH (50 mmol/l) and 10 ml of sterile human albumin (2·5 g/100 ml) were added. The solution was sterilized by passing it through a Millipore filter [Swinnex-13 Filter Unit (0942), Millipore Corp., Bedford, Mass. 01730, U.S.A.]: a sample was set aside for counting and a known volume of the remainder of the solution (2·5 $\times 10^5$ to $5 \times 10^6$ d.p.m.) was injected into the patient.

Procedure for plasma $[^{14}C]$bilirubin clearance studies. No drugs were administered on the day of the test, which was commenced between 09.00 and 10.00 hours after a light breakfast at 07.30 hours, and terminated 5 h later. An intravenous infusion of iso-osmotic saline with a three-way tap was set up for repeated blood sampling from an ante-cubital vein. The dose of $[^{14}C]$bilirubin was injected intravenously into the opposite arm and 10 ml blood samples were then taken at 2, 5, 10, 15, 20, 25, 30, 40, 50, 60, 70, 90, 120, 150, 180, 210, 240, 270 and 300 min. Additional samples were taken at less frequent intervals from the patients with congenital non-haemolytic jaundice up to 72 h. The samples were centrifuged at 2500 rev/min for 10 min and the plasma removed. Hourly specimens were immediately analysed for bilirubin concentration and the pigment was extracted from all the specimens on the day of the test. In the majority of patients no significant changes in serum bilirubin were observed during the study and the mean value was used for the calculations; in three patients, however, it was necessary to correct for changing levels.

Determination of specific radioactivity of $[^{14}C]$bilirubin in the plasma. The unconjugated bilirubin was extracted from the plasma by the method of Weber & Schalm (1962) and then recrystallized after co-precipitation with 'carrier' bilirubin. Plasma (4 ml) was added to 26·4 ml of extraction reagent (prepared by mixing 200 ml of ethyl acetate and 320 ml of lactic acid, and adding 9·65 ml of fresh diazo reagent [0·6 ml of 2% sodium nitrite added to 20 ml of 0·4% sulphanilic acid in HCl (0·6 mol/l)]. Chloroform (8 ml) was immediately added to the tube and the contents mixed by inverting rapidly fifty times; the specimens were then centrifuged at 2000 rev/min for 5 min. (It is important that there is no delay in extracting the bilirubin with chloroform after the addition of the diazo reagent, otherwise some of the bilirubin will be diazotized and extracted into the upper layer.) A portion (14 ml) of the lower layer was transferred to a glass-stoppered tube, and 2 ml of bilirubin solution were added. The bilirubin solution was prepared by dissolving 24 mg of bilirubin, obtained from British Drug Houses, Poole, Dorset, in 50 ml of chloroform with warming and then filtering; its exact concentration was determined spectrophotometrically. The chloroform extract was washed three times with water to eliminate any remaining extraction reagent, and then decreased in volume to approximately 7 ml under nitrogen. This was filtered into a graduated centrifuge tube through a fluted Whatman no. 1 filter paper, decreased in volume to 1 ml under nitrogen, and 2 ml of methanol added. Further decrease in volume to 1 ml under nitrogen resulted in precipitation of the bilirubin. The specimen was left at $-10^\circ$C for 20 min, and then centrifuged at $5^\circ$C for 10 min at 2000 rev/min; the supernatant fluid was decanted off and the precipitate washed with ice-cold methanol and dried under nitrogen; it was then left in the deep-freeze at $-12^\circ$C until the next day.
To determine the specific radioactivity of bilirubin in each specimen, the precipitate was first dissolved in 3 ml of chloroform. A portion (0.05 ml) was diluted with 3 ml of chloroform and the absorbance at 450 nm determined. The remaining 2.95 ml were transferred quantitatively to a counting vial with several chloroform washings. The vials were evaporated to dryness under nitrogen on a hot plate, after which 0.3 ml of NCS solubilizer (Amersham/Searle Corp., Radiochemical Centre, Bucks.) and 0.3 ml of hydrogen peroxide (100 vol.) were added, and the vials then heated at a temperature not greater than 60°C for 1 h to achieve almost complete decoloration of the bilirubin. A portion (0.1 ml) of the injected dose was treated in similar fashion. The vials were then cooled and 15 ml of scintillator fluid, \( \{ \text{prepared by dissolving 4 g of PPO (2,5-diphenyloxazole) and 50 mg of POPOP} \} \) in 1 litre of toluene and then adding 500 ml of Triton X-100; the mixture was left 24 h before use} \} was added with sufficient water (0.6 ml) to give a one-phase solution at 4°C. Specimens were kept in the dark at 0°C for 24 h or more in order to decrease chemiluminescence and counted in a Nuclear-Chicago scintillation counter, together with appropriate background vials for 20 min periods; the counting efficiency ranged from 60 to 70%.

In calculations it was assumed that the molar extinction coefficient for bilirubin at 450 nm was 60 000 litre mol\(^{-1}\) cm\(^{-1}\), and that the recrystallized bilirubin had been derived from both plasma bilirubin present in the lower layer (equivalent to 3.9 ml of plasma containing the \([^{14}C]\)bilirubin) and the added unlabelled bilirubin, and that losses for both components would be proportional.

In order to test the precision of the analytical procedure, a small quantity of \([^{14}C]\)bilirubin was dissolved in 1 ml of (5 mmol/l) NaOH and divided between two tubes containing pooled plasma at two concentrations of bilirubin (4.3 mg/100 ml and 0.45 mg/100 ml respectively). Several 4 ml aliquots from each tube were then treated as plasma specimens as described above, and the \([^{14}C]\)bilirubin specific radioactivity determined. Recovery of isotope ranged from 83.7 to 91.9% in six samples taken from icteric plasma, and from 80.6 to 88.7% in five samples taken from the non-icteric plasma.

**Determination of radioactivity in faeces.** Homogenates of 6 days combined specimens were prepared; an aliquot was weighed and its \([^{14}C]\)bilirubin radioactivity determined as described for plasma samples.

**Data analysis**

Bilirubin metabolism can be considered as a model system composed of interacting compartments, containing both labelled and unlabelled pigments, for which a series of mass balance equations can be written (Carson & Finkelstein, 1970). These equations can be linearized using a Taylor's series expansion (Head, 1964) and then dynamic reduction of the model may be carried out in a systematic fashion. Since unconjugated, but not conjugated, bilirubin is estimated in the samples, it is possible to reduce the model to that of an unconjugated plasma bilirubin compartment equilibrating with an unconjugated extravascular bilirubin compartment and a liver receptor compartment; unconjugated bilirubin is lost from this receptor compartment following its conversion to conjugated bilirubin in the endoplasmic reticulum and subsequent excretion in the bile (Fig. 1).

An injection of \([^{14}C]\)bilirubin into the plasma can be regarded as the application of a small impulse to the system. Since the dynamics of the compartments of the reduced model can be described by linearized first-order differential equations, then the impulse response obtained
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from the test data may be represented by the summation of exponential components, i.e.

$$\sum_{i=1}^{n} A_i e^{-a_i t}$$

In order to estimate the parameters of the exponential components, a digital computer curve-fitting technique was carried out. Initial estimates were made for the parameters $A_i$ and $a_i$ and the resulting model impulse response was compared with the experimental test data. Using the Rosenbrock hill-climbing technique (Rosenbrock & Storey, 1966) it was possible to vary the parameters $A_i$ and $a_i$ so as to obtain a minimal value for the sum of the squares of the differences from the experimental data and thus a 'best-fit' curve, which is described by the equation:

$$q(t) = A_1 e^{-a_1 t} + A_2 e^{-a_2 t} + A_3 e^{-a_3 t}$$

where $A_2$, $A_3$ and $A_1$ are the intercepts on the $y$ axis and $a_1$, $a_2$ and $a_3$ are the respective rate constants.

![Diagram of bilirubin transport compartments]

**Fig. 1.** Compartments in reduced model of transport of unconjugated bilirubin.

The following clearance constants, which are independent of the assumptions made in the selection of the model system (Donato, Matthews, Nosslin, Segre & Vitek, 1966), were calculated.

(i) Percentage retention of $[^{14}\text{C}]	ext{bilirubin}$ in the plasma at 4 h (expressed as percentage of the computed plasma concentration at zero time).

(ii) The half-life ($t_\text{1/2}$) of $[^{14}\text{C}]	ext{bilirubin}$, derived from the slope of the terminal exponential of the clearance curve.

(iii) The fraction of the plasma unconjugated bilirubin pool irreversibly cleared/min by the liver ($k_e$):

$$k_e = 1 \left( \frac{A_1}{a_1} + \frac{A_2}{a_2} + \frac{A_3}{a_3} \right)$$

where $A_1 + A_2 + A_3 = 1$.

$[^{14}\text{C}]	ext{Bilirubin}$ is rapidly cleared from the plasma by normal subjects, and by 5 h the corrected number of counts in 4 ml aliquots of plasma ranged from 67 to 85 c.p.m. Accordingly, it was
decided to limit the study to a 5 h period. Our values for $t_\frac{1}{4}$ are therefore of the same order as those reported by Barrett et al. (1968) but differ from those reported by Berk et al. (1970), who continued their studies for 24–30 h.

Student's t-test was used to test for significant differences of results.

RESULTS

Plasma $[^{14}C]$bilirubin clearance curves before phenobarbitone treatment

Representative curves of the plasma clearance of $[^{14}C]$bilirubin in the four groups of patients before the commencement of phenobarbitone treatment are shown in Fig. 2. Clearance of isotope was significantly slower in patients with Gilbert's syndrome (Group I) than in normal subjects (Group IV), and was slowed to an even greater extent in the patients with congenital non-haemolytic jaundice (Group II). The patient with haemolytic jaundice (Group III) had a normal clearance.

![Normalized computed plasma $[^{14}C]$bilirubin clearance curves for: (I) patient with Gilbert's syndrome (no. 7); (II) patient with congenital non-haemolytic jaundice (no. 15); (III) patient with haemolytic jaundice (no. 17); (IV) normal subject (no. 21).](image)

Analysis of the clearance data is shown in Table 2. Comparison of Groups I and II with the normal subjects (Group IV) showed that for both groups the 4 h retention of isotope and $t_\frac{1}{4}$
were significantly greater (38·8% and 322 min for Group I, 57% and 924 min for Group II compared with 11·3% and 109 min for Group IV; \( P < 0·001 \)), and \( k_e \) was significantly decreased (0·0035 and 0·0011 respectively compared with 0·0112; \( P < 0·001 \)). For all indices examined, the results in Group II were more abnormal than those for Group I. The patient with haemolytic jaundice (Group III) had values for 4 h retention, \( t_{\frac{1}{2}} \) and \( k_e \), which were within the normal range, in spite of a plasma bilirubin of 2·1 mg/100 ml at the time of the test.

**Table 2.** Kinetics of \([^{14}\text{C}]\)bilirubin clearance before and during phenobarbitone (PB) treatment. \( t_{\frac{1}{2}} \) is the half-life of the terminal exponential of the 5 h curve. \( k_e \) is the fraction of the plasma unconjugated bilirubin pool irreversibly cleared by the liver/min. The values in parentheses for patient 15 were obtained after 9 months treatment with phenobarbitone. Some of the early data on patient 18 were inadequate for analysis and are marked *.

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<td></td>
<td>38·8 ± 10·6</td>
<td>9·7 ± 6·8</td>
<td>322 ± 117</td>
</tr>
</tbody>
</table>

**Effect of phenobarbitone administration**

Phenobarbitone administration to patients with Gilbert's syndrome resulted in lowering of the plasma bilirubin levels, as reported elsewhere (Black & Sherlock, 1970). In agreement with other studies of congenital non-haemolytic jaundice (Whelton, Krustev & Billing, 1968; Crigler & Gold, 1969; Arias et al., 1969), phenobarbitone therapy was strikingly successful in
lowering the plasma bilirubin; it was noted that 4 weeks therapy to patients 15 and 16 failed to lower plasma levels entirely to normal, and indeed 9 months therapy with this medication at the same dose level had no further effect. The plasma bilirubin level of the patient with haemolytic jaundice was slightly lower at the end of the 2 weeks therapy with phenobarbitone than at the outset, but had not fallen outside the range observed in this patient over a prolonged period of observation, during which he received no medication.

![Graph](image)

FIG. 3. Effect of phenobarbitone (180 mg/day for 2 weeks) on plasma $[^{14}\text{C}]$bilirubin clearance in a patient with Gilbert's syndrome (no. 6).

Assay of hepatic bilirubin glucuronyl transferase activity in patients 3, 4 and 6 with Gilbert's syndrome both before and after phenobarbitone administration (Table 1) only demonstrated a significant increase in enzyme activity in one patient (no. 6). In the two patients who only had assays carried out after phenobarbitone therapy, the values recorded were greater than those previously recorded in untreated patients with Gilbert's syndrome (Black & Billing, 1969). Hepatic bilirubin glucuronyl transferase activity was not detected in the four patients with congenital non-haemolytic jaundice prior to drug therapy, and was still undetectable in patients 15 and 16 after 4 weeks phenobarbitone therapy.

The effect of 2 weeks phenobarbitone therapy (180 mg/day) upon the plasma $[^{14}\text{C}]$bilirubin clearance curve of a patient with Gilbert's syndrome is illustrated in Fig. 3. Treatment greatly enhanced the clearance of isotope from the plasma so that the curve became indistinguishable from normal (cf. Fig. 2). Table 2 shows the results for analysis of clearance data after pheno-
Pheno barbitone and $[^{14}C]$bilirubin clearance

barbitone therapy. The patients with Gilbert's syndrome all showed marked improvement in their previously abnormal indices, and with the exception of patients 8, 9 and 10 the values fell within the normal range. Thus the mean value for the 4 h retention fell from 38.8% to 9.7%, that for the $t_+$ fell from 322 to 110 min, and the fractional clearance ($k_e$) increased from 0.0035 to 0.0135.

The post-phenobarbitone data in the two patients with congenital non-haemolytic jaundice (Table 2) who underwent the complete study showed improvement in all indices of $[^{14}C]$bilirubin clearance, the results now closely resembling those observed in patients with untreated Gilbert's syndrome. These values were essentially unchanged in patient 15 when studied again 9 months later.

Analysis of post-treatment data in the normal subjects revealed a variable effect of phenobarbitone administration. Subjects 18 and 19 showed little change in the indices of clearance, whereas in subjects 20 and 21 there appeared to be enhancement of clearance of the isotope from the plasma with an increase in $k_e$. Patient 17 with haemolytic jaundice and a normal pre-treatment study showed an intermediate response.

Effect of glutethimide administration on $[^{14}C]$bilirubin clearance

Plasma $[^{14}C]$bilirubin clearance studies were obtained in three patients (nos. 1 and 3 with Gilbert's syndrome, and no. 17 with haemolytic jaundice) after therapy with glutethimide. A decrease in plasma bilirubin levels was noted in the two patients with Gilbert's syndrome, as reported elsewhere (Black & Sherlock, 1970), which was of the same magnitude as that obtained with phenobarbitone. This was paralleled by a corresponding improvement in the clearance data as shown in Table 3. In the patient with haemolytic jaundice, the results after 2 weeks on glutethimide, unlike those on phenobarbitone, were not significantly different from the pretreatment values.

Additional studies in patients with congenital non-haemolytic jaundice

The improved clearance of $[^{14}C]$bilirubin from the plasma during phenobarbitone treatment was accompanied by an increase in the 6 day cumulative faecal excretion of radioactivity (expressed as percentage of the dose administered) from 32 to 60% for patient 15, and from 52 to 80% for patient 16.

Azopigment analysis (Heirwegh, Van Hees, Leroy, Van Roy & Jansen, 1970) on duodenal samples obtained from patient 15 revealed the presence of small quantities of conjugated bilirubin (bilirubin monoglucuronide) before treatment (H. Jansen, unpublished observations); it was not possible to obtain a comparable specimen after treatment.

DISCUSSION

The name Gilbert's syndrome has been applied to a benign condition characterized by a persistent mild unconjugated hyperbilirubinaemia (range 0.8–5.0 mg/100 ml) in the absence of overt haemolysis or coexistent hepatic or systemic disease. The condition usually presents in adolescence or early adult life, affects males more commonly than females (Foulk, Butt, Owen, Whitcomb & Mason, 1959; Powell et al., 1967), and is discovered either accidentally during routine laboratory investigations or after presentation with one of a variety of non-specific symptoms (Powell et al., 1967). The incidence of the condition in the general population is not
TABLE 3. Comparison of effect of phenobarbitone (PB) and glutethimide (G) on [14C]bilirubin kinetics. $k_e$ is the fraction of the plasma unconjugated bilirubin pool irreversibly cleared by the liver/min. Patient 1 was unable to attend for control studies. Patients received phenobarbitone (180 mg/day in divided doses) for 2 weeks. Patients 1 and 3 received glutethimide (500 mg nocte) for several months and patient 2 for 2 weeks.

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>Treatment</th>
<th>Plasma bilirubin (mg/100 ml)</th>
<th>Retention of bilirubin at 4 h (%)</th>
<th>$t_s$ (min)</th>
<th>$k_e$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control PB G treatment</td>
<td>Control PB G treatment</td>
<td>Control PB G treatment</td>
<td>Control PB G treatment</td>
<td>Control PB G treatment</td>
</tr>
<tr>
<td>1</td>
<td>Gilbert's syndrome</td>
<td>2.4 0.9 0.7</td>
<td>8.4 13.9</td>
<td>103 123</td>
<td>0.0110 0.0065</td>
</tr>
<tr>
<td>3</td>
<td>Gilbert's syndrome</td>
<td>2.5 0.6 0.7</td>
<td>44.3 13.2 21.5</td>
<td>485 128 118</td>
<td>0.0022 0.0090 0.0064</td>
</tr>
<tr>
<td>17</td>
<td>Haemolytic jaundice</td>
<td>2.1 1.7 1.6</td>
<td>9.0 2.5 10.5</td>
<td>100 61 112</td>
<td>0.0121 0.0153 0.0103</td>
</tr>
</tbody>
</table>
Phenobarbitone and $[^{14}C]$bilirubin clearance

known for certain, but values ranging from 0.4 to 1% (Powell, 1972) and 8% (Kornberg, 1942) have been cited. Occasionally more severe forms of unconjugated hyperbilirubinaemia are encountered in which the plasma bilirubin level is in the range 15–20 mg/100 ml; these patients (as exemplified by our Group II patients) are usually recognized shortly after birth and have been referred to as cases of congenital non-haemolytic jaundice (or Crigler–Najjar syndrome, Type II; nomenclature suggested by Arias et al., 1969). A survey of several of these patients (Arias et al., 1969) revealed a very high incidence of mild unconjugated hyperbilirubinaemia in parents or siblings; it was therefore of interest that both parents of patients 15 and 16 were found to have raised plasma unconjugated bilirubin levels, and the mother had a $[^{14}C]$bilirubin clearance curve compatible with Gilbert’s syndrome; the parents of patients 13 and 14 were not examined. This pattern of inheritance does not appear to occur in the most severe form of chronic unconjugated hyperbilirubinaemia (Crigler–Najjar syndrome, Type I), in which kernicterus is common and phenobarbitone treatment ineffective. It is generally accepted that all these conditions are associated with decreased amounts of hepatic bilirubin glucuronyl transferase and indeed the enzyme activity was found to be undetectable in the four patients with congenital non-haemolytic jaundice. These findings are in keeping with the graded enzyme-deficiency concept of inherited chronic unconjugated hyperbilirubinaemia (Black & Sherlock, 1970).

The precise cause for the hyperbilirubinaemia of Gilbert’s syndrome is still a matter of dispute. Though some patients have been shown to have minor decreases in erythrocyte survival (Powell, Billing & Williams, 1967), the calculated bilirubin loads have never been sufficient to account for the raised plasma bilirubin levels. Previous investigations have shown that patients with Gilbert’s syndrome show an abnormal clearance from the plasma of intravenously administered ‘cold’ bilirubin loads (Billing et al., 1964) or tracer doses of isotopically labelled bilirubin, even in the presence of normal bilirubin production rates (Barrett et al., 1968; Berk et al., 1970). That it reflects a fundamental disturbance in the hepatic transport of bilirubin and is not merely a manifestation of an enlarged bilirubin pool is evident, since in our patient with haemolytic jaundice (pure ‘overload’ hyperbilirubinaemia) the plasma clearance of $[^{14}C]$bilirubin was entirely normal despite the presence of a similarly elevated plasma bilirubin level, and hence an increased bilirubin pool. Also, two of the patients with Gilbert’s syndrome with normal serum bilirubin concentrations at the time of the test, nevertheless had abnormal clearance values. Berk et al. (1970) have made similar observations.

On the basis of multicompartmental analysis of $[^{14}C]$bilirubin clearance data, it has been inferred that patients with Gilbert’s syndrome have both a defect in bilirubin conjugation and inability of the liver cell to ‘take up’ bilirubin circulating in the plasma (Billing et al., 1964; Barrett et al., 1968), the site of the uptake defect being placed at the cell membrane or within the cytoplasm of the liver cell. If, however, the transfer of bilirubin across the liver cell is considered as a linked process embracing uptake of the pigment by the cell, its transfer to the endoplasmic reticulum, conjugation by bilirubin glucuronyl transferase, and finally passage of the conjugated pigment into the bile, then interruption of this process at any point up to, and including, the conjugation step will produce similar perturbations of the kinetics of $[^{14}C]$-bilirubin clearance from the plasma. At present it is not possible to differentiate abnormalities of bilirubin transfer due to cell membrane (or cytoplasmic) defects from those arising from defective function of the endoplasmic reticulum on the basis of multicompartmental analysis of plasma $[^{14}C]$bilirubin clearance data (Berk et al., 1970).
The precise mechanism whereby bilirubin enters the liver cell is still a matter for conjecture; however, it appears to be a process which is shared by other organic anions which are not excreted as glucuronides such as bromosulphthalein and Indocyanine Green (Nosslin, 1968). Investigation of the plasma disappearance of these compounds in patients with Gilbert's syndrome has revealed that this is usually normal (Foulk et al., 1959), although Berk, Blaschke & Waggoner (1972) have recently shown marginally increased bromosulphthalein retention (with normal Indocyanine Green clearance) in about one-third of the patients with Gilbert's syndrome. There is therefore no definite evidence to support the hypothesis that the mechanism for the hepatic uptake of bilirubin is impaired; on the other hand, assays of glucuronyl transferase activity in vitro have demonstrated that bilirubin conjugation is defective (Metge, Owen, Foulk & Hoffman, 1964; Black & Billing, 1969).

This investigation was designed to further our understanding of the relationship between bilirubin glucuronyl transferase activity and bilirubin clearance in patients with unconjugated hyperbilirubinaemia. Chronic phenobarbitone administration has been shown to increase the activities of many hepatic microsomal enzymes (Conney, 1967) including bilirubin glucuronyl transferase, both in animals (Catz & Yaffe, 1968) and in man (Black et al., 1973). A similar effect with glutethimide has been shown in experimental animals by Blaschke & Berk (1972). While Yaffe, Levy, Matsuzawa & Baliah (1966), Whelton et al. (1968) and Crigler & Gold (1969) have suggested that the administration of phenobarbitone to patients, whose unconjugated hyperbilirubinaemia resulted from decreased hepatic bilirubin glucuronyl transferase activity, might lower plasma bilirubin, by increasing the amount of enzyme, no previous data on enzyme levels in this situation have been presented. Only Crigler & Gold (1969) have previously reported on the effects of phenobarbitone on bilirubin clearance (in a child with congenital non-haemolytic jaundice).

The increases in hepatic bilirubin glucuronyl transferase in patients with Gilbert's syndrome on phenobarbitone treatment were small, though three of the five determinations fell within 2 standard deviations of the mean of a normal control group (Black & Billing, 1969), whereas none did in the pre-phenobarbitone-treatment group. Bilirubin clearance, as reflected by the results for $k_\varepsilon$, was significantly enhanced by phenobarbitone treatment and became indistinguishable from that of normal subjects. Surprisingly, studies in the patients with congenital non-haemolytic jaundice failed to demonstrate detectable bilirubin glucuronyl transferase activity after phenobarbitone therapy despite impressive decreases in plasma bilirubin levels, a more rapid clearance of $[^{14}C]$bilirubin from the plasma and enhanced excretion of the isotope in the faeces. We attribute this failure either to insufficient sensitivity of the assay procedure at very low levels of enzyme activity, or the possibility that digitonin is not a suitable activator for the enzyme in these particular patients. Indeed, in occasional adult patients with Gilbert's syndrome and plasma bilirubin concentrations of 3–4 mg/100 ml, enzyme activity has been undetectable (M. Black, unpublished observations). It may be therefore that data obtained from assay of bilirubin glucuronyl transferase activity on small amounts of hepatic tissue under only theoretically optimum in vitro conditions gives, at most, an approximation of the activity of enzyme in vivo.

Alternative explanations for the beneficial effects of phenobarbitone and glutethimide in chronic unconjugated hyperbilirubinaemia have to be considered. One possibility concerns $Y$ and $Z$, the organic anion-binding proteins present in the cytoplasm of the liver cell, which have been described by Levi, Gatmaitan & Arias (1969). Though a role for these proteins in the
Phenobarbitone and [14C]bilirubin clearance

uptake of bilirubin by the liver cell remains speculative, investigations in experimental animals have shown that Y (ligandin) increases in amount after phenobarbitone administration (Reyes, Levi, Gatmaitan & Arias, 1969); no comparable observations have been made with glutethimide. In the absence of evidence that these proteins can be rate-limiting for the transport of bilirubin or other organic anions, it is questionable whether increasing their amount, without simultaneously increasing the activity of the microsomal enzymes, would lead to an improvement in plasma [14C]bilirubin clearance kinetics. A complementary role for protein Y, acting in association with enhanced bilirubin glucuronyl transferase activity levels, cannot, however, be excluded.

Another factor to be considered is bile flow. It is known that in animals phenobarbitone, unlike a number of other enzyme inducers (Klassen, 1971), can cause an increase in bile flow which is bile-salt independent (Berthelot, Erlinger, Dhumieux & Preaux, 1970). It has been suggested that this is the means whereby the drug decreases plasma concentrations of conjugated bilirubin (Thompson & Williams, 1967) in primary biliary cirrhosis, and of bile salts and bilirubin in biliary atresia (Stiehl, Thaler & Admirand, 1972). If phenobarbitone has this action in man, and this is still unproven, then it may be that bilirubin excretion is increased by changes in bile flow and in this way its removal from the plasma is stimulated. No information is currently available on this aspect of glutethimide administration.

The effect of phenobarbitone administration upon the kinetics of [14C]bilirubin clearance in the normal subjects and in the patient with haemolytic jaundice was variable. In two of the normal subjects little change was seen but in the two other normals, and to a lesser extent in the patient with haemolytic jaundice, [14C]bilirubin clearance appeared to be significantly enhanced. Phenobarbitone had a greater effect than glutethimide on plasma [14C]bilirubin clearance in the patient with haemolytic jaundice, despite evidence that the latter drug is better at increasing hepatic bilirubin glucuronyl transferase in man (Black et al., 1973). This observation should be extended to other patients with normal bilirubin clearance.

An interesting aspect of this study concerns the limited response of the Group II patients (congenital non-haemolytic jaundice) to phenobarbitone therapy. Despite the administration of substantial doses of phenobarbitone, plasma bilirubin levels did not fall below 3 mg/100 ml, and the plasma clearance of [14C]bilirubin did not improve beyond that observed in untreated patients with Gilbert’s syndrome. A similar limited response of this type of patient to phenobarbitone has been seen in other published cases (Crigler & Gold, 1969; Arias et al., 1969). These observations lend further support to the concept that congenital non-haemolytic jaundice and Gilbert’s syndrome have a similar pathogenesis, differing only in severity.

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REFERENCES


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