The effect of repeated phlebotomy on bilirubin turnover, bilirubin clearance and unconjugated hyperbilirubinaemia in the Crigler–Najjar syndrome and the jaundiced Gunn rat: application of computers to experimental design

P. D. BERK, B. F. SCHARSCHMIDT, JEANNE G. WAGGONER AND S. C. WHITE

Section on Diseases of the Liver, Digestive Diseases Branch, National Institute of Arthritis, Metabolism and Digestive Diseases, and Metabolism Branch, National Cancer Institute, National Institutes of Health, Bethesda, Maryland, U.S.A.

(Received 14 November 1975)

Summary

1. A multicompartmental model of erythrokinetics and bilirubin production has been developed to predict the consequences of chronic phlebotomy on daily bilirubin turnover.

2. Control values for four physiological variables including bilirubin turnover were determined in a 20-year-old woman with type I congenital non-haemolytic jaundice (Crigler–Najjar syndrome). With these base-line data, the model predicted the following changes during phlebotomy: a 34% fall in bilirubin turnover; a 240% increase in the haemoglobin content of bone-marrow erythroid precursors; a 25% fall in the half-life of 51Cr-labelled erythrocytes; a characteristic alteration of the erythrocyte survival curve after labelling with [2-14C]glycine.

3. On the assumption, previously validated in normal volunteer subjects and patients with Gilbert's syndrome, that hepatic bilirubin clearance was independent of turnover and would therefore remain unchanged, a fall in plasma unconjugated bilirubin concentration during phlebotomy from 436 to 282 \( \mu \text{mol/l} \) was expected.

4. Accordingly, the patient underwent phlebotomy 350 ml/week for 2 months, and 500 ml/week during an additional 3 months. Appropriate studies during phlebotomy confirmed each of the predictions in paragraph 2 above. In particular, turnover fell by 31%. Unexpectedly, plasma unconjugated bilirubin remained essentially unchanged. Analogous results were observed in phlebotomized jaundiced Gunn rats.

5. Kinetic studies in both the patient and the rats demonstrated that the failure of plasma unconjugated bilirubin to fall in parallel with bilirubin turnover resulted from a prolongation of the terminal half-life of radioactively labelled bilirubin and a fall in bilirubin clearance in every instance.

6. These studies indicate that (a) in congenital non-haemolytic jaundice, bilirubin clearance is uniquely influenced by bilirubin turnover and (b) compartmental modelling and kinetic studies are useful for predicting and interpreting the results of both physiological experiments and experimental therapeutic regimens.

Key words: bilirubin, compartmental models, computers, Crigler–Najjar syndrome, jaundice, phlebotomy.

Introduction

The concentration of unconjugated bilirubin in the plasma (BR)\(^{(1)}\) varies directly with bilirubin production as estimated from measurements of plasma bilirubin turnover, and inversely with bilirubin clearance (\(C_{\text{BR}}\)), according to the relationship BR \(\simeq\) bilirubin turnover/\(C_{\text{BR}}\) (Berk, Berlin & Howe, 1974a). Several lines of evidence, to be

\(^{(1)}\) Abbreviations: BR, mean plasma unconjugated bilirubin concentration; \(C_{\text{BR}}\), bilirubin clearance.
reviewed below, indicate that, under most circumstances, bilirubin clearance is independent of the rate of bilirubin turnover. Hence, in a given individual, the plasma concentration of unconjugated bilirubin will rise or fall in parallel with any increase or reduction in plasma bilirubin turnover. We have attempted to exploit this relationship by using chronic phlebotomy to decrease bilirubin turnover in a patient with type I congenital non-haemolytic jaundice (Crigler–Najjar syndrome) in whom a number of attempts to reduce the plasma concentration of unconjugated bilirubin by accelerating bilirubin clearance had been ineffective (Blaschke, Berk, Scharschmidt, Guyther, Vergalla & Waggoner, 1974). In the course of this study, a computer-based multicompartmental model of erythrokinetics and bilirubin production was developed to predict the effect of phlebotomy on a number of physiological variables including plasma bilirubin turnover, and to serve as a basis for evaluating the clinical response to therapy. The success with which this simple model predicted a number of the responses to phlebotomy suggests an important potential role for this technique in the design of future physiological studies.

Methods

Bilirubin turnover studies

Unconjugated $^{14}$C]bilirubin (Barrett, Mullins & Berlin, 1966) and specifically labelled unconjugated $^{3}$H]bilirubin (Howe, Berk, Bloomer & Berlin, 1970) were prepared biosynthetically in dogs with bile fistulae as previously described. The biological behaviour of these two preparations has been shown to be identical (Howe et al., 1970), and they were used interchangeably in this study, depending on availability.

Plasma labelled bilirubin disappearance studies were performed as previously described (Berk, Howe, Bloomer & Berlin, 1969), except that daily blood sampling in the patient with Crigler–Najjar syndrome was continued for a minimum of 7 days, in order to define the terminal exponential of the plasma labelled bilirubin disappearance curve with precision. Four labelled bilirubin studies were performed during an 18 months control period and one during a period of weekly phlebotomy. For each study, the fractional rate constant ($k$) and half-life ($t_{0.5}$) of the terminal exponential of the plasma labelled bilirubin disappearance curve (d.p.m./ml of plasma) were determined by the method of least squares from all points obtained after the initial 24 h; the total volume of distribution of injected labelled bilirubin was calculated by means of the usual isotope-dilution equation from the injected dose and the extrapolated value of the terminal exponential at zero time; $C_{BR}$ was calculated as: $C_{BR} = k \times \text{total volume of distribution}$. Finally, plasma bilirubin turnover was calculated from $C_{BR}$ and $BR$ according to the basic relationship (Berk et al., 1969) shown in eqn. (1).

$$\text{Bilirubin turnover (umol day}^{-1} \text{ kg}^{-1}) = \frac{B R (\mu \text{mol} / \text{l}) \times 1440 (\text{min/day})}{1000}$$

$BR$, the mean plasma unconjugated bilirubin concentration, represents the average of at least ten measurements obtained during the course of each labelled bilirubin study, the method of Weber & Schalm (1962) being used.

Data for the initial control study, in which multiple samples were obtained during the first 24 h, were also analysed by the plasma curve integral technique of Nosslin (1964), after fitting the complete disappearance curve to a sum of three exponential functions on a Univac 1108 digital computer. The Simulation, Analysis and Modelling (SAAM) program of Berman & Weiss (1967) was employed for this purpose. Values for $C_{BR}$ and plasma bilirubin turnover obtained by the more general plasma curve integral method were virtually identical with those derived from the simpler ‘terminal slope’ technique, which was employed for all subsequent studies because it requires much less blood sampling. Although the terminal slope method cannot be used in subjects with normal or only moderately reduced hepatic function (Schmid & Hammaker, 1963; Donato, Matthews, Nosslin, Segre & Vitek, 1966), it can be shown to be valid in the unique situation represented by the Crigler–Najjar syndrome (see Appendix 1 to this paper).

Labelled bilirubin disappearance studies in jaundiced Gunn rats were performed and analysed by a previously described micromodification of the procedure employed in the patient (Scharschmidt, Plotz, Berk, Waggoner & Vergalla, 1974), and analysed by the ‘terminal slope’ method.

$^{51}$Cr-labelled erythrocyte volume and total circulating haemoglobin mass

The patient's total circulating erythrocyte volume was determined from the dilution of autotransfused
Phlebotomy in Crigler–Najjar syndrome

$^{51}$Cr-labelled erythrocytes, by the method of Sterling & Gray (1950) as modified by Read (1954). Whole blood volume was estimated from total erythrocyte volume and the peripheral venous haematocrit. The total circulating haemoglobin mass was calculated as the product of total erythrocyte volume and the mean corpuscular haemoglobin concentration (g/ml).

$^{51}$Cr-labelled erythrocyte half-life and mean erythrocyte life span

The $^{51}$Cr-labelled erythrocyte half-life was determined by the method of least squares from blood samples obtained at least twice weekly over the 3–4 weeks after determination of total erythrocyte volume. This measurement was performed once during the control period and once during the period of chronic phlebotomy. In addition, mean erythrocyte life span was calculated on each of these occasions from plasma bilirubin turnover, total erythrocyte volume and mean corpuscular haemoglobin concentration by the method of Berk, Bloomer, Howe, Blaschke & Berlin (1972b).

$[^{14}C]$Haemin erythrocyte survival curve

During the phlebotomy period, the patient was injected intravenously with $[2-^{14}C]$glycine. Multiple blood samples were obtained over the subsequent 7 weeks. Erythrocyte haemin was isolated from these samples by the method of Labbe & Nishida (1957), and the specific radioactivity of $[^{14}C]$haemin determined with a low-background, thin-window $\beta$-counter after plating at infinite thin-ness on aluminium planchets.

Total body haemoglobin and marrow precursor haemoglobin

The carbon monoxide dilution space, a measure of both circulating and marrow precursor haemoglobin, was determined as previously reported (Berk, Rodkey, Blaschke, Collison & Waggoner, 1974b), using a closed rebreathing system similar to that originally described by Coburn, Blakemore & Forster (1963). Since 1 g of haemoglobin binds 1.39 ml of carbon monoxide (Sundström, 1972), total body haemoglobin was calculated by dividing the measured carbon monoxide dilution space by 1.39. The haemoglobin content of marrow erythroid precursors was then estimated as shown in eqn. (2).

Haemoglobin of marrow erythroid precursors = total body haemoglobin – total circulating haemoglobin

(2)

Case summary

M.E.H.K., a 20-year-old woman with type I congenital non-haemolytic jaundice, a relative of the original patients with this disorder described by Crigler & Najjar (1952), has been the subject of three previous reports (Blaschke et al., 1974; Childs & Najjar, 1956; Childs, Sidbury & Migeon, 1959). Her clinical course has been unique in that, despite life-long severe unconjugated hyperbilirubinaemia of 340–600 $\mu$mol/l (20–35 mg/100 ml), she developed normally to age 18. However, in January 1972, during her senior year in high school, she was stricken with bilirubin encephalopathy (Blaschke et al., 1974). Acute, transient reduction of bilirubin concentration by exchange plasmapheresis was associated with a cessation of neurological deterioration and some subsequent improvement. During the 12 months after the episode of bilirubin encephalopathy, her plasma bilirubin concentration returned to its previous range of 340–600 $\mu$mol/l. Although the patient’s overall course remained stable, with no downward trend, her neurological status fluctuated somewhat, and correlated roughly with her bilirubin concentrations. Accordingly, we were concerned that the persistently high amounts of unconjugated bilirubin imposed a risk of recurrent encephalopathy.

Both before and immediately after her initial episode of bilirubin encephalopathy, attempts to reduce her plasma bilirubin concentration by administration of phenobarbital, glutethimide, cholestyramine and oral agar, and phototherapy—methods aimed at accelerating directly or indirectly the clearance of bilirubin from plasma—had been uniformly unsuccessful (Blaschke et al., 1974; Berk, Martin, Blaschke, Scharschmidt & Plotz, 1975). Because of the linear relationship between plasma bilirubin turnover and bilirubin concentration observed in other patients (Berk et al., 1974a, 1975) (vide infra), a method was sought to reduce plasma bilirubin turnover, in the expectation that this would result in a parallel reduction in $BR$.

Model of erythrokinetics and bilirubin production: effects of phlebotomy

Rationale for consideration of chronic phlebotomy.
In 1938, Hawkins & Whipple demonstrated, in dogs with biliary-renal fistulae, that a series of large phlebotomies within a short period of time was followed by a fall in bilirubin production which persisted for approximately 100 days, until the cohort of young cells formed in response to the phlebotomy reached senescence. The rate of bilirubin production was thus shown to be highly dependent on the size of the pool of senescent erythrocytes, rather than the total circulating erythrocyte mass.

Since patients with haemolysis have an erythrocyte age distribution highly skewed toward younger cells, we considered the possibility that a similar age distribution, with a large population of young and a small population of senescent cells, could be produced in patient M.E.H.K. by chronic, weekly phlebotomy. It was thought likely that such a change in erythrocyte age distribution would be accompanied by a fall in bilirubin turnover and, in consequence, a reduction in $B_E$.

**Model of erythrokinetics and bilirubin production.**

In order to test this hypothesis more critically, a model of erythrokinetics and bilirubin production was proposed, in which the life cycle of the erythrocyte, from its earliest differentiation in the marrow to its ultimate senescent death and conversion into bilirubin, was represented by a multicompartmental model consisting of twenty compartments arranged in series (Fig. 1). As described in Appendix 2, measurements of mean corpuscular haemoglobin concentration, total circulating haemoglobin, total body haemoglobin and bilirubin turnover (and the resulting derived value of mean erythrocyte life span) during a control period permitted calculation of all of the pool sizes and intercompartmental transfer rates of the model. Knowledge of these variables in turn permitted computer simulation of the effects of chronic phlebotomy on bilirubin turnover and several aspects of erythrokinetics, including the shape of the erythrocyte survival curve after $[2-^{14}C]$glycine labelling and the haemoglobin content of bone marrow erythroid precursors.

**Protocols for phlebotomy**

In patient M.E.H.K. Experimental measurements of total circulating erythrocyte volume and carbon monoxide dilution space were performed on three
occasions and studies of labelled bilirubin kinetics on four occasions in patient M.E.H.K. during 1971 and 1972. The mean values of each of the parameters calculated from these studies, and a single experimental measurement of the $^{51}$Cr-labelled erythrocyte half-life, constitute the base-line data used for the initial model solution (see Appendix 2). The model was then perturbed by addition to the compartments representing the circulating erythrocyte mass of new pathways whose effect was to simulate a loss of erythrocytes from the circulation equivalent to a 350 ml/week phlebotomy. This perturbation led to prediction by the model of a significant fall in erythrocyte mass whose effect was to simulate a loss of erythrocytes from the circulation equivalent to a 350 ml/week phlebotomy. This perturbation led to prediction by the model of a significant fall in bilirubin turnover and BR. Accordingly, phlebotomy was commenced, and between 22 January and 3 April 1973 a total of 350 ml of whole blood was removed once weekly. Phlebotomy was increased to 500 ml/week from 9 April to 2 July 1973.

Throughout the period of phlebotomy, ferrous sulphate (900 mg) and folic acid (1 mg) were given daily by mouth. On six occasions during the programme the oral iron was supplemented with intravenous Imferon (250 mg) because of falling serum iron concentration. However, at no time was actual iron deficiency present, as indicated by the lack of such manifestations as hypochromia, microcytosis or an elevation of the total serum iron-binding capacity. In particular, the serum iron was in excess of 100 μg/100 ml for the month before and throughout the duration of the phlebotomy period isotope studies. On two occasions, because of vitamin B$_{12}$ concentrations which were at the lower limit of normal, parenteral vitamin B$_{12}$ was administered. At each phlebotomy, 25 g of human albumin was administered in order to prevent both volume and albumin depletion.

On 4 June 1973, after more than 4 months of phlebotomy had failed to reduce the plasma bilirubin concentration, injections of $^{51}$Cr-labelled erythrocytes, $[^{3}H]$bilirubin and $[2^{-14}C]$glycine were given in order to determine CBR, bilirubin turnover, total circulating erythrocyte volume, $^{51}$Cr-labelled erythrocyte half-life and the $[^{14}C]$haemoglobin specific radioactivity curve. The carbon monoxide space was also measured. These studies were performed in an attempt to determine why the predicted beneficial effects of phlebotomy on BR had not been observed. All studies were performed with the fully informed consent of the patient and her family, after approval of the protocol by the appropriate Radiation and Clinical Research Committees.

Effects of chronic phlebotomy in jaundiced Gunn rats. During a control period, studies of labelled bilirubin kinetics and serial measurements of the plasma bilirubin concentration were performed in six homozygous jaundiced male Gunn rats, whose initial weights were 360–430 g. These data were used to calculate base-line values for BR, CBR and plasma bilirubin turnover. In addition, the plasma volume was determined by extrapolation to zero time of multiple samples obtained in the first few minutes after injection of the labelled bilirubin, as previously described (Scharschmidt et al., 1974). Total circulating haemoglobin was estimated from the experimental measurements of plasma volume, the peripheral venous haematocrit and mean corpuscular haemoglobin concentration. Mean erythrocyte life span was calculated by the bilirubin turnover method of Berk et al. (1972).

After completion of these studies, the phlebotomy regimen was begun. In five animals, a blood sample equivalent to an average of 0.7% of body weight was obtained twice weekly from the tail, by the ‘farmer’s wife’ technique (Enta, Hockey & Reed, 1968). After 4 weeks of this regimen had produced no significant change in bilirubin concentration, the volume removed was increased to 1.0–1.1% of body weight (approx. 15% of blood volume) twice weekly. In the sixth animal the larger bleedings were started directly. During phlebotomy, animals were given intramuscular injections of iron, folic acid and vitamin B$_{12}$ at average rates of 4.3 mg, 1.2 mg and 30 μg respectively per week.

Three of the six animals died during or shortly after a phlebotomy procedure at 1, 3 and 4 months into the study. At the time of death, the plasma bilirubin concentration averaged 98% of base line in these three animals. In the remaining three animals, studies of labelled bilirubin kinetics were repeated after a total of 4 months (two rats) and 1 month of phlebotomy, after which phlebotomy was discontinued. Plasma bilirubin concentration was measured periodically for a minimum of 3 months after cessation of phlebotomy. In one animal, a repeat study of labelled bilirubin kinetics was performed 6 months after cessation of the phlebotomy.

The computer was also used to predict the effects of phlebotomy in the Gunn rats. Although no experimental values for carbon monoxide space and total body haemoglobin were determined in the rats during the control period, it was possible to estimate
the necessary base-line model parameters usually derived from these data from previously published studies of rat erythrokinetics (see Appendix 2). The carbon monoxide space was determined experimentally in the rats during the phlebotomy studies.

Results

Effect of phlebotomy in patient M.E.H.K.

Basic experimental data during the control period, assumed or model-predicted data during the phlebotomy period, and actual experimental measurements during the phlebotomy period are summarized in Table 1. Certain aspects of the data are illustrated in Fig. 2.

During the control period total circulating erythrocyte volume averaged 21.4 ml/kg, mean corpuscular haemoglobin 32.43% and total circulating haemoglobin 6.93 g/kg. Total body haemoglobin, estimated from measurements of carbon monoxide space, averaged 7.43 g/kg, or 1.07 times total circulating haemoglobin. From these data, the mean value of bilirubin turnover (5.98 μmol day$^{-1}$ kg$^{-1}$), and the base-line configuration of the model (Fig. 2a), in which there are no erythrocyte losses due to phlebotomy, the model calculations indicate a uniform size (0.46 g of haemoglobin/kg body weight) for each of the circulating erythrocyte haemoglobin compartments (Fig. 2b), and a hypothetical $^{14}$C-haemin erythrocyte survival curve (Fig. 2c) which closely resembles published curves in subjects with normal erythropoiesis (Shemin & Rittenberg, 1946; Gray, Neuberger & Sneath, 1950).

During the phlebotomy period, from the model configuration indicated in Fig. 2(d) and assuming no change in either total circulating erythrocyte volume or CBR, the model and eqn. (1) above [and eqn. (7) of Appendix 2], lead to the following predictions: (a) a fall in $^{51}$Cr-labelled erythrocyte half-life to 19.8 days; (b) an increase in total body haemoglobin to 124% of total circulating haemoglobin; (c) a marked increase in the haemoglobin mass in young circulating erythrocytes (0.95 g/kg body weight in compartment 5) with a corresponding decrease in that in senescent erythrocytes (0.17 g/kg body weight in compartment 19) (Fig. 2e), leading to (d) a correspondingly rapid fall-off in the 'plateau' phase of the $^{14}$C-haemin specific radioactivity curve (Fig. 2f), and (e) a 36% fall in bilirubin turnover to 3.97 μmol day$^{-1}$ kg$^{-1}$. As a result of this fall, assuming that CBR was unchanged, BR was expected to fall from 436 to 287 μmol/l.

In fact, there was a slight decline in total circulating erythrocyte volume during phlebotomy. Nevertheless, the observed changes in (a) $^{51}$Cr-labelled erythrocyte half-life, (b) the ratio of total

### Table 1. Effects of phlebotomy on selected haematological and hepatic parameters in patient M.E.H.K.

Mean values±SEM are shown where indicated. $^{51}$Cr vol. = $^{51}$Cr-labelled erythrocyte volume; $^{51}$Cr $t_{0.5}$ = $^{51}$Cr-labelled erythrocyte half-life; TBHb = total body haemoglobin, determined from carbon monoxide dilution space; TCHb = total circulating haemoglobin, determined from $^{51}$Cr-labelled erythrocyte volume; BRT = plasma bilirubin turnover; CBR = hepatic bilirubin clearance; BR = mean plasma unconjugated bilirubin concentration.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>Phlebotomy</th>
</tr>
</thead>
<tbody>
<tr>
<td>$^{51}$Cr vol. (ml/kg)</td>
<td>21.4±0.7</td>
<td>21.4</td>
</tr>
<tr>
<td>$^{51}$Cr $t_{0.5}$ (days)</td>
<td>26.5</td>
<td>19.8</td>
</tr>
<tr>
<td>TBHb/TCHb</td>
<td>1.07</td>
<td>1.24</td>
</tr>
<tr>
<td>BRT ($\mu$mol day$^{-1}$ kg$^{-1}$)</td>
<td>5.98±1.03</td>
<td>3.97</td>
</tr>
<tr>
<td>BRT (% of control)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(a) From senescent erythrocytes</td>
<td>37</td>
<td></td>
</tr>
<tr>
<td>(b) 'Early labelled peak'</td>
<td>233</td>
<td></td>
</tr>
<tr>
<td>(c) Total</td>
<td>66</td>
<td>69</td>
</tr>
<tr>
<td>CBR (ml min$^{-1}$ kg$^{-1}$)</td>
<td>0.009±0.001</td>
<td>0.009</td>
</tr>
<tr>
<td>BR ($\mu$mol/l)</td>
<td>436±15</td>
<td>287</td>
</tr>
</tbody>
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Phlebotomy in Crigler-Najjar syndrome

(a) Control (d) Phlebotomy

FIG. 2. Model-derived predictions of certain effects of phlebotomy. See the text for details of (a)–(f). [Reproduced from Berk et al. (1975) by permission of Annals of Internal Medicine.]

(b) (e)

Comportment

Time (doyd)

FIG. 3. Patient M.E.H.K.'s erythrocyte [14C]haemin specific radioactivity (d.p.m./mg) curve, after the intravenous injection of [2-14C]glycine during the trial of phlebotomy. The experimental points (○) are virtually superimposable on the curve (continuous line) predicted by the computer. [Reproduced from Berk et al. (1975) by permission of Annals of Internal Medicine.]

Effect of phlebotomy in jaundiced Gunn rats

The results of phlebotomy in jaundiced Gunn rats are summarized in Table 2. Base-line values for the labelled bilirubin t0.5 and plasma bilirubin turnover are similar to data reported by others (Schmid & Hammaker, 1963; Robinson, 1971). Furthermore, the calculated value of 1·10 ± 0·24 (SEM) μmol day−1 100 g−1 for bilirubin turnover is similar to a recently reported value of 1·21 ± 0·10 (SEM) μmol day−1 100 g−1 for bile bilirubin excretion in Sprague-
TABLE 2. Effects of chronic phlebotomy in jaundiced Gunn rats

Mean values ± SEM are shown where indicated. TRCV = total circulating erythrocyte volume. For definitions of other abbreviations see Table 1. The value of TBHb/TCHb during the control period was calculated from an assumed marrow maturation time in the rat of 3-0 days (see Appendix 2). This value of TBHb/TCHb, in turn, was used for subsequent predictions.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>Phlebotomy</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(n = 6)</td>
<td>Predicted</td>
</tr>
<tr>
<td>TRCV (ml/100 g)</td>
<td>2.6 ± 0.2</td>
<td>2.6</td>
</tr>
<tr>
<td>TBHb/TCHb</td>
<td>1.06</td>
<td>1.16</td>
</tr>
<tr>
<td>BRT (μmol day⁻¹ 100 g⁻¹)</td>
<td>1.10 ± 0.24</td>
<td>0.71</td>
</tr>
<tr>
<td>BRT (% of control)</td>
<td>—</td>
<td>65</td>
</tr>
<tr>
<td>[¹⁴C]Bilirubin t₀.₅ (h)</td>
<td>37 ± 3</td>
<td>37</td>
</tr>
<tr>
<td>C_re (μl min⁻¹ 100 g⁻¹)</td>
<td>5.2 ± 0.4</td>
<td>5.2</td>
</tr>
<tr>
<td>BR (μmol/l)</td>
<td>142 ± 19</td>
<td>92</td>
</tr>
</tbody>
</table>

Dawley rats (Gisslebrecht & Berk, 1974). When mean erythrocyte life span was calculated by the method of Berk et al. (1972b) from the base-line values of total circulating erythrocyte volume and plasma bilirubin turnover, the resulting mean value of 57 days is very similar to published values for survival of erythrocytes in the rat obtained by several independent techniques (Berlin & Berk, 1975; Landaw & Winchell, 1970).

As indicated in Table 2, the model accurately predicted the increase in the ratio total body haemoglobin/total circulating haemoglobin, and the fall in plasma bilirubin turnover resulting from phlebotomy in the Gunn rats. As with the patient, the fall in bilirubin turnover was accompanied by a prolongation of the labelled bilirubin t₀.₅ and a decrease in bilirubin clearance, so that values for plasma unconjugated bilirubin concentration during phlebotomy were not significantly different from those obtained during the control period (Student's t-test, P > 0.6).

In the one animal studied before, during and 6 months after cessation of phlebotomy, values for total circulating erythrocyte volume, plasma labelled bilirubin t₀.₅, plasma bilirubin turnover and BR were virtually identical during the two control studies (Fig. 4). During phlebotomy there was a marked decline in bilirubin turnover, but as a result of a corresponding decrease in Cₜᵢᵢ, the plasma concentration of unconjugated bilirubin remained unchanged (Fig. 4).

Discussion

The studies described in this report represent one of a series of attempts to reduce the plasma bilirubin concentration in a unique patient with Crigler–Najjar syndrome, who had developed bilirubin encephalopathy at 18 years of age (Blaschke et al., 1974). Previous approaches had focused on various techniques for accelerating bilirubin clearance, either by augmenting normal hepatic bilirubin metabolism (phenobarbital or glutethimide), by decreasing a hypothetical enterohepatic circulation (cholestyramine and oral agar), or by degrading the bilirubin to other, presumably less-toxic, products.
(phototherapy). Each of these attempts proved unsuccessful (Blaschke et al., 1974). Accordingly, as the plasma bilirubin concentration also depends in linear fashion on plasma bilirubin turnover, an attempt was made to effect a reduction in the former by decreasing the latter. The rationale for our approach derived from the classic study of Hawkins & Whipple (1938) described above.

In terms of any therapeutic benefit to our jaundiced patient, the critical assumption involved in these studies was that bilirubin clearance \( (C_{BR}) \) would be independent of bilirubin production and plasma bilirubin turnover. Under this circumstance, any reduction in bilirubin turnover would be followed by a proportional decrease in the plasma unconjugated bilirubin concentration \( (B_R) \). We have previously reported that \( C_{BR} \) in a group of patients with normal hepatic function and uncomplicated haemolysis \( (0.68 \pm 0.15 \text{ (sp) ml min}^{-1} \text{ kg}^{-1}) \) was virtually identical with that in normal volunteer subjects without haemolysis \( (0.65 \pm 0.18 \text{ ml min}^{-1} \text{ kg}^{-1}) \), despite an increase in plasma bilirubin turnover in the former group to 330% of normal (Berk, Bloomer, Howe & Berlin, 1970; Berk, Blaschke & Waggoner, 1972a). Similarly, among patients whose clinical data (Berk et al., 1970), histological features (Barth, Grimley, Berk, Bloomer & Howe, 1971) and, in some cases, enzyme assays (Black & Billig, 1969) were indicative of Gilbert's syndrome, \( C_{BR} \) in those with concomitant haemolysis \( (0.20 \pm 0.08 \text{ ml min}^{-1} \text{ kg}^{-1}) \) was similar to values in patients without haemolysis \( (0.19 \pm 0.04 \text{ ml min}^{-1} \text{ kg}^{-1}) \) despite an increase in plasma bilirubin turnover to 220%, of that in the non-haemolysing patients (Berk et al., 1970, 1972a). In a further unpublished study, \( C_{BR} \) in a single patient with hereditary spherocytosis was 0.66 ml min\(^{-1}\) kg\(^{-1}\) before and 0.65 ml min\(^{-1}\) kg\(^{-1}\) after splenectomy, despite a fall in plasma bilirubin turnover from 552 to 6.8 \( \mu \text{mol day}^{-1} \text{ kg}^{-1} \). Hence, up to an eightfold variation in bilirubin turnover seemed to have no effect on \( C_{BR} \) in both individuals with normal hepatic bilirubin metabolism and patients with Gilbert's syndrome. We therefore generalized from these data, and assumed that \( C_{BR} \) was independent of plasma bilirubin turnover in the Crigler–Najjar syndrome as well.

Our current results clearly indicate that this assumption is not, in fact, applicable to patients with Crigler–Najjar syndrome. In both the patient and in the Gunn rat studies, a fall in plasma bilirubin turnover was associated with a corresponding decrease in \( C_{BR} \), with very little change in \( B_R \). The rat studies are important not only in confirmation of the patient data, but also because they indicate that the decrease in \( C_{BR} \) is an inherent feature of the disease, and not related to the albumin infusions which were performed after each phlebotomy in the patient (vide supra), but not in the rats. Therefore, although our original goal of reducing \( B_R \) was not achieved, these studies have contributed new knowledge about the physiology of bilirubin excretion in the Crigler–Najjar syndrome.

The mechanism by which plasma bilirubin turnover and \( C_{BR} \) are linked in Crigler–Najjar syndrome and the Gunn rat, as opposed to normal subjects or patients with Gilbert's syndrome, is unknown. It is almost certain that bilirubin removal in the former situations occurs to a considerable extent by processes which make only a minor contribution to overall bilirubin excretion under normal circumstances (Schmid & Hammaker, 1963; Ostrow, 1971). Furthermore, there is considerable evidence that, at least in the Gunn rat, 50% of bilirubin excretion occurs at extrahepatic sites (Schmid & Hammaker, 1963; Ostrow, 1971). Since the mathematical determination of \( C_{BR} \) is independent of any particular model of bilirubin metabolism (Berk et al., 1969), as well as of the site and biochemical mechanism of the irreversible removal of bilirubin from plasma, our own data furnish no helpful information about this point. As a first assumption, one might have predicted that the various alternative pathways of bilirubin excretion, first described by Schmid & Hammaker (1963), would follow first-order kinetics. Under these circumstances, \( C_{BR} \) via these alternative pathways would be independent of plasma bilirubin turnover and plasma bilirubin concentrations would vary directly with turnover just as in normal individuals. Our data strongly suggest that the alternative pathways of bilirubin excretion do not follow first-order kinetics. However, any further hypothesis about the mechanism and kinetics of the alternative pathways would be purely speculative at this point.

One further aspect of these studies merits further comment: the use of a compartmental model to predict the effect of a physiological perturbation. A compartment model of a biological system, as defined by its associated differential equations, is, in effect, a concise description mathematical terms of how the modeller believes the system works.
Such models have been applied to studies of bilirubin (Berk et al., 1969), iron (Wasserman, Sharney, Gevirtz, Schwartz, Weintraub, Tendler, Dumont, Dreiling & Witte, 1965), calcium (Neer, Berman, Fisher & Rosenberg, 1967), magnesium (Avioli & Berman, 1968), iodide (Berman, Hoff, Barandes, Becker, Sonenberg, Benua & Koutras, 1968), glucose (Kronfeld, Ramberg & Shames, 1971), protein (Berlin, Berman, Berk, Phang & Waldman, 1969) and lipid (Quarfordt, Frank, Shames, Berman & Steinberg, 1970) metabolism. The most common application of such models is the analysis of experimental curves in terms of numerical values for specific biological variables. In the present study, the use of a model has been extended—under certain limited restrictive assumptions—to predict the behaviour of the system in response to a stress or perturbation. The success of this approach in predicting the behaviour of a number of important biological variables, with a conceptually simple compartmental model, suggests an important potential role for this technique in the design of future physiological studies.

APPENDIX 1

Validity of terminal slope method for estimation of plasma bilirubin turnover

In the first publication describing the use of radioactively labelled bilirubin in man, plasma bilirubin turnover in a patient with Crigler–Najjar syndrome was calculated by the terminal slope method (Schmid & Hammaker, 1963). Although this technique appeared to provide reasonable results in the Crigler–Najjar patient, it led to a marked overestimate in subjects with normal rates of bilirubin clearance (Schmid & Hammaker, 1963). The terminal slope method is only valid if the rate of exchange between internal compartments is rapid compared with the rate of excretion, so that uniform specific radioactivity is achieved throughout the body before any significant excretion of isotope (Donato et al., 1966). Hence, Schmid & Hammaker (1963) concluded that uniform specific radioactivity was presumably achieved in the patient with Crigler–Najjar syndrome, but not in normal subjects. The terminal slope method has been
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...widely accepted without further verification for the estimation of plasma bilirubin turnover in both Crigler–Najjar syndrome patients (Billing, Gray, Kulczycka, Manfield & Nicholson, 1964; Crigler & Gold, 1969; Bloomer, Berk, Howe & Berlin, 1971) and Gunn rats (Schmid & Hammaker, 1963; Robinson, 1971).

Subsequently, we have applied the less-restrictive plasma curve integral method of Nosslin (1964) to this estimation in normal subjects and subjects with a wide range of hepatic and haematological disorders. Validity of this method has been demonstrated by: (a) its close correlation \( r = 0.99 \) with independent estimates of carbon monoxide production (Berk et al., 1974b); (b) its agreement with a faecal isotope dilution method for estimating bilirubin production (Berk et al., 1972b); (c) the fact that values for plasma bilirubin turnover derived by this technique lead to accurate calculated values for the life span of erythrocytes (Berk et al., 1972b). In contrast, no independent verification of the terminal slope method for calculation of plasma bilirubin turnover has become available, although its validity for determining the turnover rates of specific proteins has had extensive independent validation (Donato et al., 1966).

In order to compare the two methods directly, plasma bilirubin turnover was estimated during the first of the four control studies in patient M.E.H.K. by both the terminal slope method (5.15 \( \mu \text{mol day}^{-1} \text{kg}^{-1} \)) and plasma curve integral method (5.04 \( \mu \text{mol day}^{-1} \text{kg}^{-1} \)). The excellent agreement supports the validity of the former technique. In addition, the plasma labelled bilirubin disappearance curve of patient M.E.H.K. was subjected to compartmental analysis in terms of a previously described three-compartment model of bilirubin metabolism (Berk et al., 1969), which has also been extensively validated (Berk, 1975). As indicated in Fig. A1, computer-predicted specific radioactivity curves for the hepatic and extravascular pools become virtually identical with the plasma specific radioactivity curve by 24 h, at a time when appreciably more than 90% of the injected isotope is still in the body, and the whole-body retention curve behaves as a single exponential from zero time onward. Under such circumstances, the simple ‘terminal slope’ method provides accurate estimates of bilirubin turnover (Donato et al., 1966). This is in marked contrast to the situation in a normal subject (Fig. A2), in whom specific radioactivity of the extravascular pool constantly exceeds that of the hepatic and plasma pools after 6 h, and the whole-body retention curve cannot be expressed as a single
exponential function. Under these circumstances, the 'terminal slope' method of calculation is invalid (Donato et al., 1966).

These data provide both a theoretical and experimental validation for the use of the terminal slope method for estimation of plasma bilirubin turnover in patients with Crigler-Najjar syndrome, and, by analogy, in the Gunn rat.

APPENDIX 2

A model of erythrokinetics and bilirubin production

In order to assess the effects of phlebotomy on bilirubin production, it was considered useful to represent the endogenous synthesis of haemoglobin and its subsequent conversion into bilirubin by a compartmental system which could be quantitatively defined from a limited number of simple experimental measurements, such as circulating erythrocyte mass and erythrocyte life span.

In the model illustrated in Fig. 1 of the main paper, each of the compartments numbered 1–19 represents haemoglobin in erythroid cells of increasing age. Erythrocyte precursors in the marrow are represented by compartments 1–4, and the sum of these compartments—the total marrow erythroid precursor haemoglobin mass—is represented by compartment 21. The total circulating haemoglobin mass is depicted by the compartments enclosed in the rectangle and numbered 5–19. Each of these fifteen compartments is considered to represent haemoglobin in circulating erythrocytes of successively older age, with compartment 19 representing the haemoglobin in senescent erythrocytes. Compartment 22 is the sum of compartments 5–19 and, as such, is the compartmental equivalent to total circulating haemoglobin. In essence, the use of the linear arrangement of compartments 5–19 is an application of a standard modelling concept—that of the ‘delay line’, to represent the finite life span of circulating erythrocytes. The choice of fifteen compartments to represent the circulating haemoglobin mass has no particular biological significance. Rather, in testing various potential models against normal 14C-labelled erythrocyte survival curves obtained in several individuals after the administration of [2-14C]glycine, a fifteen-compartment 'delay line' was the smallest number of compartments which generated survival curves closely approximating the experimental data.

Similarly, no biological significance is attached to the choice of four compartments to represent the bone marrow. Again, this represents the smallest number of pools mathematically consistent with the early 'uptake' phase of 14C- or 59Fe-labelled erythrocyte survival curves.

In conformity with the notation established for the SAAM computer (Berman & Weiss, 1967), the mass of haemoglobin in any compartment i is indicated by $M_i$, the fraction of compartment i transferred per day to any other compartment j by the constant $\lambda_{ij}$, and, for compartments 5–19, the fraction of the $i^{th}$ pool eliminated from the system per day by phlebotomy by $\lambda_i$. In accordance with the above definitions, $R_{ij}$, the mass of haemoglobin transferred per day from compartment i to compartment j, is calculated as $\lambda_i M_i$.

The conversion of haemoglobin into bilirubin is considered to occur in compartment 20. Thus the pathway denoted by $\lambda_{20,19}$ represents the death of senescent erythrocytes, and that denoted by $\lambda_{20,4}$ represents ineffective erythropoiesis, i.e. the catabolism of haemoglobin in developing precursors of erythrocytes. In the general case random haemolysis of circulating erythrocytes would be indicated by the fifteen pathways $\lambda_{2i,1}$ for $i = 5–19$. In the specific case under consideration, the rate of random haemolysis was assumed to be insignificant on the basis of experimental erythrokinetic measurements including a normal 51Cr-labelled erythrocyte half-life and mean erythrocyte life span, and these pathways are omitted from the model. Accordingly, the model accounts for bilirubin production from two sources: the $R_{20,19}$ g of haemoglobin resulting daily from death of senescent erythrocytes, and the $R_{20,4}$ g derived from ineffective erythropoiesis. Since 1 g of haemoglobin is converted stoichiometrically into 61.9 μmol of bilirubin, bilirubin turnover is given by eqn. (1).

\[
\text{Plasma bilirubin turnover (μmol day}^{-1} \text{kg}^{-1}) = 61.9 \left[R_{20,19} + R_{20,4}\right]
\] (1)

It has been shown that not all 'early labelled' bilirubin is derived from ineffective erythropoiesis, some being of hepatic origin (Berk et al., 1974a; Ibrahim, Schwartz & Watson, 1966; Robinson, Tsong, Brown & Schmid, 1966). However, previous studies in our laboratory have shown that, in the presence of normoblastic erythropoiesis, the total quantity of 'early labelled peak' bilirubin from all sources can be satisfactorily approximated by 15% of plasma bilirubin turnover, irrespective of the
rate of erythropoiesis (Berk et al., 1972a). Since the haemoglobin of all erythrocyte precursors leaving the most mature marrow precursor pool (compartment 4) is, under normal circumstances, ultimately converted into bilirubin, an ‘early labelled peak’ bilirubin production representing 15% of the total was provided for by setting $\lambda_{20,4}(\lambda_{5,4} + \lambda_{20,4}) = 0.15$, or $\lambda_{20,4} = 0.1765 \lambda_{5,4}$.

**Determination of model parameters from base-line experimental data**

Under the definitions given above, the mean transit time ($t$) of a cohort of cells or haemoglobin through any of the compartments 5–19 will be equal to $t = 1/\lambda_{i1}$. If all fifteen $\lambda_{i1}$ from $i = 5–19$ are assumed to be equal, then the mean erythrocyte life span may be calculated as 15 (1/\lambda_{i1}). In actual practice, since erythrocyte life span was determined experimentally, the expression was used to calculate the values of $\lambda_{i1}$ for $i = 5–19$.

Similarly, values for the $\lambda_{i1}$ of the marrow precursor compartments ($i = 1–4$) were calculated in the patient from the measured base-line experimental value for the marrow precursor haemoglobin mass (eqn. 2 of the main paper), again assuming that $\lambda_{2,1} = \lambda_{3,2} = \lambda_{4,3} = \lambda_{5,4}$. Because of the loss of haemoglobin from compartment 4 due to ineffective erythropoiesis ($\lambda_{10,4}$) the steady-state pool sizes of compartments 1–4 are related as follows (see above): $M_1 = M_2 = M_3 = 1.1765M_4$. Therefore, marrow precursor haemoglobin mass may be represented as in eqn. (2) here.

**Marrow precursor haemoglobin mass**

$$M_1 = 4.5295M_4$$

These equations permit determination of each of the marrow $M_1$ values. The quantity of haemoglobin entering or leaving the circulation in the steady state is equal to total circulating haemoglobin/mean erythrocyte life span. This is the same quantity transferred daily from compartment 4 to 5. Hence this ratio = $\lambda_{5,4} M_4$. Solution of this expression provides a value for $\lambda_{5,4}$ and hence for $\lambda_{2,1}, \lambda_{3,2}$ and $\lambda_{4,5}$ as well.

Hence, in the patients, all the variables (pool sizes and intercompartmental fractional transfer rates) of the twenty-two-compartment model of steady-state erythrokinetics could be determined from experimental values for total circulating haemoglobin (i.e. total circulating erythrocyte volume and mean corpuscular haemoglobin concentration), total body haemoglobin (i.e. carbon monoxide dilution space) and mean erythrocyte life span (calculated from plasma bilirubin turnover). Although no measurement of carbon monoxide space or total body haemoglobin was available for the rats during the control period, values for $\lambda_{2,1}$ through $\lambda_{5,4}$ were estimated from published data indicating that the mean maturation time (stem cell to emergence time) of developing erythrocytes in rat marrow is 3-0 days (Stohlman, Brecher & Moores, 1962). Thus the value of $t$ is 0.750 day for each of pools 1–4, with the corresponding value of $\lambda_{2,1} = \lambda_{5,4}$ being 1.333. In all other respects, computer analysis of the rat studies was identical with that of the patient data. Carbon monoxide space and total body haemoglobin were determined during the phlebotomy studies by placing the three surviving rats simultaneously in the closed rebreathing system previously described by Rodkey, Collison & O’Neill (1972).

If, after the injection of a tracer dose of [2-14C]-glycine, a unit pulse of [14C]haemoglobin is produced in compartment 1, the subsequent [14C]haemoglobin content $f_i(t)$ of each of the primary compartments (1–20) of the model at various times after injection will be described by the set of twenty simultaneous linear differential equations (eqn. 3).

$$\frac{df_i}{dt} = -\lambda_{i1}f_i + \lambda_{ii}f_1, \text{ for } i = 1–20, j = 1–19, j \neq i$$ (3)

By convention, $\lambda_{i1}$ is the sum of all the $\lambda$ values leading away from the $i$th compartment. Determination of each $f_i$ value is carried out by computer integration of the corresponding differential equation. The isotope content of the circulating erythrocyte mass, $f_{22}(t)$ is simply

$$f_{22}(t) = \sum_{i=5}^{19} f_i(t)$$ (4)

The curve of $f_{22}(t)$ is equivalent to the haemoglobin or [14C]haemin erythrocyte survival curve obtained after injection of [2-14C]glycine as an erythrocyte precursor.

**Prediction of effects of chronic phlebotomy**

In order to assess the effects of phlebotomy on the system, the following simplifying assumptions were
made: (a) as a result of increased marrow production of erythrocytes, the total circulating erythrocyte volume and total circulating haemoglobin mass would rapidly return to base-line values between individual phlebotomies, and for the purposes of pool size calculations, could be considered to be constant; (b) the mean transit time of haemoglobin through each of the compartments 1–19 would not be altered appreciably from their basal values, so that numerical values for each of the \( \lambda_i \), determined during base-line studies could be used to simulate the effects of phlebotomy. Phlebotomy was simulated by adding to each of compartments 5–19 a new pathway, \( \lambda_{oi} \), the value of which, per day, was equal to \( V/\text{whole blood volume} \), where \( V \) is equal to one-seventh of the volume of blood removed at each weekly phlebotomy. From the values for each of \( \lambda_{oi} \) and \( \lambda_{si} \), and the experimental value of total circulating haemoglobin, the computer calculated (1) the new steady-state pool sizes of each of the compartments of the model during phlebotomy, including total body haemoglobin, (2) plasma bilirubin turnover, as well as the separate components thereof derived from senescent erythrocyte death and ineffective erythropoiesis, and (3) the new predicted \( [^{14}C] \) haemin erythrocyte survival curve after injection of \( [2-{ }^{14}C] \) glycine during phlebotomy. The predicted value of BR was calculated from the predicted value of plasma bilirubin turnover by eqn. (1) of the main paper, on the assumption that \( C_{BR} \) was unchanged from base-line value.

One additional prediction was performed, independent of the model. The general equation describing the survival curve of \( ^{51} \)Cr-labelled erythrocytes is shown in eqn. (5).

\[
N(t) = N_0 \left( 1 - \frac{t}{T} \right) e^{-\left(k_0 + k_\text{e}\right)t}
\]

In this equation \( N(t) \) is the quantity of circulating erythrocyte \( ^{51} \)Cr at time \( t \), \( N_0 \) the initial quantity of circulating erythrocyte-bound \( ^{51} \)Cr, \( T \) the maximum or potential life span of the erythrocyte, \( k_\text{e} \) the fractional daily rate of \( ^{51} \)Cr elution from erythrocytes and \( k_0 \) the fractional loss of labelled erythrocytes from the circulation by either random (age-independent) haemolysis or, in the present case, by phlebotomy (Berlin & Berk, 1975). Since both the \( ^{51} \)Cr-labelled erythrocyte half-life and erythrocyte life span during the control period were entirely normal, it was assumed that there was no random haemolysis in the patient. Under these circumstances, during the control period, \( k_0 = 0 \) and mean erythrocyte life span = \( T \) (potential life span). By definition, when \( t = ^{51} \)Cr-labelled erythrocyte half-life, \( N(t)/N_0 = 0.5 \). Substitution into eqn. (5) gives eqn. (6).

\[
0.5 = \left[ 1 - \left( ^{51} \text{Cr-labelled cell half-life}/\text{mean life span} \right) \right] \times e^{-\left(k_\text{e} + \frac{V}{\text{whole blood volume}}\right) \times \left( ^{51} \text{Cr-labelled cell half-life} \right)}
\]

(6)

From the experimental values for \( ^{51} \)Cr-labelled erythrocyte half-life and mean erythrocyte life span, eqn. (6) was solved for \( k_\text{e} \).

In order to predict the \( ^{51} \)Cr-labelled erythrocyte half-life during phlebotomy, it was assumed that: (a) phlebotomy produced no changes in the values of either \( T \) (i.e. erythrocyte life span) or \( k_0 \) determined during the control period; (b) the rate of any random haemolysis was very small compared with the loss of labelled cells by phlebotomy and could therefore be neglected. Thus the value for \( k_0 \) was taken to be the same as \( \lambda_{si} \), i.e. as \( V/\text{whole blood volume} \). Hence the predicted value of \( ^{51} \)Cr-labelled erythrocyte half-life during phlebotomy was determined from an iterative numerical approximation solution of the relationship in eqn. (7), where the values for erythrocyte life span and \( k_0 \) were those determined during the control period.

\[
0.5 = \left[ 1 - \left( ^{51} \text{Cr-labelled cell half-life}/\text{mean life span} \right) \right] \times e^{-\left(k_0 + \frac{V}{\text{whole blood volume}}\right) \times \left( ^{51} \text{Cr-labelled cell half-life} \right)}
\]

(7)

Acknowledgments

 Portions of these results were presented at a Combined Clinical Staff Conference of the National Institutes of Health, Bethesda, Maryland, on 7 February 1974, and subsequently published (Berk et al., 1975).

The authors are indebted to Dr F. L. Rodkey and Mr H. A. Collison for all of the carbon monoxide measurements and to Ms Phyllis Cromwell for the rapid and accurate typing of the manuscript.

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