INCREASED GLUCURONIDATION OF BILIRUBIN IN MAN AND RAT BY ADMINISTRATION OF ANTIPYRINE (PHENAZONE)

M. L'E. ORME, L. DAVIES AND A. BRECKENRIDGE

Department of Clinical Pharmacology, Royal Postgraduate Medical School, London

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SUMMARY

1. Antipyrine in a dose of 3.2 mmol (600 mg) daily for 6 weeks produced a significant fall in both total and unconjugated serum bilirubin concentrations in six patients with Gilbert's syndrome. The maximum reduction in serum bilirubin concentration was seen after 2 weeks of treatment.

2. In the rat, administration of antipyrine in doses of 0.42 and 1.27 mmol 24 h⁻¹ kg⁻¹ (80 and 240 mg 24 h⁻¹ kg⁻¹) for 84 h caused a significant increase in the apparent maximal velocity (Vₘₐₓ) for the glucuronidation of bilirubin by liver microsomal preparations when the concentration of either uridine diphosphate glucuronic acid (UDPGA) or bilirubin was altered. There was no significant difference between the apparent Vₘₐₓ values attained with the two doses of antipyrine in either set of experiments. Neither the microsomal protein content nor the apparent affinity constant (Kₘₐₓ) was altered in these studies.

3. In contrast, administration of phenobarbitone in doses of 0.34 mmol 24 h⁻¹ kg⁻¹ (80 mg 24 h⁻¹ kg⁻¹) caused a significant increase in the microsomal protein content but there was no significant change in the values for the apparent Vₘₐₓ or apparent Kₘₐₓ for the glucuronidation of bilirubin with various concentrations of both UDPGA and bilirubin.

Key words: antipyrine, phenobarbitone, bilirubin, enzyme induction, glucuronosyltransferase.

Gilbert's syndrome represents the most common form of chronic unconjugated hyperbili- rubinaemia not associated with overt signs of haemolysis. The cause of this hyperbilirubinaemia has been postulated to be an abnormality in the hepatic uptake of bilirubin (Powell, Hemingway, Billing & Sherlock, 1967) but recently a significant reduction in the glucuronosyltransferase activity has been demonstrated in these patients (Black & Billing, 1969; Felsher, Craig

Correspondence: Dr A. Breckenridge, Department of Clinical Pharmacology, Royal Postgraduate Medical School, Du Cane Road, London W12 0HS.
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& Carpio, 1973). Glucuronosyltransferase is localized in the endoplasmic reticulum of the liver cell (Zeidenberg, Orrenius & Ernster, 1967) and drugs such as phenobarbitone, which are known to increase the activity of microsomal drug-metabolizing enzymes, have been successfully used to lower the serum bilirubin concentration (Trolle, 1968; Kreek & Sleisenger, 1968). However, patients taking phenobarbitone often complain of the sedation produced, at least in the early stages of treatment. We have shown that antipyrine (phenazone) is an inducer of hepatic microsomal drug-metabolizing enzymes in man and in animals; further, its administration is associated with few side-effects (Breckenridge, Orme, Thorgeirsson, Davies & Brooks, 1971). The aim of the studies reported here was to examine the effect of the administration of antipyrine on the serum bilirubin of patients with unconjugated hyperbilirubinaemia and to study the mechanism of the changes produced by experiments in the rat.

MATERIALS AND METHODS

Patients studied

Six male patients between the ages of 17 and 35 years were studied over a 3 month period. Each patient fulfilled the accepted criteria for the diagnosis of Gilbert's disease, namely an unconjugated hyperbilirubinaemia on at least four occasions over a period of 3 months, with normal serum transaminase and alkaline phosphatase concentrations, bromosulphthalein retention at 45 min, haemoglobin concentration and reticulocyte count. In two cases hepatic histology by light-microscopy was normal; in the other four patients no liver biopsy was performed. One patient (S.G.) was found to have hereditary spherocytosis 1 year before the study, with a high serum unconjugated bilirubin (2.4 μmol l⁻¹; 14 mg/100 ml) and increased erythrocyte osmotic fragility. Splenectomy was performed at that time and at the time of this study his erythrocyte osmotic fragility had been normal for 6 months, with a normal reticulocyte count, but his unconjugated serum bilirubin concentration remained elevated. Both this patient and patient S.B. noticed scleral icterus at the start of the study. No other patient, apart from patient R.G. who had non-specific upper abdominal pain unresponsive to antacids, had any symptomatic complaint. Clinical examination failed to reveal any abnormal clinical signs. Informed consent was obtained from these patients for the procedures involved.

Plan of studies

Each patient was seen once weekly at the same time of day (17.00–18.00 hours) and 15 ml of blood was taken by plastic syringe into a dry glass tube and allowed to clot. The blood samples were stored in the dark at 4°C overnight and the serum was separated next morning. Each sample was analysed within 4 h for both total and conjugated bilirubin and thus, by difference, the unconjugated bilirubin. Bilirubin was measured by an automated procedure with bilirubin standards used as described by Billing, Haslam & Wald (1971).

The patients were seen once weekly for 6 weeks and at the end of this time antipyrine was prescribed in a dose of 1.57 mmol (300 mg) twice daily for 8 weeks and weekly estimations of bilirubin were continued. The concentrations of total and unconjugated serum bilirubin over the last 6 weeks of this antipyrine treatment period were compared with the values over the 6 weeks of the control period and the results were analysed by the use of Student's t-test.

Animal studies

Antipyrine (0.42 and 1.27 mmol 24 h⁻¹ kg⁻¹) and phenobarbitone (0.34 mmol 24 h⁻¹ kg⁻¹)
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were administered by intraperitoneal injection to groups of 100 g male Wistar rats. Two doses of antipyrine were administered to rats since it has recently been shown that the degree of induction of the drug-hydroxylating enzymes is dependent on the dose of the inducing agent (Breckenridge, Orme, Davies, Thorgeirsson & Davies, 1973). Injections of 0.9% sodium chloride solution were given to control animals. The drug under study was given twice daily for 3 days and once on the fourth day. On the fifth day, the rats were killed by cervical dislocation. The livers were removed and washed in ice-cold 154 mmol/l KCl solution containing 0.02 mol/l Tris–HCl buffer, pH 7.4 (Tris–KCl buffer). The livers were homogenized in a Teflon–glass homogenizer with 4 volumes of ice-cold Tris–KCl buffer. The homogenates from individual rats were pooled so that each pool consisted of the livers from four rats which had received either phenobarbitone, antipyrine or NaCl injections.

Microsomal preparations were prepared by differential centrifugation as described previously (Davies, Gigon & Gillette, 1969) except that the microsomal pellet was washed once with ice-cold Tris–KCl buffer before recentrifugation at 156 000 g for 30 min.

Microsomal protein was determined by the method of Lowry, Rosebrough, Farr & Randall (1951). Each microsomal suspension was diluted to contain 5 mg of protein ml⁻¹ and was used immediately for the determination of enzyme activity.

Assay of enzyme activity

The assay of activity of bilirubin–uridine diphosphate glucuronosyltransferase (EC 2.4.1.17) was performed as described by Black, Billing & Heirweigh (1970) with minor modifications. The incubations were performed in a total volume of 990 μl consisting of 500 μl of the microsomal suspension (to give a final concentration of 2-5 mg of protein ml⁻¹), 300 μl of bilirubin–albumin (2-4:1) solution, 150 μl of a solution of uridine 5′-diphosphoglucuronic acid (UDPGA) and 40 μl of 0-125 mol l⁻¹ MgCl₂. Digitonin was not added to the incubations as an activating agent and no ammonium sulphamate was added to the diazotization reagent.

The molar extinction coefficient for the azo pigment of bilirubin was determined by the method of Van Roy & Heirweigh (1968) at 530 nm and was found to be 44.2 × 10³ mol⁻¹ cm⁻¹. From the molar extinction coefficient and the absorbance of the sample at 530 nm minus the blank value, the number of nmol of bilirubin conjugated 30 min⁻¹ mg⁻¹ of microsomal protein can be calculated.

Two sets of experiments were performed. In the first set of experiments, the concentration of bilirubin was kept constant at 256 nmol ml⁻¹ of incubate, and the concentration of UDPGA was varied to give final concentrations of 0-83, 1-24, 2-48, 4-96 and 7-46 μmol ml⁻¹ of incubate. After a preliminary experiment to establish the apparent Kₘ for UDPGA, separate experiments were performed with microsomal suspensions prepared from phenobarbitone- and antipyrine-treated rats and from control rats.

In the second series of experiments the concentration of UDPGA was kept constant at 7-46 μmol ml⁻¹ while the concentration of bilirubin was varied to give final concentrations of 57-0, 85-5, 120, 188, 221 and 256 nmol of bilirubin ml⁻¹ of incubate. After one preliminary experiment to establish the apparent Kₘ for bilirubin, sets of experiments were performed on separate occasions with hepatic microsomal suspensions from the rats treated with phenobarbitone, antipyrine (0.42 or 1.27 mmol 24 h⁻¹ kg⁻¹) and from control rats.

The apparent affinity constants (Kₘ) and apparent maximal velocities (Vₘₐₓ) for glucuronidation of bilirubin were calculated for each microsomal preparation in each experiment by
using the computer program described by Davies et al. (1969). Variations among the experiments were evaluated by Student’s t-test.

RESULTS

Human studies

Table 1 shows the total and unconjugated serum bilirubin concentrations in the control and antipyrine-treatment periods in the six patients. In each patient there was a highly significant

<table>
<thead>
<tr>
<th>Patient</th>
<th>Total</th>
<th>Unconjugated</th>
<th>Total</th>
<th>Unconjugated</th>
</tr>
</thead>
<tbody>
<tr>
<td>S.B.</td>
<td>0.50±0.07</td>
<td>0.43±0.06</td>
<td>0.23±0.03**</td>
<td>0.17±0.03**</td>
</tr>
<tr>
<td>S.C.</td>
<td>0.20±0.07</td>
<td>0.14±0.03</td>
<td>0.08±0.01**</td>
<td>0.07±0.01*</td>
</tr>
<tr>
<td>R.G.</td>
<td>0.25±0.04</td>
<td>0.19±0.04</td>
<td>0.11±0.02**</td>
<td>0.07±0.02**</td>
</tr>
<tr>
<td>S.G.</td>
<td>0.40±0.08</td>
<td>0.34±0.06</td>
<td>0.19±0.02**</td>
<td>0.14±0.03**</td>
</tr>
<tr>
<td>C.S.</td>
<td>0.20±0.02</td>
<td>0.18±0.01</td>
<td>0.12±0.02**</td>
<td>0.09±0.03**</td>
</tr>
<tr>
<td>P.W.</td>
<td>0.22±0.04</td>
<td>0.17±0.02</td>
<td>0.10±0.02**</td>
<td>0.09±0.02**</td>
</tr>
</tbody>
</table>

*P<0.01; **P<0.001.

fall in both the total and the unconjugated serum bilirubin concentrations during the 6 weeks of antipyrine therapy. Patients S.B. and S.G. both noted a disappearance of the scleral icterus. No patient noted any side-effects during the period of antipyrine administration.

Fig. 1 shows the time-course of the change in serum bilirubin in a typical patient (S.B.). The maximum effect was seen by the second week of antipyrine administration.

Animal studies

Microsomal protein content and liver weight. After pretreatment with antipyrine (0.42 mmol 24 h⁻¹ kg⁻¹) the mean liver microsomal protein content was 15.8±2.4 mg g⁻¹ of liver and after antipyrine, 1.27 mmol 24 h⁻¹ kg⁻¹, 17.9±2.6 mg g⁻¹ of liver. Neither of these values was significantly greater than control (14.1±2.9 mg g⁻¹; P>0.05). Liver weight was not increased by antipyrine administration (6.2±0.3 and 6.1±0.5 g 100 g⁻¹ body weight compared with 5.4±0.4 g 100 g⁻¹ body weight in the controls).

After phenobarbitone administration, there was a significant increase in both liver microsomal protein content and in liver weight. Mean liver microsomal protein content was 18.1±1.2 mg g⁻¹ of liver and mean liver weight 7.8±0.3 g 100 g⁻¹ body weight, both of which are significantly above control values (P<0.05).

Enzyme kinetics. Table 2 shows the results for the glucuronidation of bilirubin in vitro by the various rat liver microsomal preparations. In the experiments where the concentration of UDPGA was varied, there was a significant increase in the apparent Vₘₐₓ after both doses of antipyrine, from 15.4±0.4 to 23.7±0.8 nmol of bilirubin conjugated 30 min⁻¹ mg⁻¹ of
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Figure 1. Time-course of change in serum bilirubin (○, total and ■, unconjugated) in patient S.B. given antipyrine 3.2 mmol (600 mg) daily (1 μmol 1⁻¹ of bilirubin = 5.8 mg/100 ml).

The studies reported here show that in patients with Gilbert’s syndrome a significant reduction in the serum bilirubin occurs during treatment with antipyrine, without any side-effects such as are found with phenobarbitone. After the start of treatment the serum bilirubin falls quite rapidly so that the maximum reduction is achieved by 2 weeks and, after this time, the serum bilirubin remains relatively constant. Some patients who have more marked reduction in their bilirubin–glucuronosyltransferase activity are more markedly jaundiced (Arias, Gartner, Cohen,
<table>
<thead>
<tr>
<th>Drug</th>
<th>Dose (mmol 24 h(^{-1}) kg(^{-1}))</th>
<th>No. of experiments</th>
<th>Microsomal protein (mg g(^{-1}) of liver ± se)</th>
<th>Liver wt. (g 100 g(^{-1}) body wt. ± se)</th>
<th>(V_{max}) (nmol of bilirubin conjugated 30 min(^{-1}) mg(^{-1}) of protein ± se)</th>
<th>(K_m) (mmol 1(^{-1}) ± se)</th>
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</thead>
<tbody>
<tr>
<td><strong>(a) Variable UDPGA concentrations</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>—</td>
<td>6</td>
<td>14·1 ± 2·9</td>
<td>5·4 ± 0·4</td>
<td>15·4 ± 0·36</td>
<td>2·4 ± 0·3</td>
</tr>
<tr>
<td>Antipyrine</td>
<td>0·42</td>
<td>5</td>
<td>15·8 ± 2·4</td>
<td>6·2 ± 0·5</td>
<td>23·7 ± 0·82**</td>
<td>2·56 ± 0·32</td>
</tr>
<tr>
<td>Antipyrine</td>
<td>1·27</td>
<td>3</td>
<td>17·9 ± 2·6</td>
<td>6·1 ± 0·5</td>
<td>24·5 ± 1·39***</td>
<td>2·04 ± 0·22</td>
</tr>
<tr>
<td>Phenobarbitone</td>
<td>0·34</td>
<td>4</td>
<td>18·1 ± 1·2*</td>
<td>7·8 ± 0·3*</td>
<td>15·5 ± 0·38</td>
<td>2·57 ± 0·09</td>
</tr>
<tr>
<td><strong>(b) Variable bilirubin concentrations</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>—</td>
<td>5</td>
<td>14·4 ± 1·48</td>
<td></td>
<td>67·7 ± 10·5</td>
<td></td>
</tr>
<tr>
<td>Antipyrine</td>
<td>0·42</td>
<td>4</td>
<td>19·3 ± 1·48*</td>
<td></td>
<td>64·9 ± 3·2</td>
<td></td>
</tr>
<tr>
<td>Antipyrine</td>
<td>1·27</td>
<td>3</td>
<td>25·0 ± 4·28*</td>
<td></td>
<td>85·6 ± 20·0</td>
<td></td>
</tr>
<tr>
<td>Phenobarbitone</td>
<td>0·34</td>
<td>4</td>
<td>14·9 ± 0·49</td>
<td></td>
<td>62·3 ± 5·9</td>
<td></td>
</tr>
</tbody>
</table>
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Ben Ezzer & Levi, 1969). These patients, also referred to as Crigler–Najjar type II, have been treated successfully in the past with phenobarbitone. It is this group of patients who might derive benefit from antipyrine administration.

The studies in vitro of rat liver microsomal preparations reported here show that antipyrine caused an increase in the apparent $V_{\text{max}}$ for the glucuronidation of bilirubin but no change in the apparent $K_m$. These findings are interpreted as indicating an increase in the activity of bilirubin–glucuronosyltransferase without any change in the apparent affinity of the enzyme for either bilirubin or for UDPGA. It is of interest that increasing the dose of antipyrine caused no further increase in bilirubin glucuronidation. In contrast, a dose–response relationship has been shown with antipyrine-induced rates of drug oxidation (Breckenridge et al., 1973). In contrast, phenobarbitone caused no change in the activity of bilirubin–glucuronosyltransferase. This latter finding is in agreement with Potrepka & Spratt (1971), who found no rise in the activity of bilirubin–glucuronosyltransferase after the administration of phenobarbitone to guinea-pigs. Similarly, Wong (1972) found only very small increases (less than 10%) in the activity of bilirubin–glucuronosyltransferase in rat liver after treatment of the animal in vivo with phenobarbitone. Phenobarbitone caused an increase in both the liver microsomal protein content and liver weight, whereas antipyrine caused only an increase in specific enzyme activity.

If these results can be extrapolated to the situation in man, they suggest that the fall in serum unconjugated bilirubin seen after treatment with phenobarbitone may not be due to an increase in the activity of bilirubin–glucuronosyltransferase, as has been suggested by Black & Sherlock (1970). It is known that administration of phenobarbitone may cause several other changes relevant to this situation, e.g. an increase in liver blood flow (Ohnhaus, Thorgeirsson, Davies & Breckenridge, 1971), an increase in bile flow (Klaassen & Plaa, 1968) and an increase in organic anion hepatic binding protein (Reyes, Levi, Gatmaitan & Arias, 1969), all of which may contribute to changes in serum bilirubin. Antipyrine would appear to exert its effect by causing a specific increase in enzyme activity, whether or not it causes these other effects; in patients with low bilirubin–glucuronosyltransferase activity, it would appear to be a more acceptable as well as a more logical form of treatment than phenobarbitone.

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