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STUDIES ON THE BILIARY EXCRETION MECHANISMS OF DRUGS—II

BILIARY EXCRETION OF THIAMPHENICOL, CHLORAMPHENICOL AND THEIR GLUCURONIDES IN THE RAT*

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Abstract—Thiamphenicol (TP) or chloramphenicol (CP) administered intravenously (14 μmoles) to rats with ligated renal pedicles is rapidly excreted in bile mostly as the glucuronide (about 23 and 75 per cent in 7 hr respectively). Increasing the dose of either drug does not result in an increased excretion of glucuronide, indicating that the excretion process is saturable. TP glucuronide (TPG) or CP glucuronide (CPG) administered intravenously to rats with ligated renal pedicles is rapidly excreted into bile in high concentration as unchanged glucuronide. The maximal excretion rate of TPG or CPG (about 14·0 and 18·0 μmoles/10 min respectively) when each glucuronide (100 μmoles) was administered is much higher than that (about 5·1 and 8·5 μmoles/10 min respectively) when each aglycone (200 μmoles) was administered. The results suggest that the transport maxima (Tms) for the biliary excretion of TP and CP are due to a saturation of the conjugating process. CPG used in this study is isolated by a new method.

INVESTIGATORS have reported that several organic acid compounds, such as sulfobromophthalein, penicillin and the bile acids, are transported into bile against concentration gradients, that the transfer process is saturable, and that certain acids compete for transfer. These observations indicate that the process involved is one of active transport.[1,2]

Certain endogenous compounds, such as bilirubin and thyroxine, and a large number of foreign compounds are known to be excreted in the bile as glucuronides.[3,4] These glucuronides may be actively secreted into bile by the same mechanism that transports a variety of organic acids. In general, relatively little is known about the biliary excretion mechanism of glucuronides, because it is difficult to obtain them in a pure crystalline form. Thiamphenicol glucuronide (TPG) has been isolated in pure crystalline form from urine and bile of guinea pigs, and chloramphenicol glucuronide (CPG) has been successfully isolated in pure crystalline form from human urine, using a new method described in this report. The main purpose of this study, however, was to investigate the hepatic excretion mechanisms of

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these glucuronides after administration of thiamphenicol (TP) and chloramphenicol (CP) in rats.

METHODS*

Drugs. TP, m.p. 165–166°, was recrystallized from water, and CP, m.p. 150–151°, was the commercially available product. UDP-glucuronic acid was obtained from Sigma Chemical Co. TPG, m.p. 190–192°, was isolated from guinea pig bile and urine. The isolation procedure will be reported in detail elsewhere. CPG was isolated by the method described below.

Isolation of CPG. CPG was isolated from human urine. Five male volunteers each took 2 g CP orally and their urine was collected for 12 hr. The urine was adjusted to pH 4 with HCl. Activated charcoal (200 g) was added to the urine and the mixture was stirred for 12 hr at 4°. Then the charcoal was filtered, washed with water (500 ml), and dried under reduced pressure at room temperature. The metabolite was eluted from the charcoal with methanol (2 liters) containing 0·1% (v/v) of 28% NH_4OH. The eluate was evaporated to dryness under reduced pressure at 45°. The residue was then dissolved in water (100 ml). The solution was adjusted to pH 4 and passed through an adsorbent column (3 × 40 cm; Amberlite XAD-2). After a washing with water (200 ml), the metabolite was eluted with methanol containing 0·1% (v/v) of 28% NH_4OH. The eluate was evaporated to dryness under reduced pressure at 45° to give crude ammonium chloramphenicol glucuronide. The salt solution in methanol was banded on thin-layer plates (40 × 40 cm) of Silica gel (Merck, Kieselgel HF_{254}) and developed in $CHCl_3$–methanol–AcOH–H_2O (10:4·5:1:1). The spot corresponding to the glucuronide gave a purple color when sprayed with naphthoresorcinol and a yellow color with stannous chloride followed by *p*-dimethylaminobenzaldehyde. The band corresponding to the glucuronide (identified by examining the plate under ultraviolet light, 254 nm) was scraped from the plates and eluted with methanol. The eluate was evaporated to dryness under reduced pressure to give the ammonium salt of chloramphenicol glucuronide, which was 80 per cent pure. This ammonium salt was converted to the sodium salt in accordance with a general method, and the salt recrystallized from absolute ethanol and petroleum ether; the salt, m.p. 200–210°, was 99 per cent pure. The amounts of glucuronic acid[5] and chloramphenicol in the sodium salt of the glucuronide were determined. The glucuronic acid/CP molar ratio was 1·0. The ultraviolet absorption in methanol showed a peak at 274 nm as observed with CP (274 nm). From these results, this glucuronide could be considered to be the same compound as CPG isolated by Glazko *et al.*[6]

Procedure in animals. Male Wistar rats, weighing 340–360 g, were anesthetized by an intraperitoneal injection of sodium pentobarbital (40 mg/kg). Through an abdominal incision, the renal pedicles were ligated and the bile duct was cannulated with polyethylene tubing. After the incision was closed, bile was collected for a 10 min period prior to an intravenous injection (femoral vein) of TP (100–200 μmoles) CP (100–200 μmoles), or their glucuronides (100 μmoles). Each drug was injected over a 5-min period, and bile was collected for 60 min at 10-min intervals. In a few longer-term experiments, bile was collected for seven consecutive 60-min periods in

* All melting points were uncorrected. Ultraviolet spectra were obtained with a Hitachi model 3T spectrophotometer. A Hitachi 124 spectrophotometer was used for the determination of drugs.

rats in which the renal pedicles were not ligated. Since it has been shown that hypothermia develops under the conditions of these experiments,[7] a heating lamp was used over each animal in order to maintain body temperature at 38°.

Enzyme assay. Male Wistar rats, weighing 210–230 g, were used. Animals were decapitated, and their livers were removed and homogenized with cold isotonic KCl. An incubation mixture was prepared containing 25% homogenate from 250 mg liver, 1 μmole UDP-glucuronic acid, 0·33 μmole substrate and 200 μmoles Tris (hydroxymethyl) aminomethane buffer, pH 7·4, to a final volume of 3 ml. The mixture was incubated for 30 min at 37°. After the reaction, the mixture was cooled in ice-water, and 1 ml of 5% $NaHCO_3$ was added to it. The amount of glucuronide formed was determined by measuring the concentration of the unchanged drug as described below.

Determination of TP. Unchanged TP was extracted with 20 ml ethyl acetate for 10 min. After the tube had been centrifuged for 5 min, 15 ml of the organic phase was transferred to a flask and evaporated to dryness under reduced pressure. The residue was dissolved in 5 ml of 0·2 N NaOH, heated in a boiling water bath for 10 min, and then cooled to room temperature. One ml of 1 M NaH_2PO_4, 5 ml ethylene dichloride and 2 ml of 0·5% $NaIO_4$ solution were added to the mixture and the flask was shaken mechanically for 10 min. After centrifugation, 4 ml of the organic phase was treated by the procedure described in a previous paper.[8] The absorbance of the color obtained was measured at 415 nm.

Determination of unchanged CP. Unchanged CP was extracted from the reaction mixture with 20 ml ethylene dichloride. The extraction was carried on for 10 min mechanically and 15 ml of the clear organic phase was evaporated to dryness under reduced pressure. The residue was dissolved in 5 ml of 0·2 N NaOH and reduced by the addition of 0·5 ml of 2% hydrosulfite solution. After 30 min, the reaction was stopped by the addition of 1 ml of 4 N HCl; then the mixture was shaken mechanically with 5 ml ethyl acetate for 5 min. After centrifugation, 5 ml of aqueous phase was transferred into a test tube and 0·5 ml of 1% $NaNO_2$ solution, 1 ml of 1·5% ammonium sulfamate solution and 0·5 ml of 0·2% Tsuda's reagent[9] were added to the tube. The mixture was kept in a water bath at 50° for 30 min and then cooled to room temperature. The absorbance was measured at 558 nm.

Determination of drugs in bile. TP and TPG were measured by the colorimetric assay technique described previously.[8] CP and CPG were measured by the method of Glazko *et al.*[10]

Statistical analysis. Statistical comparisons were made using Student's *t*-test. Unless otherwise specified, the term significant is taken to mean a P value of less than 0·05.

RESULTS

Recovery of TP, CP and their glucuronides in bile. During 7 hr after a single dose of CP, 75·1 ± 1·9 per cent of the administered CP was excreted in the bile of rats with intact kidneys. In these animals, 96·8 ± 3·5 per cent of the total amount recovered in the bile was a glucuronide, and little unchanged CP was excreted into bile, as shown in Table 1.

On the other hand, 23·0 ± 0·9 per cent of the administered TP was excreted in

TABLE 1. BILIARY EXCRETION OF TP, CP AND THEIR METABOLITES AFTER ADMINISTRATION OF TP AND CP IN RAT

Drug*	% Dose excreted in 7 hr	Excretion of metabolites† Unchanged (%)	Conjugated (%)	Arylamines (%)
TP	23·0 ± 0·9	7·4 ± 0·1	92·6 ± 4·0	
CP	75·1 ± 1·9	0·0	96·8 ± 3·5	3·2 ± 0·3

* Drugs, 14 μmoles, were given intravenously. Results are expressed as mean ± S.E. for four to six animals.

† Excretion is given as the per cent of total metabolites excreted.

the bile. Of the total amount recovered in the bile, 92·6 ± 4·0 per cent was a glucuronide, and 7·4 ± 0·1 per cent was unchanged TP.

Saturation of the excretion process of glucuronides. Evidence that TPG and CPG are excreted by a saturable transport process was obtained by measuring the excretion rates at different doses of TP and CP. When the dose of CP was increased from 100 to 150 μmoles, the maximal excretion rate (μmoles/10 min) of CPG increased from 5·9 ± 0·2 to 8·5 ± 0·3, as shown in Fig. 1. Moreover, when the dose was increased to 200 μmoles, the maximal excretion rate of CPG did not increase further; at these doses there is no significant difference in the amount of CPG excreted (44·1 for 150 μmoles vs 42·2 μmoles/60 min for 200 μmoles). This indicates that the CPG synthesizing and/or transport process is saturated at high doses.

Figure 2 shows the maximal excretion rates (μmoles/10 min) of TPG at the various doses of TP. When the dose of TP was increased to 175 μmoles or more, an apparen

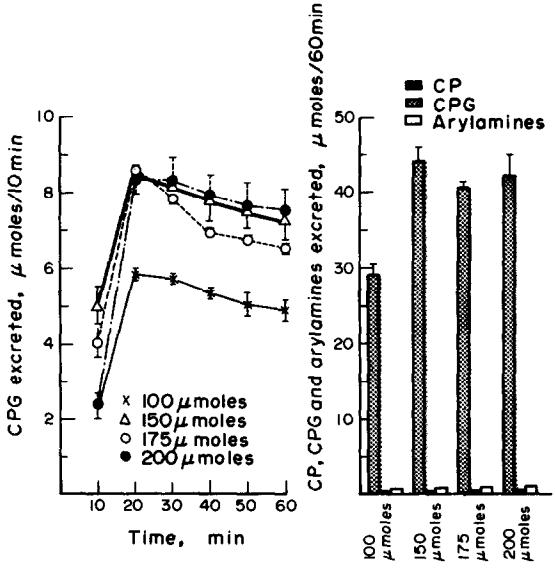

FIG. 1. Biliary excretion of CP metabolites at various doses of CP in rats with ligated renal pedicles. Drug were administered intravenously. Results are the means of four experiments. The bracketed vertical line show the standard error of the mean.

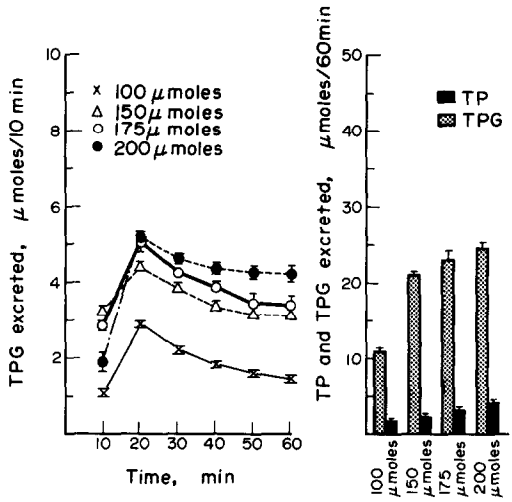

FIG. 2. Biliary excretion of TP metabolites at various doses of TP in rats with ligated renal pedicles. Drugs were administered intravenously. Results are the means of four experiments. The bracketed vertical lines show the standard error of the mean.

transport maximum (Tm) of TPG was obtained at a second 10-min time period; at doses of 175 and 200 μmoles, there was no significant difference in the maximal excretion rates of TPG (5·0 vs 5·1 μmoles/10 min). Furthermore, at these two doses, there was no significant difference in the total amount of TPG excreted in 60 min (23·1 vs 24·6 μmoles/60 min). This indicates that the TPG synthesizing and/or transport process is also saturated at high doses.

Maximal excretion rates of TPG and CPG after administration of TPG and CPG. It was then investigated whether the maximal excretion rates of these glucuronides

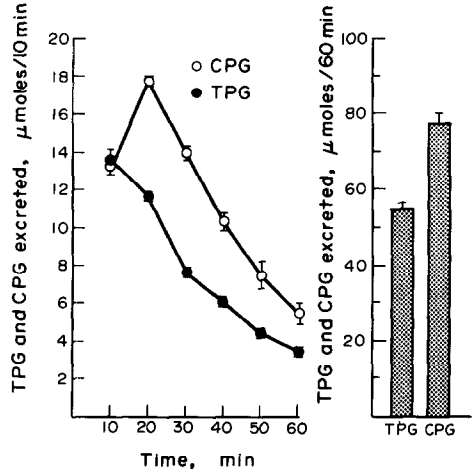

FIG. 3. Biliary excretion of TPG and CPG after administration of TPG and CPG in rats with ligated renal pedicles. Drugs, 100 μmoles, were administered intravenously. Results are the means of four experiments. The bracketed vertical lines show the standard error of the mean.

were determined by saturation of the glucuronide synthesizing process or by saturation of the glucuronide transport process. When 100 μmoles CPG or TPG was administered intravenously to rats with ligated renal pedicles, large amounts of the drug readily appeared in the bile. The maximal excretion rates of CPG and TPG were observed during the second 10-min time period after drug administration, as shown in Fig. 3. The maximal excretion rates of CPG and TPG were 17·9 ± 0·3 (μmoles/10 min) and 13·6 ± 0·6 (μmoles/10 min) respectively. These rates are considerably higher than the Tm obtained when CP or TP was administered. Moreover, the amount of CPG excreted in 60 min was considerably more than that obtained after a dose of 200 μmoles CP (78·7 vs 42·2 μmoles/60 min). The amount of TPG was also considerably more than that obtained after a dose of 200 μmoles TP (55·1 vs 24·6 μmoles/60 min). These findings suggest that the Tm observed after CP or TP administration was not due to saturation of the glucuronide transport process but to saturation of the glucuronide formation process.

TABLE 2. RATES OF GLUCURONIDE SYNTHESIS BY RAT LIVER HOMOGENATE WITH TP AND CP AS ACCEPTOR*

Acceptor	Amount (μmoles)	Acceptor conjugated (μmoles/g wet liver wt.)
TP	0·33	0·056 ± 0·006 (7)
CP	0·33	0·245 ± 0·003 (9)

* Results are given as mean ± S.E. The number of animals used is shown in parentheses.

Formation of glucuronide of TP and CP by rat liver homogenate. Conjugation of CP in liver homogenate of male rats is a considerably more rapid process than that of TP (Table 2); the rate of CP conjugation is about 4·4 times higher than that of TP conjugation. These observations suggest the possibility that the rapid rate of CP conjugation is one factor responsible for the high Tm and the high amount of biliary excretion of CPG when CP is administered to rats.

DISCUSSION

It is known that certain endogenous compounds, such as bilirubin and thyroxine, and a large number of foreign compounds are excreted in the bile as glucuronides. However, relatively little is known about the biliary excretion mechanism of the glucuronides.

Organic compounds having carboxylic or sulfonic groups are in general excreted actively into bile by a common process in rats.[1,2,11] In the present study, CP and TP were excreted into rat bile in large quantities, mostly as the glucuronide. Since CPG and TPG are organic acids, it may be anticipated that they are also actively excreted into bile. Furthermore, it may be anticipated that the glucuronides are excreted in the bile by a saturable process. In this investigation, a maximal excretion rate (8·6 ± 0·3 μmoles/10 min) of CPG was observed after administration of CP in doses greater than 150 μmoles. A maximal excretion rate of TPG (5·1 ± 0·1 μmoles/10 min) was observed after administration of TP in doses greater than 175 μmoles. These data

are shown in Figs. 1 and 2. On the other hand, the maximal excretion rates of CPG and TPG (about 18·0 and 14·0 μmoles/10 min respectively) obtained after administration of isolated CPG and TPG, 100 μmoles, were observed to be much higher than those obtained after CP and TP administration. This result might lead to the assumption that the Tms seen in Figs. 1 and 2 were due to the glucuronide conjugating process. In a study of the biliary excretion of phenolphthalein, 4-methylumbelliferone and 8-hydroxychinoline, Mulder[12] suggested that the rate-limiting step in the biliary excretion of the compounds was not the conjugation process but the transport process of the formed glucuronides. Our results indicate that the transport maxima for the biliary excretion of the glucuronides of such compounds may be nearly equal to or less than those obtained when the aglycones are administered. Actually, in our laboratory, it was observed that the Tm of phenolphthalein glucuronide obtained after its aglycone was administered was nearly equal to that obtained after the glucuronide was administered to rats with ligated renal pedicles (unpublished results). It is considered, however, that more detailed studies using glucuronides themselves are necessary for the elucidation of the biliary excretion mechanism of glucuronides.

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