Comparative Metabolism of Chloramphenicol in Germfree and Conventional Rats

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The action of gut microflora on the metabolism of chloramphenicol (CP) was studied in germfree (GF) and conventional (CV) rats after administration of single oral doses of tritiated CP. There were similarities in the metabolic pathways of CP in the GF and CV animals, i.e., rapid absorption, hepatic glucuroconjugation, and biliary excretion of the CP conjugate. CP, CP-oxamic acid, CP-alcohol, and CP-base were present in similar proportions in the urine of both GF and CV rats. Differences observed included the slow elimination of total radioactivity and a reduced proportion of the urinary excretion versus the fecal excretion in the GF rats and different patterns of urinary and fecal metabolites for the two groups. Reduction products which were present in much greater quantities in the urine and feces of CV rats are compatible with the generally described hydrolysis of the CP-glucuronide, followed by a nitroreduction of the CP by the gut microflora and the reabsorption of a part of the products formed. In GF rats, CP-glucuronide was the major fecal metabolite, a portion of it having been reabsorbed and excreted in the urine. Although in lesser amounts, reduction products were still present as urinary metabolites in GF rats. Such a reduction in the tissues might produce active intermediate that could be related to CP toxicity.

Chloramphenicol (CP) is one of the oldest and most potent antibiotics, but its use is limited because of its toxic side effects. CP produces two types of adverse effects on bone marrow-derived cells: a dose-related reversible suppression of erythropoiesis due to the inhibition of mitochondrial protein synthesis (1a), and a rare dose-independent idiosyncratic response resulting in aplastic anemia. The mechanism inducing this latter adverse effect is still unknown. Yunis (19) provided data on inherited susceptibility of DNA synthesis to CP or one of its metabolites. Several researchers, such as Weisburger et al. (17) and more recently Corbitt and Chipko (3), have suggested that CP-induced aplastic anemia may be due to CP reduction metabolites. In humans and rats, reduction of the nitro moiety of CP in vivo produces arylamine or acetylated arylamine. Although potentially toxic intermediate reduction products have not yet been isolated or identified, they are assumed to be metabolites and have been synthesized and tested for toxicity by Pazdernik and Corbitt (11) and more recently by Yunis et al. (20). The latter authors emphasized the role of the p-NO2 group as the determining structural feature in the pathogenic mechanism of aplastic anemia induced by CP. They clearly showed the high toxicity of the synthetic hydroxylamine and nitroso derivatives. These are much more potent inhibitors of DNA synthesis than CP and cause irreversible stem cell damages.

Since the first experiments of Glazko et al. (7), it was evidenced that the nitroreductase activities of some isolated microorganisms (14), as well as of the whole gastrointestinal microflora, may reduce the CP molecule. Goldman (8) and Scheline (13), reviewing drug-gut microflora interactions, related CP toxicity to the formation of reduction products in the gut. However, direct in vivo evaluation of the specific role of nitroreductase activity in the gut flora, in connection with other metabolic CP reduction processes, has not yet been made. Moreover, to be related to the CP toxicity mechanism, the role of the gut flora has to be integrated into the metabolic fate of CP in the animal, namely, the absorption, elimination, and biotransformation processes with which it may interact.

The purpose of this work was to determine the exact role of the gut flora on the metabolism of CP by using conventional (CV) and germfree (GF) rats. The identification and quantitation of the major metabolites (not limited to the reduction products) and their elimination kinetics in the urine and feces were thus performed in both types of animals.

Although some of the main metabolic pathways in CV rats were described several years ago by Glazko (5) and then completed by identi-
fication of some new metabolites (4, 15), we achieved a more complete metabolic pattern in CV and GF rats by using a high-specific-activity tritiated CP molecule, in association with analysis by high-pressure liquid chromatography (HPLC) of the different urinary and fecal extracts.

MATERIALS AND METHODS

Reagents and apparatus. CP (B-grade) purchased from Calbiochem was used in all stages of the experiments.

All solvents used were analytical grade and came from E. Merck AG. HPLC was performed on a Spectra-Physics model SP 3500 B chromatograph equipped with a 280-nm detector and a stainless steel column (250 by 4.6 mm [inner diameter]) packed with Merck RP-18 (5 μm). Fractions were collected on a Gilson microcollector.

Radioactivity measurements were performed in an SL 32 liquid scintillation spectrometer (Intertechnique), using Aqualuma (Kontron) as a scintillation mixture. [1-3H]CP (185 mCi/mmol) was synthesized by reduction of the corresponding ketone derivative of CP with NaBH₄, in accordance with Pohl and Krishna (12), and the d-threo isomer was then purified by HPLC. The stability of the labeling was then regularly controlled by HPLC during storage.

Animals, drug administration, and collection of samples. The animals used were male OFA, 6-week-old rats (IFFA CREDO). Four GF rats were maintained in a plastic Trexler-type isolator. Four CV rats were housed in another isolator and maintained in exactly the same conditions as the GF ones. Each rat was kept in an individual stainless steel cage inside the isolator, and fed ad libitum a commercial diet (Usine d’Alimentation Rationelle) sterilized by irradiation (4 Mrad). They were adapted to these conditions for 2 weeks before starting the experiment. The sterility of the GF rats was checked every day during the experiment.

Each rat received by oral intubation 0.7 ml of a solution of 1,2-propanediol containing 7 mg of CP and 85.7 μCi of labeled CP, sterilized by passage through a membrane filter (0.22-μm pore size; Millipore Corp.).

Feces and urine were collected from each rat for 4 and 3 days, respectively. The feces were ground and hydrolyzed overnight at 90°C with 2 N HCl. After centrifugation, the filtered supernatant was measured for its radioactivity and then lyophilized. The urine was measured for its total radioactivity and then lyophilized. Tritiated water was determined by counting urine and feces portions before and after lyophilization and combustion in an automatic oxidizer apparatus (Oxymat, Intertechnique).

Urine and feces analysis. The lyophilized urine was solubilized in 1 ml of 0.1 N HCl. Extraction of the acid urine was carried out by shaking three times with 30 ml of ethyl acetate. The organic phase was evaporated to dryness under vacuum, and the residue was dissolved in a 1-ml water-methanol (1:1) mixture; 10 μl was chromatographed on the octadecyl reverse-phase column, using methanol–0.05 M Na₂HPO₄ (30:70), pH 5.3, as mobile phase at a 0.6-ml/min flow rate. Fractions (150 μl) were collected in disposable 3-ml scintillation counting tubes with a microcollector. After addition of 2.5 ml of the fluor mixture, each fraction was counted in a scintillation spectrometer. Results were computerized and read out as diagrams constituting metabolic profiles.

The residual urine aqueous phase was neutralized (pH 7.0) by using 1 N sodium hydroxide. Ion-pair HPLC was performed on the same octadecyl reverse-phase column, using methanol–0.05 M heptane sulfonic acid sodium salt in distilled water (30:70), adjusted to pH 3.3 with H₃PO₄, as mobile phase. Fraction collection and radioactivity measurements were operated as described for simple reverse-phase analysis.

The lyophilized fecal extracts were dissolved in 1 ml of a water-methanol (1:1) mixture, and 10 μl was chromatographed on the normal reverse-phase column as described for the organic phase from urine.

The quantitation was performed by summation of the radioactivity in the fractions belonging to the same peak. When the separation was incomplete, the quantitation of each peak was obtained by summation of the well-separated fractions and distribution of the common fraction in proportion to peak heights. Recovery rates were evaluated by comparison with the total radioactivity injected into the chromatograph. Analyses were performed on urine and feces samples every day and for each rat individually.

The peaks were identified at once from the HPLC fractions, using a combination of separative techniques (thin-layer chromatography or gas-liquid chromatography) and structural analysis (mass spectrometry) as previously described (1).

RESULTS

The kinetics of the urinary and fecal elimination of total radioactivity (CP and metabolites) are presented in Table 1. Tritiated water represented about 8.5 and 18.5%, respectively, of the total radioactivity excreted in the urine and feces of CV or GF rats.

The urinary metabolites that were isolated (Fig. 1) were identified as CP, CP-oxamic acid, CP-glucuronide conjugate, CP-base, CP-alcohol, CP-arylamine, and CP-acetylarylamine (Fig. 2). The fecal metabolites identified after hydrolysis were collected in two major fractions. One, identified as CP-base, may result either from CP, CP-alcohol, CP-oxamic acid, CP-base, or CP-glucuronide that is supposed to be present in fresh feces. The second one gathers as CP-base alylamine all the reduction products of CP present in the fresh feces. About 10 and 25% of the total radioactivity excreted in the CV and GF rat feces, respectively, remained unidentified.

The proportions of each metabolite excreted in the urine and feces of both CV and GF rats, expressed as a percentage of the total daily urinary or fecal radioactivity, are given in Table 2. The sum of the radioactivity of the acidic extract plus the residual aqueous phase was found to be equal to 97.6 ± 5.6% of the radioactivity of the original urine. On the other hand,
TABLE 1. Daily excretion of radioactivity in the urine and feces of CV and GF rats*  

<table>
<thead>
<tr>
<th>Day</th>
<th>Urine Radioactivity (mean % of dose ± SD)</th>
<th>Feces Radioactivity (mean % of dose ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CV</td>
<td>GF</td>
</tr>
<tr>
<td></td>
<td>GF</td>
<td>CV</td>
</tr>
<tr>
<td>1</td>
<td>19.8 ± 1.2</td>
<td>11.6 ± 1.8</td>
</tr>
<tr>
<td>2</td>
<td>2.6 ± 0.2</td>
<td>2.2 ± 0.5</td>
</tr>
<tr>
<td>3</td>
<td>1.0 ± 0.3</td>
<td>1.4 ± 0.3</td>
</tr>
<tr>
<td>4</td>
<td>0.9 ± 0.3</td>
<td>4.5 ± 0.5</td>
</tr>
<tr>
<td>Total</td>
<td>23.3 ± 0.9</td>
<td>15.2 ± 1.2</td>
</tr>
</tbody>
</table>

* Determined after administration of a single oral dose of 85.7 μCi of [1-3H]CP in 7 mg of CP.

The sum of the radioactivity of the peaks corresponding to identified and unidentified metabolites was equal to 96.9 ± 1.3% of the radioactivity for each extract.

DISCUSSION

CP is well known to be absorbed from the gut of the rat and then conjugated by the liver to CP-glucuronide. This compound is excreted in the bile and readily hydrolyzed by the gut microflora β-glucuronidase, resulting in free CP. Part of it is reabsorbed, whereas a large amount is reduced to aromatic amines by bacterial action, with subsequent reabsorption of a portion of these products. However, since this original metabolic scheme was established by Glazko (5), improved analytical methods have been developed which determine specifically CP-arylamine and CP-acetylarylamine among the reduction products and identify CP-oxamic acid (15), CP-base, and CP-alcohol (4) as new urinary metabolites.
TABLE 2. Patterns of urinary and fecal CP metabolites in CV and GF rats.*

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Location</th>
<th>CV rats* (Total Radioactivity of total urine or feces ± SD)</th>
<th>GF rats* (Total Radioactivity of total urine or feces ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Day 1</td>
<td>Day 2</td>
</tr>
<tr>
<td>CP-oxamic acid</td>
<td>Urine</td>
<td>25.1 ± 0.2</td>
<td>24.1 ± 0.9</td>
</tr>
<tr>
<td>CP-glucuronide</td>
<td>Urine</td>
<td>6.8 ± 0.4</td>
<td>6.6 ± 0.3</td>
</tr>
<tr>
<td>CP-alcohol</td>
<td>Urine</td>
<td>8.3 ± 0.2</td>
<td>7.7 ± 0.0</td>
</tr>
<tr>
<td>CP-acetylamylamine</td>
<td>Urine</td>
<td>18.1 ± 0.3</td>
<td>18.2 ± 0.3</td>
</tr>
<tr>
<td>CP-base</td>
<td>Urine</td>
<td>15.6 ± 0.2</td>
<td>13.9 ± 0.4</td>
</tr>
<tr>
<td>Feces (after hydrolysis)</td>
<td></td>
<td>3.8 ± 0.8</td>
<td>3.3 ± 0.0</td>
</tr>
<tr>
<td>CP-base arylamine</td>
<td></td>
<td>88.1 ± 2.6</td>
<td>86.1 ± 1.4</td>
</tr>
<tr>
<td>CP-oxamic acid</td>
<td></td>
<td>5.5 ± 0.2</td>
<td>4.9 ± 0.0</td>
</tr>
</tbody>
</table>

An extensive discussion of the analytical methods used for the urine can be found in another paper (1), but extractions and analyses carried out for urine were not applicable as such for feces. In spite of the possible degradation of some of the metabolites, we completed an acidic hydrolysis before the analysis. It was checked after complete hydrolysis to ascertain that all of the metabolites with a nitro moiety were gathered in one peak (CP-base) and that all of the reduced metabolites were in another well-separated peak (CP-base arylamine). Except for one peak corresponding to an unidentified metabolite(s) in the feces of GF rats (25.5% of the total fecal radioactivity), little radioactivity remained unexplained or was lost over the whole analytical procedure.

The results in Table 1 show that fecal excretion is slower in GF rats than in CV rats. This is a classical observation, related to a slow passage time in the GF animal (9). On the other hand, the proportion of the total radioactivity excreted daily in urine versus that in the feces was higher in CV than in GF rats. This result is in accordance with observations by Glazko et al. (6) of a rapid cecal reabsorption of CP and aromatic amines in CV rats, whereas the intact glucuronide conjugate does not readily pass through the intestinal mucosa but reaches the cecum to be excreted in the feces with no bacterial degradation occurring in the GF animals. This specific point concerning the absorption and routes of elimination of the CP-glucuronide will be discussed below with the data obtained from the GF rats. All the radioactivity excreted, as analyzed in Table 2, was attributable to specific compounds (except for one fecal peak in the GF rats). The same metabolites were present in both GF and CV rats in relative proportions shown in Table 2. As described previously for rats (15), CP-oxamic acid appeared to be the major urinary metabolite in GF rats also. CP-base, CP-alcohol, and CP-glucuronide were present in urine from GF rats as well as in that of CV rats, but in higher daily proportions, particularly at days 2 and 3. The difference might be due to the high proportion of reduction products, CP-arylamine and CP-acetylamylamine, in CV urine. The main difference between GF and CV rats was in the amounts of these amines in feces and urine (Table 2). The urinary excretion of the reduced metabolites in GF rats was slower than in CV rats, but the total amount was similar at the end in both groups. In addition, a high proportion of free CP in the urine from GF rats appeared during days 1, 2, and 3. This raised the questions of the existence, extent, and localization of glucuron conjugation in GF rats as well as of the elimination routes and possible reabsorption of the CP glucuronide in relation to the
significant amount of CP glucuronide in urine from GF rats at day 3. Thus, complementary experiments were carried out. [1-3H]CP or [1-3H]CP-glucuronide was administered intraduodenally under halothane anesthesia to GF rats. In a first experiment, portal blood was collected after 30 min. In a second experiment, arterial blood, urine, and bile were collected for 1 h after administration. It was thus demonstrated that, when administered in the duodenum, CP was absorbed unchanged, as only one radioactive peak of CP was observed in the portal blood. Moreover, CP was then rapidly and almost completely glucuron conjugated in the liver and then mainly excreted in the bile (99% of the radioactivity absorbed) as CP-glucuronide (96% of the bile radioactivity). A much smaller proportion of the absorbed radioactivity was found in the arterial blood and corresponded to both CP and glucuronide. On the other hand, when CP-glucuronide was administered under the same conditions, it was absorbed, although to a lesser extent than CP (1/10), without being hydrolyzed by the intestinal cells. CP-glucuronide was the only compound found in the portal blood; no CP was observed. This observed CP-glucuronide was excreted mainly in the bile (93% of the absorbed radioactivity) and partly in the urine. These results confirm the data obtained during the metabolic study on conscious animals (for a longer period) and suggest the following scheme. Absorption of CP and hepatic glucuron conjugation is followed by a major biliary excretion of the CP-glucuronide, which is believed to occur in the same way and to the same extent in both GF and CV rats. In the CV animals, as classically described, the CP-glucuronide is then hydrolyzed and reduced by the gut microflora. CP and its reduction products are partly excreted in the feces or reabsorbed, being recycled and CP reduction products being excreted in the urine. In GF rats, the major part of the CP-glucuronide could be excreted in the feces and appear as CP-base, whereas a significant part is thought to be reabsorbed and then excreted in the bile and urine.

As assumed by Glazko et al. (7) and by several authors (10, 14) on the basis of in vitro experiments, this in vivo metabolic study of GF rats confirms the major role of the nitroreductase activity of the gut microflora in the formation of CP reduction products. This could be an argument for the hypothesis of a direct role of the gut microflora in CP toxicity (8). At the same time, however, this comparative study shows that, even though in much lesser amounts, reduction products are still present in GF rats, confirming previous in vitro observations on tissue nitroreductase activity toward CP (5, 18) and giving the first in vivo evaluation of the extent of this phenomenon. As CP-induced aplastic anemia is described as being dose independent and as no data are available about the CP toxicity on GF animals as compared to CV ones, the question is raised of the pharmacological and toxicological significance of such a local nitroreduction besides the main bacterial action. The reduction products, namely CP-arylamine and CP-acetylarylamine, must be considered end products of a reductive process involving nitroso and hydroxylamine derivatives as highly reactive intermediary metabolites (3). These metabolites, which cause in vitro irreversible damage to the DNA synthesis process in bone marrow cells (20), can be assumed to be toxic only if they are produced locally by the target cells or tissues. If formed in the digestive tract, they are rapidly inactivated and eliminated. According to Yunis et al. (20), nitroreduction could be an important step in the activation of some carcinogens and mutagens. The authors stated that it is highly probable that, in the predisposed subject, CP undergoes an extensive cellular nitroreduction, leading to mutagenicity and ultimately to aplastic anemia. The present study gives direct in vivo evidence of nitroreduction by tissues and supports the assumption of Yunis et al. Unfortunately, the nitroso derivative has not been identified as such in feces or urine, even in CV rats, because of either its high reactivity and very short lifetime in the intestinal lumen or the efficiency of the microbial reductases that lead rapidly to amines. On the other hand, in some species, especially humans, CP is not excreted in the bile, and thus no action of the gut microflora is to be expected if CP is not administered orally. However, aplastic anemia has been described after parenteral administration of CP (2, 16). Thus, the formation of active intermediates such as CP reduction products in target cells or tissues could contribute to the explanation of CP toxicity in bone marrow cells. The GF rat then offers an excellent experimental tool to determine the respective roles of gut microflora and host tissue nitroreductase activity and to study the pharmacological significance and mechanism of CP reduction and reduced metabolites-cell interactions.

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LITERATURE CITED


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