Metabolism of \( p \)-aminobenzoic acid in the perfused livers of chronically uraemic rats

M. B. HOWIE AND E. BOURKE

Department of Clinical Medicine, Trinity College, Meath Hospital, Dublin, Ireland

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Summary

1. Chronic renal failure was induced in rats by surgical removal of thirteen-sixteenths of renal tissue.

2. The metabolism of \( p \)-amino\(^{14} \)C\)benzoic acid was studied in the isolated perfused livers of control and chronically uraemic rats. There was a slower rate of clearance and decreased overall metabolism of the compounds in the uraemic rats.

3. Conjugation of \( p \)-amino\(^{14} \)C\)benzoic acid with glycine and the formation of \( p \)-aminobenzoic acid glucuronide were both significantly decreased in the uraemic rats, but there was a significant increase in acetylation of both \( p \)-aminobenzoic acid and its glycine conjugate.

Key words: \( p \)-aminobenzoic acid, \( p \)-aminobenzoylglycine, \( p \)-aminohippuric acid, liver, uraemia.

Introduction

Hepatic detoxication mechanisms are potentially important in renal failure as excretion is impaired. Prolongation of life with maintenance haemodialysis adds to the significance of any resultant alterations in these mechanisms. Nine synthetic conjugation reactions have been so far established in man which presumably reduce toxicity (Williams & Millburn, 1975; Davis, 1977) and, of these, glucuronide formation, acetylation and glycine conjugation are amongst the more important.

\( p \)-Aminobenzoic acid undergoes all three of these conjugation reactions both in man and in the rat (Tabor, Freeman, Baily & Smith, 1951; Kitamura, Nakao & Yanagisawa, 1960), although the rates at which the reactions proceed differ in both species. We have studied the metabolism of \( p \)-aminobenzoic acid in the perfused livers of control and chronically uraemic rats.

Materials and methods

Ten male Wistar rats (275–325 g) with chronic renal failure were studied, with seven control animals. Under ether anaesthesia, through a midline incision, a right nephrectomy was performed leaving the adrenal gland intact, and the left kidney was incised below its pedicle, with an upward cut towards its lateral margin leaving approximately three-eighths of the kidney. The exposed surface was immediately covered with surgical gauze (Ethicon Ltd, Scotland, U.K.). The abdomen was sutured and the animals were housed (as were the control rats) in individual metabolism cages for approximately 3 weeks before liver perfusion. They were fed on a standard Purina chow diet and water ad libitum.

The isolated rat livers were perfused in situ by the technique of Hems, Ross, Berry & Krebs (1966). After pentobarbitone (6 mg/100 g body weight) anaesthesia, heparin (0.1 ml, 1000 i.u./ml) was administered via the saphenous vein. Inflow to the liver was via a Frankis–Evans cannula in the portal vein, and outflow via a polythene cannula.
inserted through the right atrium into the inferior vena cava. The first 5 ml of perfusate emerging from the outflow cannula was discarded before its connection to the perfusion system. The perfusate (150 ml) consisted of Krebs & Hensleit (1932) bicarbonate saline, with aged human erythrocytes to carry oxygen, and bovine serum albumin (Hems et al., 1966), to which was added 5 ml of p-aminobenzoic acid solution, making a total of 92 μmol of substrate in the final perfusion medium. The perfusate was continuously gassed with O₂/CO₂ (95:5, v/v). Bile was collected throughout the perfusion via a cannula inserted in the bile duct.

Bile was collected in weighed tubes at 30 min intervals and medium samples were taken at the same intervals for a total of 150 min. Evaporation of medium was observed to occur at a rate of 2 ml/h and, in subsequent calculations of metabolite concentrations, corrections were made for this as well as the volume of sample previously removed.

**Co-chromatography and quantification of metabolites**

Equal volumes of perchloric acid (7% solution) were added to samples of perfusion medium (2 ml) and, after centrifugation, 3 ml of supernatant was neutralized with measured volumes of potassium buffer (500 mmol/l), pH 7-5, and KOH (2 mol/l). KClO₄ was removed by centrifugation after freezing and thawing and 2 ml of supernatant was freeze-dried and reconstituted to 0.5 ml with water. Recovery of medium p-aminobenzoic acid after freeze-drying was 97.4% (SE 0.2).

Chromatography was based on that of El-Makarem, Millburn, Smith & Williams (1967), except that: (a) Whatman ET 81 was used instead of Whatman no. 1, giving more discrete spots and better separation of acetyl-p-aminobenzoate and acetyl-p-aminobenzoylglycine; (b) butanol/acetic acid/water was used in the proportions 120:30:50. Aliquots of reconstituted freeze-dried material (100 μl) were spotted in duplicate. Standards (5 μl) of the sodium salts of p-aminobenzoic acid, acetyl-p-aminobenzoate, p-aminobenzoylglycine and acetyl-p-aminobenzoylglycine were superimposed to allow ready identification in U.V. light. Chromatography was carried out for 12 h in a Panglass descending Chromotank (Shandon Scientific Co., London). A standard of p-aminobenzoic acid glucuronide was not available, but in preliminary experiments a fluorescent spot containing radioactivity was consistently observed with Rₚ 0.23 on both Whatman no. 1 and ET 81 paper, corresponding to the location of p-aminobenzoic acid glucuronide identified by El-Makarem et al. (1967). This was confirmed by radioautography.

After localization of the metabolites under u.v. light, they were circled, cut out and placed in liquid-scintillation vials containing toluene/Triton X-100 (2:1) scintillation fluid with PPO (2.7 g/l) (Turner, 1968). Samples were counted for radioactivity in a Packard liquid-scintillation counter (model 3375; Packard Instrument Co., Illinois, U.S.A.) and the d.p.m. calculated from the channels ratio. Standards of p-aminobenzoic acid spotted on ET 81 paper and then counted for radioactivity gave quantitative recoveries comparable with standards pipetted directly into the scintillation fluid. The total recovery of radioactivity from the five chromatographic spots was 89.7% (SE 3-7) of that in prechromatographed freeze-dried material in preliminary experiments. Portions of untreated bile (100 μl) were similarly chromatographed and the recovery was 90% (±2.7) of that in portions of bile pipetted directly into scintillation vials. The concentration (in μmol) was calculated from the initial specific radioactivity of the added p-aminobenzoic acid and the radioactivity of each metabolite.

At the end of the perfusion the liver was weighed, and about 50 mg accurately weighed, and added to scintillation vials containing 1.0 ml of Protosol (New England Nuclear Corp.). After 12 h digestion at 55°C the sample was counted for radioactivity in toluene/Triton X-100 at 4°C to reduce chemiluminescence.

**Substrates and standards**

Acetyl-p-aminobenzoylglycine was synthesized by the method of Newman, Kattus, Genecin, Genest, Calkins & Murphy (1949). p-Aminobenzoic acid and acetyl-p-aminobenzoate were obtained from Eastman Kodak Ltd, London, and p-aminobenzoylglycine from Merck Sharpe and Dohme (Rahway, New Jersey, U.S.A.). p-Aminobenzoic acid was from Micro-Bio Laboratories (London W.11).

**Statistics**

Comparisons were made by the paired or unpaired Student's t-test. Results are given as mean ± SEM.
Liver detoxications in uraemia in rats

Results

The mean serum urea nitrogen in the uraemic rats was 31.4 ± 2.4 mmol/l, significantly higher than in the control group, which was 8.4 ± 1.0 mmol/l ($P < 0.01$) and similar to that reported for chronically uraemic rats by Avioli, Sharpe & Bridge (1969). The mean perfusion flow rate was 19.7 ± 0.4 ml/min in the uraemic group, identical with that in the control group (20.0 ± 0.7). Biliary flow rate in the uraemic group (62 ± 3.4 mg h⁻¹ g⁻¹) was not significantly different from that in the control group (67 ± 4.8 mg h⁻¹ g⁻¹). The liver wet weight of the uraemic group (8.70 ± 0.57 g) was also similar to that of the control group (9.10 ± 0.82 g).

There was a highly significant negative correlation when the amount of p-aminobenzoic acid in the medium was plotted against time in both the control and the uraemic rats (Fig. 1). The extrapolated intercepts were similar in both but the slope of the regression line was significantly steeper in the control rats ($P < 0.025$). The mean biliary excretion of p-aminobenzoic acid in the uraemic group was 3.91 ± 0.40 nmol/min, exceeding that in the control group, which was 2.37 ± 0.33 nmol/min ($P < 0.01$).

The principal metabolite of p-aminobenzoic acid was p-aminobenzoylglycine, which rose to 30% of the concentration of the initial p-aminobenzoic acid by the end of the perfusion in the control group. The amount of p-aminobenzoylglycine in the medium increased linearly throughout perfusion in both groups but rose less in the uraemic group than in the control group, and this was reflected in biliary excretion of p-aminobenzoylglycine (Table 1). In the control group this biliary excretion (7.42 ± 0.38 nmol/min) exceeded biliary excretion of p-aminobenzoic acid (2.37 ± 0.37 nmol/min) ($P < 0.01$). Since the concentrations of both compounds in the perfusion medium are changing in opposite directions direct comparisons are not possible. However, the results indicate a higher biliary excretion of the glycine conjugate than of p-aminobenzoic acid itself.

The pattern of glucuronide formation in the medium was similar to that for p-aminobenzoylglycine, in that its concentration increased progressively throughout perfusion, but total glucuronide formation was less than one-sixth of that of p-aminobenzoylglycine at the end of perfusion (Table 1). The mean biliary excretion rate of the glucuronide (5.81 ± 1.31 nmol/min) was similar to that of p-aminobenzoylglycine ($P > 0.1$), indicating a higher hepatic clearance of the glucuronide than of the glycine conjugate. Glucuronide formation in the medium was less in the uraemic group than in the control group and this was again reflected in a lower biliary excretion (Table 1).

Both p-aminobenzoic acid and p-aminobenzoylglycine were acetylated. In the control group acetyl-p-aminobenzoylglycine concentration (1.86 ± 0.48 μmol) exceeded that of acetyl-p-aminobenzoate (0.97 ± 0.33 μmol) ($P < 0.01$). In both instances, however, the rate of acetylation fell after 60 min and was approaching a plateau towards the end of perfusion. Acetyl-p-aminobenzoylglycine appeared to have a higher biliary excretion. In contrast to the previous metabolites, acetylation of both compounds was significantly higher in the uraemic animals than in the control rats ($P < 0.01$) (Table 1), and this was again reflected in their biliary excretions.

The amount of p-aminobenzoic acid added to the perfusion medium was the same in both groups (92 μmol). At the end of the experiment the total amount of this compound and its metabolites was calculated from the sum of radioactivity in the individual chromatographic spots. Values in control animals (63.23 ± 4.27 μmol) and in uraemic animals (66.80 ± 5.17 μmol) were similar. Total biliary excretion in the uraemic group was 4.24 ± 1.01 μmol, similar to that in the control group: 4.07 ± 1.23 μmol. The liver contained 6.41 ± 2.33

Fig. 1. Effects of chronic uraemia on the disappearance of p-aminobenzoic acid (PABA) in the isolated perfused rat liver. In the uraemic group (■) $y = -0.27x + 62.1$ ($r = -0.94$), and in the control group (●) $y = -0.34x + 58.7$ ($r = -0.88$). $t = 2.57; P > 0.025$. 

Table 1. Biliary Excretion of p-aminobenzoic acid and its Metabolites

<table>
<thead>
<tr>
<th>Compound</th>
<th>Control</th>
<th>Uraemic</th>
</tr>
</thead>
<tbody>
<tr>
<td>p-aminobenzoic acid</td>
<td>3.91 ± 0.40</td>
<td>2.37 ± 0.33</td>
</tr>
<tr>
<td>p-aminobenzoylglycine</td>
<td>1.31 ± 0.31</td>
<td>1.37 ± 0.37</td>
</tr>
<tr>
<td>Acetyl-p-aminobenzoylglycine</td>
<td>1.86 ± 0.48</td>
<td>1.37 ± 0.37</td>
</tr>
<tr>
<td>Acetyl-p-aminobenzoate</td>
<td>0.97 ± 0.33</td>
<td>0.97 ± 0.33</td>
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</tbody>
</table>

Table 2. Hepatic Clearance of p-aminobenzoic acid and its Metabolites

<table>
<thead>
<tr>
<th>Compound</th>
<th>Control</th>
<th>Uraemic</th>
</tr>
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<td>Acetyl-p-aminobenzoate</td>
<td>0.97 ± 0.33</td>
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M. B. Howie and E. Bourke

**Table 1. Effects of experimental uraemia on glycine conjugation, glucuronide formation and acetylation of p-aminobenzoic acid in the perfused rat liver**

Biliary excretion rates are mean values obtained during the period of perfusion. Perfusion medium values are the concentrations obtained at the end of the perfusion. The results represent the mean values ± SEM. PAH, p-aminobenzoic acid; PABA, p-aminobenzoic acid.

<table>
<thead>
<tr>
<th></th>
<th>PAH</th>
<th>PABA</th>
<th>Acetyl-PABA</th>
<th>Acetyl-PAH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Perfusion medium</td>
<td></td>
<td></td>
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<tr>
<td>Control</td>
<td>31-1 ± 2.2</td>
<td>4.8 ± 0.4</td>
<td>0.97 ± 0.33</td>
<td>1.86 ± 0.48</td>
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<tr>
<td>Uraemic</td>
<td>22.7 ± 2.4</td>
<td>3.2 ± 0.4</td>
<td>2.87 ± 0.48</td>
<td>3.58 ± 0.31</td>
</tr>
<tr>
<td>Significance of difference (control vs uraemic)</td>
<td>$P &lt; 0.02$</td>
<td>$P &lt; 0.01$</td>
<td>$P &lt; 0.01$</td>
<td>$P &lt; 0.01$</td>
</tr>
<tr>
<td>Biliary excretion</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>7.42 ± 0.38</td>
<td>5.81 ± 1.31</td>
<td>0.74 ± 0.14</td>
<td>3.45 ± 0.28</td>
</tr>
<tr>
<td>Uraemic</td>
<td>5.60 ± 0.40</td>
<td>2.06 ± 1.17</td>
<td>1.22 ± 0.19</td>
<td>5.70 ± 0.84</td>
</tr>
<tr>
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μmol in the uraemic animals, and 6.39 ± 1.91 μmol in the control animals. Mean recovery in the uraemic animals (84-28%, range 74.82-96.96) and control rats (86.71%, range 72.07-98.82) was again similar.

**Discussion**

Significant differences have been observed in the metabolism of p-aminobenzoic acid by the perfused livers of control and chronically uraemic rats. The clearance of the compound from the medium was reduced in the uraemic group, and as this group had a higher rate of biliary excretion this suggests that the overall metabolism of p-aminobenzoic acid was reduced in uraemia. This was also reflected in a significant reduction in both the formation and biliary excretion of the glycine conjugate (p-aminobenzoylglycine) and of the glucuronide in the uraemic animals.

Uraemic serum depresses the transport of the derivatives of benzoic acid, p-aminobenzoic acid and o-iodobenzoic acid into kidney tissue *in vitro*, and this inhibition is partly competitive (Bourke, Frindt, Rose, Preuss & Schreiner, 1970). The difference in hepatic metabolism that we observed in the two groups of rats occurred in the presence of an identical perfusion medium. More complex adaptations than direct inhibition by substances circulating in uraemic serum must therefore account for these findings. Increased gluconeogenesis has also been reported in the isolated perfused livers of acutely uraemic rats (Frohlich, Scholmerich, Hoppe Seyler, Maier, Talke, Schollmeyer & Gerok, 1974).

Glycine conjugation requires ATP and coenzyme A, as well as availability of intracellular glycine (Williams & Millburn, 1975). The conjugation reaction occurs with the carboxyl group of p-aminobenzoic acid, and the glycine-N-acetylase enzyme (EC 2.3.1.13) involved is located in the hepatocytes (Schacter & Taggart, 1954). In man, administered p-aminobenzoic acid is rapidly cleared from the serum, principally by its conjugation with glycine to form p-aminobenzoylglycine which is eliminated through the kidneys (Deiss & Cohen, 1950; Tabor et al., 1951). In premature babies this conjugation reaction is markedly impaired (Vest & Salzberg, 1965), as has been reported for newborn rats (Brandt, 1960).

Glucuronide formation requires that uridine diphosphate glucuronic acid is synthesized first. It occurs with either carboxyl or hydroxyl groups and the enzyme involved, glucuronyl transferase (EC 2.4.1.17), is located in the microsomes (Davis, 1977). In premature babies, where glucuronide synthesis is also at a low level, chloramphenicol, which is detoxicated by this route, is particularly toxic (Kauffman, 1960). We cannot explain the depression of both glycine conjugation and glucuronide formation observed in our uraemic rats by a single biochemical mechanism.

Both p-aminobenzoic acid and p-aminobenzoylglycine were significantly acetylated in the perfused liver. Unlike the previous reactions, acetylation tended to reach a plateau towards the end of the perfusion. Despite the addition of acetate to the medium, optimum conditions for progressive acetylation were not achieved in this study. With a concentration of p-aminobenzoic acid in the perfusate about one-third of that of our study, Kvetina & Fendrich (1968) found that acetylation tended to
Liver detoxications in uraemia in rats

level off at 60 min. In contrast to glycination and glucuronide formation, however, acetylation was significantly increased in the uraemic animals as compared with the control rats. Unlike the former reactions, acetylation occurs with the amino groups of p-aminobenzoic acid and p-aminobenzoylglycine, the acetyltransferase involved in these reactions being located in the cytosol (Webber, 1970), and it is located in the reticuloendothelial cells rather than in the hepatocytes (Govier, 1965).

There are a number of acetylating enzymes (Franz & Krisch, 1968) but that involved in the hepatic acetylation of p-aminobenzoic acid (EC 2.3.1.5) has a broad specificity for acetyl acceptors including sulphonamide. Studies have strongly suggested that sulphonamide acetylation is enhanced in patients with chronic renal failure (Fine & Sumner, 1975). The mechanism of the enhanced acetylation by the livers of the uraemic rats, compared with the control rats, despite identical perfusion media, remains obscure.

The alterations to metabolism of p-aminobenzoic acid in the livers of the uraemic rats, as compared with the control rats, are similar in pattern to that of metabolism of the compound in premature infants, as compared with adult humans (Vest & Salzberg, 1965). In the premature infant the plasma clearance of the compound given intravenously is delayed, associated with a reduced rate of glycination and glucuronide formation, but with relatively greater acetylation.

Our findings are of potential relevance to clinical uraemia for three reasons. First, benzoic acid derivatives are quantitatively the most important non-nitrogenous organic acids found in normal human urine, resulting principally from the metabolism of intestinal micro-organisms (Asatoor, Chamberlain, Emmerson, Johnson, Levi & Milne, 1967); they are markedly elevated in uraemic serum (Bourke et al., 1972), but their metabolic fate is unknown. Secondly, the three conjugation reactions that p-aminobenzoic acid undergoes in the rat liver, acetylation, glycination and glucuronide formation, also occur in man (Tabor et al., 1951). One or other of these reactions is also shared by a variety of pharmacological agents and other foreign compounds. Knowledge of the effects of uraemia on such detoxication mechanisms may be useful in the choice or dosage of drugs in man. Finally the enzymes involved in these reactions have both endogenous and exogenous substrates, and the conjugating agents themselves have roles in normal intermediary metabolism apart from those involving foreign compounds.

Acknowledgments

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References


