Nephrotoxic Activity in Rats Fed Diets Containing DL-3-(N-Phenylethlamino)-Alanine


A School of Sciences, Deakin University, Vic. 3217.
B Veterinary Research Institute, Parkville, Vic. 3052; present address:
Australian Animal Health Laboratory, Geelong, Vic. 3219.
C Division of Protein Chemistry, CSIRO, Parkville, Vic. 3052.

Abstract
Rats were fed DL-3-(N-phenylethlamino)-alanine which resulted in kidney lesions histologically identical with those produced by the structurally related compound lysinoalanine. Possible mechanisms for nephrotoxicity are discussed.

Introduction
Woodard and Alvarez (1967) described a renal lesion, characterized by cyto-megalic changes in the straight portion of the proximal tubules, in rats which had been fed an industrial grade alkali-treated soy protein. The lesion was similar to the renal alteration reported by Newberne and Young (1966) who used alkali-treated soy protein to study the effects on rats of diets with marginal levels of methionine and choline. Subsequent studies (Woodard and Short 1973; De Groot et al. 1976) showed that the lesions were associated with the presence of lysinoalanine (N^N-DL-(2-amino-2-carboxyethyl)-L-lysine, LAL) in the alkali-treated proteins and that as little as 250 mg/kg of synthetic LAL in the diet of rats produced similar effects within 1 week. LAL does not occur naturally but is produced from cystinyl- or glycosidically bound seryl residues during heating or alkali treatment via an unsaturated intermediate, dehydroalanine (DHA). This latter compound couples with the free e-amino group of lysine residues to give LAL. However, DHA may react with compounds other than lysine and in earlier work we demonstrated that a variety of biogenic amines can react to give different N-substituted diaminopropionic acid derivatives (Rivett 1980; Jones et al. 1981).

It has been postulated (Hayashi 1982) that the nephrotoxicity of LAL could be due to its ability to chelate metals and inactivate metallo-enzymes. Friedman et al. (1985) subsequently found that alkali-treated, lysinoalanine-containing and lysinoalanine-free food proteins were able to inactivate the metallo-enzyme carboxypeptidase A. The inhibition was considered by Friedman et al. (1985) to be possibly due to the presence of D-amino acids produced by the alkali treatment binding non-specifically to the enzyme's active sites.

In an independent series of experiments D-serine and derivatives of D-serine were shown to cause acute nephrotoxicity in rats (Fishman and Artom 1942; Kaltenbach et al. 1979). Since there are structural similarities between all β-diaminopropionic
acid derivatives and serine we considered it appropriate to establish the nephro-toxicity or otherwise of a typical derivative, phenylethylaminoalanine (PEAA) (Jones et al. 1981; Tucker et al. 1983), and so go some way towards establishing the generality of the toxic effects of this class of compound. This paper reports the results of acute and prolonged toxicity tests on rats fed PEAA and compares them with the reported activities of LAL and D-serine derivatives. Some data are also presented on the ability of PEAA to inactivate a metallo-enzyme in order to determine whether this was a possible mechanism for toxicity.

Experimental

Preparation of 3-(N-phenylethlamino)-N-(α)-acetyl-DL-alanine (N-acetyl PEAA)

N-acetyl PEAA was synthesized by the method described by Jones et al. (1981) and checked for purity by elemental analysis (theoretical, C 62·4%, H 7·2%, N 11·2%; found, C 62·0%, H 7·2%, N 11·0%) and by electrophoresis at pH 1·9 and 3·5 (only one ninhydrin-positive spot was found). The infrared spectra of batches of N-acetyl PEAA were indistinguishable from each other.

Feeding Trials with DL-3-(N-Phenylethlamino)-alanine in Rats

Prior to incorporation into the rat feed, the N-acetyl PEAA was hydrolysed to PEAA by refluxing in 2 M HCl. The hydrolysate was neutralized with NaOH and the sodium chloride levels of diets with varying levels of PEAA adjusted to a constant level. The compound was mixed at various levels into powdered mouse-breeder ration (Barostoc Products, Melbourne). Water and 5% vegetable oil were added and the feed hand-formed into pellets before vacuum drying at 35°C to the water content of the original ration. Trials were conducted using Wistar rats which, although less susceptible to the nephroctomegaly produced by dietary LAL, do not exhibit the large strain variations in kidney lesions that occur in Sprague-Dawley rats (Struthers et al. 1979). Graded levels of PEAA were fed using the same design to that employed by De Groot et al. (1976). The diets were fed to 8-week-old Wistar rats.

Levels of PEAA (0, 1000, 3000 or 10 000 mg/kg) were fed to groups of five male rats for 4 weeks. Each of the rats was housed individually.

Lower levels of PEAA (0, 10, 30 or 100 mg/kg) were fed to groups of 10 males and 10 females for 13 weeks. These rats were kept in groups of five in a cage. Food intake and body weight were recorded on consecutive days at appropriate intervals during the second and fourth weeks of the acute trial and during the second and ninth weeks of the prolonged trial. Blood samples were collected terminally and measurements made for haemoglobin content, packed cell volume, mean cell haemoglobin, and red and white cell counts. Data were grouped according to dietary levels of PEAA and the means of each data set were examined for statistically significant differences using Fishers protected least significant difference procedure (Ott 1984).

Intraperitoneal Injections of PEAA

Five young male Sprague-Dawley rats (200–230 g body weight) were injected intraperitoneally with an aqueous suspension containing 100 mg PEAA/ml. N-Acetyl PEAA was hydrolysed to PEAA by refluxing in 2 M HCl and the solution was neutralized with NaOH. A sixth (control) rat was injected with a comparable volume of aqueous NaCl at a concentration equivalent to the highest dose contained in a PEAA injection. The dose rates on a molar basis, were similar to that used by Kaltenbach et al. (1979) in their study of D-serine toxicity. One rat was injected with 1·3 g PEAA/kg body weight (0·62 mmol PEAA/100 g body weight), two were injected at 1·0 g PEAA/kg body weight (0·5 mmol PEAA/100 g body weight) and two were injected at 0·8 g PEAA/kg body weight (0·4 mmol PEAA/100 g body weight). Surviving rats were killed 48 h post injection.

Autopsies and Histopathological Examinations

Rats in the feeding trials were killed by decapitation while those animals injected with PEAA were killed by carbon dioxide inhalation. Autopsies were conducted as soon as possible after death and the following organs were fixed in 10% (v/v) neutral buffered formalin: both kidneys, liver, brain, heart, lungs, stomach, small and large intestines, urinary bladder, thymus, mesenteric lymph node, spleen, testis and skeletal muscle. Tissues were immersed in paraffin wax, sectioned and stained with haematoxylin and eosin.
Experiments with the Metallo-enzyme Carboxypeptidase A

Carboxypeptidase A (EC 3.4.12.2, Worthington–Millipore, Bedford, Mass. U.S.A.) with an activity experimentally determined to be 44 units/mg protein was used initially; 0·25 ml was incubated for 1 h at 25°C with 0·15 ml PEAA at concentrations ranging from 0 to 20 mM in Tris buffer, pH 7·5. After incubation, the enzyme activity was again measured. In a second series of experiments, 0·190 ml carboxypeptidase A (a separate batch with an experimentally determined activity of 63 units/mg protein) was incubated at 25°C overnight in the presence of PEAA concentrations ranging from 0 to 66 mM before re-measuring the enzyme activity.

Table 1. Weight gain, feed intake and feed efficiency in the second and fourth week of rats fed PEAA at the rate of 0, 1000, 3000 and 10 000 mg/kg body weight

<table>
<thead>
<tr>
<th>PEAA in stock diet (mg/kg body wt)</th>
<th>Food intake per rat (g/5 days)</th>
<th>Weight gain (g/5 days)</th>
<th>Feed efficiency$^A$</th>
<th>Food intake per rat (g/5 days)</th>
<th>Weight gain (g/5 days)</th>
<th>Feed efficiency$^A$</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>84·75</td>
<td>10·09</td>
<td>0·120</td>
<td>92·27</td>
<td>15·07</td>
<td>0·162</td>
</tr>
<tr>
<td>1000</td>
<td>± 4·42</td>
<td>± 6·34</td>
<td>± 0·076</td>
<td>± 8·48</td>
<td>± 3·10</td>
<td>± 0·023</td>
</tr>
<tr>
<td>3000</td>
<td>81·81</td>
<td>13·55</td>
<td>0·165</td>
<td>94·17</td>
<td>17·27</td>
<td>0·182</td>
</tr>
<tr>
<td>10 000</td>
<td>± 11·13</td>
<td>± 3·55</td>
<td>± 0·30</td>
<td>± 8·96</td>
<td>± 4·22</td>
<td>± 0·032</td>
</tr>
<tr>
<td></td>
<td>41·87</td>
<td>± 17·96</td>
<td>± 0·46</td>
<td>73·97</td>
<td>19·35</td>
<td>0·262</td>
</tr>
<tr>
<td></td>
<td>± 5·87</td>
<td>± 9·60</td>
<td>± 0·264</td>
<td>± 21·36</td>
<td>± 5·87</td>
<td>± 0·046</td>
</tr>
<tr>
<td></td>
<td>24·92</td>
<td>± 34·69</td>
<td>± 1·50</td>
<td>63·64</td>
<td>13·02</td>
<td>± 0·220</td>
</tr>
<tr>
<td></td>
<td>± 4·66</td>
<td>± 10·85</td>
<td>± 0·660</td>
<td>± 7·08</td>
<td>± 4·62</td>
<td>± 0·064</td>
</tr>
</tbody>
</table>

$^A$ Ratio of weight gain in grams to food intake in grams.

Table 2. Body weight of rats (g) fed differing amounts of PEAA for 4 weeks

<table>
<thead>
<tr>
<th>PEAA in diet (mg/kg body wt)</th>
<th>Week 0</th>
<th>Week 1</th>
<th>Week 2</th>
<th>Week 3</th>
<th>Week 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>247·37</td>
<td>288·62</td>
<td>308·69</td>
<td>334·24</td>
<td>356·93</td>
</tr>
<tr>
<td>± 33·26</td>
<td>± 24·78</td>
<td>± 20·78</td>
<td>± 20·14</td>
<td>± 23·11</td>
<td>± 35·16</td>
</tr>
<tr>
<td>1000</td>
<td>254·58</td>
<td>278·19</td>
<td>302·36</td>
<td>333·74</td>
<td>358·16</td>
</tr>
<tr>
<td>± 22·03</td>
<td>± 24·79</td>
<td>± 27·86</td>
<td>± 29·87</td>
<td>± 31·36</td>
<td>± 35·16</td>
</tr>
<tr>
<td>3000</td>
<td>235·25</td>
<td>234·85</td>
<td>221·26</td>
<td>238·90</td>
<td>258·04</td>
</tr>
<tr>
<td>± 14·41</td>
<td>± 14·91</td>
<td>± 18·49</td>
<td>± 23·95</td>
<td>± 22·77</td>
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<tr>
<td>10 000</td>
<td>256·12</td>
<td>210·88</td>
<td>178·83</td>
<td>205·51</td>
<td>217·12</td>
</tr>
<tr>
<td>± 25·71</td>
<td>± 21·67</td>
<td>± 16·03</td>
<td>± 8·84</td>
<td>± 12·02</td>
<td>± 12·02</td>
</tr>
</tbody>
</table>

Results

Rats fed PEAA in their diets at the rate of 3000 and 10 000 mg/kg were eating significantly less ($P = 0·05$) and gaining less weight at the end of the second week than the controls or the animals fed 1000 mg/kg (Table 1). The control animals and animals fed 1000 mg/kg were not different in respect of these measurements. However, in the fourth week of feeding only the animals fed 1000 mg/kg were still eating less than the controls, indicating a greater degree of acceptance of the diet.
The body weights prior to the rats being killed showed that the animals fed 3000 and 10 000 mg/kg weighed less than controls (Table 2, \( P = 0.05 \)). In the animals given low dietary levels of PEAA, there were no significant changes in weight gains and feed efficiencies.

**Rat Mortalities**

One rat in the group fed a diet containing 10 000 mg/kg PEAA died after 10 days feeding. A rat injected with PEAA at 1.3 g/kg body weight and two rats injected with 1.0 g/kg died within 12 h of injection. All other rats survived until the planned completion of the experiments.

**Haematology**

In haemograms measured terminally there were no significant differences in haemoglobin, packed cell volume and mean corpuscular haemoglobin in rats fed PEAA at 0, 1000, 3000 and 10 000 mg/kg (\( P = 0.05 \)). There were higher levels of haemoglobin in both male and female groups fed 100 mg/kg than in controls (\( P = 0.05 \)). In addition males fed 100 mg/kg had larger packed cell volumes and consequently lower mean corpuscular haemoglobin values than controls but the biological significance of these differences, if any, is not clear.

<table>
<thead>
<tr>
<th>Rat No.</th>
<th>PEAA level in diet (mg/kg body wt)</th>
<th>Early renal lesions(^A)</th>
<th>Advanced renal lesions(^B)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>3000</td>
<td>++</td>
<td>-</td>
</tr>
<tr>
<td>12</td>
<td>3000</td>
<td>+++</td>
<td>-</td>
</tr>
<tr>
<td>14</td>
<td>3000</td>
<td>++</td>
<td>-</td>
</tr>
<tr>
<td>15</td>
<td>3000</td>
<td>+++</td>
<td>-</td>
</tr>
<tr>
<td>17</td>
<td>3000</td>
<td>+++</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>10 000</td>
<td>++++</td>
<td>++++</td>
</tr>
<tr>
<td>6</td>
<td>10 000</td>
<td>++++</td>
<td>++</td>
</tr>
<tr>
<td>11(^c)</td>
<td>10 000</td>
<td>n.a.</td>
<td>n.a.</td>
</tr>
<tr>
<td>18</td>
<td>10 000</td>
<td>++++</td>
<td>+</td>
</tr>
<tr>
<td>19</td>
<td>10 000</td>
<td>++++</td>
<td>+++</td>
</tr>
</tbody>
</table>

\(^A\) Early renal lesions were those of cellular enlargement at the cortico-medullary region and there was some minor tubule dilation.

\(^B\) Advanced changes were more widespread and there was evidence of interstitial oedema.

\(^c\) Results not available, rat died after 10 days.

**Gross Pathology**

In the feeding trials, there were no gross lesions in rats fed up to 100 mg/kg PEAA. In control rats and those fed 1000, 3000 and 10 000 mg/kg, several had pink-grey areas of consolidation in the lungs. Rats fed 3000 and 10 000 mg/kg PEAA had much less body fat and those in the latter group had paler kidneys than normal.
Nephrotoxicity of Alanine Derivative

Fig. 1. (a) Kidney from rat fed PEAA at the rate of 10,000 mg/kg body weight for 4 weeks. There was marked nephrocytomegaly (thick arrows), tubule dilation (thin arrows), proteinuria and intratubular mineral casts, and mild interstitial fibrosis. Haematoxylin and eosin. (b) Kidney from control rat fed PEAA-free diet. Tubule cell nuclei are more consistent in size. There is very little tubule dilation and tubules regularly oriented. Haematoxylin and eosin.
Fig. 2. (a) Kidney from a rat injected intraperitoneally with an aqueous suspension of PEAA at a dose rate of 1 g/kg body weight and killed 48 h later. There was severe necrosis of proximal tubules characterized by loss of nuclei and eosinophilia of cytoplasms (thick arrows), and proteinuria (thin arrows). Haematoxylin and eosin. (b) Kidney from a control rat injected with an aqueous saline solution. Tubules contain nuclei and there is very little tubule dilation and no proteinuria. Haematoxylin and eosin.
In the rats injected with PEAA at dose rates of 1·3 and 1·0 g/kg body weight (two rats), there were no significant changes. However, the two rats injected with 0·8 g/kg body weight survived for the 48 h of the experimental period and had extremely pale kidney cortices. The kidneys of the control rat were normal in colour.

**Histopathology**

Rats fed for 13 weeks on 0–100 mg/kg PEAA did not develop lesions. Table 3 summarizes the incidence of renal lesions found in the higher-dose feeding trial of 0–10 000 mg/kg PEAA. Lesions of chronic interstitial pneumonia characterized by perivascular and/or peribronchiolar accumulations of lymphocytes and masses of alveolar macrophages with some neutrophils, probably an incidental manifestation of enzootic pneumonia, were prevalent in both experimental and control rats. There were no other lesions in any organs other than the kidneys.

In summary, renal nephroses occurred in rats fed 3000 and 10 000 mg/kg PEAA with those characterized as ‘early’ occurring in all rats and those as ‘advanced’ in rats fed 10 000 mg/kg PEAA only. The ‘early’ lesions occurred near the corticomedullary junctions in the cells of the loop of the proximal tubule. They had distinctly basophilic cytoplasms and nuclei of varying sizes, mostly markedly larger than normal. Tubule lumens were frequently dilated. In one rat, Bowman’s capsules adjacent to the affected straight tubules were partially lined by cuboidal to columnar epithelium.

In ‘advanced renal lesions’, there was significant nephrosis clearly affecting at least 75% of tubule cells (Fig. 1a). Affected cells had basophilic cytoplasms and their nuclei varied in size. They often had prominent nucleoli. Tubule dilation was prominent and could extend in some nephrons to near the external surface. Basophilia and degeneration occurred mostly in the inner half of the cortex but could extend in association with tubule dilation to the external zone. There was moderate interstitial oedema, fibrosis and mononuclear cell infiltration, and some inner loop tubules were mineralized. A normal kidney from a control rat is shown in Fig. 1b for comparison.

In the three rats which died within 12 h of injections of PEAA at the rate of 1·3, 1·0 and 1·0 g/kg body weight respectively, there were no significant histological lesions other than mild autolysis. In the rats injected with PEAA at the rate of 0·8 g/kg body weight and killed 48 h later, there was severe acute renal necrosis. This was characterized by marked proteinuria, tubule dilation and, in the proximal tubules, by the almost total absence of nuclei and by eosinophilia of the cytoplasms (Fig. 2a). Distal tubules and glomeruli were normal. There were no visible lesions in any other organs. The kidneys of the control rat were normal (Fig. 2b).

**Metal Chelation Studies**

Experiments with carboxypeptidase A showed no reduction in activity even in the presence of 66 mM PEAA.

**Discussion**

Incorporation of PEAA into the diets of rats caused reduction of weight gains and feed efficiencies when fed at levels of 1000 mg/kg or above. At 3000 and
10,000 mg/kg there was marked loss of appetite, reduction in weight gains and later, distinct nephrotoxicity. At these latter levels, the losses in weight gains were clearly due, in part, to losses of appetite as well as the toxic effects, as rats ate less from the start of the trial.

The most marked effect of the consumption of the PEAA was the chronic nephrotoxicity. This was dose-dependent. Table 3 shows that rats fed 10,000 mg/kg were more severely affected than those fed 3000 mg/kg, and at the end of this trial of 4 weeks, there were no renal changes in those fed 1000 mg/kg. The renal lesions in the present paper were similar to those described by Woodard and Alvarez (1967) in rats fed alpha-protein and shown by Woodard and Short (1975) to be due to LAL. Gould and MacGregor (1977) summarized the biological effects of alkali-treated protein and LAL as follows: ‘The effects on the rat were on the kidneys causing characteristic nuclear and cytoplasmic enlargement (nephrocytomegalia), particularly in the straight portions or pars recta of the kidneys.’ This is very similar to the lesion described in this paper and it is reasonable to postulate, bearing in mind the similarities in chemical structures, that the lesions produced by LAL and PEAA are essentially the same, with the same pathogenesis.

Intraperitoneal injection with PEAA shows that it shares with D-serine a capacity to cause acute necrosis. The lesions observed in the two rats surviving 48 h were essentially similar to those described by Fishman and Artom (1942) and Morehead et al. (1945) and more recently by Ganote et al. (1974) and Kaltenbach et al. (1979). These findings suggest that there is a relationship between the chronic nephrotoxicities of PEAA and LAL and the acute nephrotoxicity of D-serine. This is supported by the lesion seen in the rats injected intraperitoneally with PEAA. These three compounds share an alanyl component in their chemical structures and the configuration of the α-carbon in this part of the molecule may be partially responsible for the toxic effects. The L-isomer of serine (Ganote et al. 1974) and of diaminopropionic acid (Kaltenbach et al. 1979) do not share the nephrotoxicity of the D-enantiomers. Similarly with LAL, the α-carbon of the alanine part of the molecule is nephrotoxic in the D- but not the L-configuration (Tas and Kleipool 1976). In our work, we have used the racemic modification of PEAA. It can be postulated that toxicity will depend on whether and to what extent the α-carbon of L-cystinyl or glycosidically bound L-serine is inverted during the formation of LAL and PEAA in proteins or protein-containing foodstuffs.

Hayashi (1982) has proposed that the nephrotoxicity of LAL could be attributed to its ability to chelate metals and inactivate metallo-enzymes such as carboxypeptidases A and B and alcohol dehydrogenase. He found that even at concentrations as low as 5 mM, LAL completely inactivated carboxypeptidase A after an overnight incubation. Hayashi (1982) reasoned that the nephrotoxicity of LAL could be due to chelation of the zinc atom of an essential kidney enzyme. We were unable to demonstrate the formation of a chelation complex between PEAA and zinc but were able to produce a kidney lesion in rats by feeding PEAA which is very similar to that reported by feeding LAL. However, this does not necessarily invalidate a common aetiology since LAL is nephrotoxic at much lower dietary levels than PEAA and it is possible that these are in accord with their metal-chelating abilities which, in the case of the latter compound, was not detectable in our experiments.
References


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