EFFECTS OF PENICILLAMINE ON DISTRIBUTION OF B₆ VITAMERS IN RAT URINE

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Summary The antivitamin B₆ effect of DL- and D-penicillamine has been studied in rats. A considerable elevation in the urinary excretion of vitamin B₆ activity (pyridoxal and its thiazolidine derivative) has been shown as a parameter of B₆ antagonism. Both DL- and D-penicillamine have been shown to have an antivitamin B₆ effect in rats, although that induced by the DL-form is considerably greater, as would be expected from previous studies. We suggest that B₆ supplementation should be included in any long term penicillamine therapy, regardless of the isomer that is employed.

Penicillamine (PeA) is now accepted as the treatment of choice of patients with Wilson's disease because of its copper-chelating properties (1), and for cystinuric patients, in whom urinary cystine stone formation is prevented by the formation of a soluble PeA-cysteinemixed disulfide (2). It is currently under investigation in the treatment of rheumatoid arthritis, where it has been shown to produce various clinical improvements (3, 4). With the first description of its use as a drug with Wilson's disease (1), it was pointed out that certain toxic reactions might be expected in view of the works of Du Vigneaud et al. (5–7) who had shown that the inclusion of L-isomer of PeA (L-PeA) in the diet of rats caused vitamin B₆ deficiency, resulting in inhibition of growth, reduced transaminase activity and abnormal excretion of xanthurenic acid following the administration of tryptophan: the D-isomer (D-PeA) appeared to have no toxic action under the conditions described (5, 8). Du Vigneaud et al. (7) also showed that the B₆ deficiency might be the result of a chemical combination between L-PeA and pyridoxal phosphate (PLP) to form a thiazolidine compound (PLP-T). Shortly

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Abbreviations: PeA, penicillamine; PLP, pyridoxal phosphate; PLP-T, thiazolidine derivative between PLP and PeA; PN, pyridoxine; PL, pyridoxal; PL-T, thiazolidine derivative between PL and PeA; PMP, pyridoxamine phosphate.
thereafter, MATSUDA and MAKINO (9-11) demonstrated that an injection of L-PeA in mice caused a decreased activity of brain glutamic acid decarboxylase and convulsions which were very similar to that caused by injection of other antivitamin B₆. This symptom was completely prevented by an injection of pyridoxine (PN), whereas D-PeA did not produce such symptoms, though D-PeA also reacted non-enzymatically with PLP to form PLP-T (11, 12). It was therefore suggested that only D-PeA be used in treatments of the disease described above.

Subsequent studies, however, showed that D-PeA also was capable of producing evidence of an antivitamin B₆ effect in the rat (13) and in man (14), using the urinary excretion of xanthurenic acid as a parameter of B₆ deficiency. TAKASHI and MATSUDA (15) also demonstrated that the administration of D-PeA to mice resulted in the decrease of PLP content in the brain which could be due to the formation of PLP-T, though the diminution of the PLP content was slight as compared to that of L-PeA. It seemed, therefore, of interest to examine whether the thiazolidine compounds were excreted in the rat urine following the administration of L-PeA or D-PeA.

MATERIALS AND METHODS

Chemicals. DL-PeA, D-PeA and B₆ vitamers were obtained from Nakarai Chemicals. Acid phosphatase was purchased from Boehringer Mannheim.

Thiazolidine compound of pyridoxal (PL) and PeA (2-(2-methyl-3-hydroxy-5-hydroxymethyl-4-pyridyl)-5,5-dimethyl-4-thiazolidinecarboxylic acid, PL-T) was synthesized according to a minor modification of the method of HEYL et al. (16). A solution of 257 mg of potassium hydroxide in 7 ml of water was added to a solution of 274 mg of DL-PeA in 40 ml of ethyl alcohol. The addition of 246 mg of PL hydrochloride produced a bright yellow colored solution. After several hours at room temperature acidification of the solution (to pH 6) caused the crystallization of the thiazolidine compound (PL-T) which was washed successively with water, alcohol and ether. The PL-T thus obtained showed the absorption spectrum distinctly different from that of the original PL but very similar to that of the reaction product between PLP and PeA (7), and showed to be chromatographically pure as shown in Fig. 2.

Injection. Male Wister rats weighing 100-120 g were used. All compounds were injected intraperitoneally. The solution of drugs were prepared daily in water, the pH being adjusted to 7.0 immediately before use. The final concentration of drugs was adjusted so that the required dosage was administered at 1% of the body weight.

Separation and determination of B₆ vitamers. Different forms of B₆ were separated by ion exchange chromatography on Amberlite CG-120 according to the method of LOO and BADGER (17). The individual B₆ vitamers in the eluates were assayed microbiologically with Saccharomyces carlsbergensis 4228 by using
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the corresponding authentic vitamers as reference standard which were treated on the column in the same way. Phosphate esters were hydrolyzed by acid phosphatase before the assay as previously reported (18). The assay method was slightly modified from that of CHIAO and PETERSON (19); the concentration of each component except that the amounts of hydrolyzed casein and sugar were half that of their basal medium (18). Thiamine was added at 500 µg/liter.

RESULTS AND DISCUSSION

B₆ vitamers levels in urine of control rats

Two rats were maintained on Oriental rat diet (Oriental Ltd.). The rats were placed at 2 p.m. in a metabolic cage and thereafter urine for 20 hr, for which the animals were fasted to avoid contamination of the urine by the diet, was collected. To the total urine (about 6 ml) an adequate amount of 5 N perchloric acid was added so as to be 1 N in its final concentration. After a small amount of insoluble material was removed by centrifugation, the supernatant was neutralized with 5 N KOH to pH 6.0 and the potassium perchlorate was removed by centrifugation in the cold. The clear supernatant (about 7 ml) was acidified with acetic acid to pH 3.5 and was applied to a column of Amberlite CG-120 (1×4 cm). The elution was performed according to the procedure of LOO and BADGER (17); PLP, the supernatant + 10 ml of water; pyridoxamine phosphate (PMP), 25 ml of 0.1 M acetate buffer, pH 4.0 after 15 ml of 0.01 M acetate buffer, pH 4.0; PL, 20 ml of 0.1 M Na phosphate buffer, pH 6.0 after 10 ml of 0.1 M acetate buffer, pH 5.0; PN, 20 ml of 0.1 M Na phosphate buffer, pH 6.5; PM, 25 ml of 0.1 M Na phosphate buffer, pH 8.5. The content of B₆ vitamer in each fraction was determined by the procedure described in MATERIALS AND METHODS.

Figure 1 shows the B₆ vitamer elution pattern of 20 hr urine of a control rat.

![Substance eluted](image)

Fig. 1. Chromatographic separation of B₆ vitamers in rat urine on a column of Amberlite CG 120 ion-exchange resin with pH-gradient elution.
Significant B₆ activity was found in the rinsed fraction corresponding to PLP. To test the possibility that the fraction might be an artifact of the column chromatography, the fraction was adjusted to pH 7.8, applied to an anion exchange column (Dowex-1-formate, 1×5 cm), and elution was performed by the procedure of Bain and Williams (20). Only a single fraction was eluted at the same buffer concentration as in the case of authentic PLP, showing clearly that the substance eluted as the first peak from the Amberlite CG 120 column was PLP itself.

Other B₆ vitamers were also distinctly separated on the column (Fig. 1). The eluate of each compound was collected in one fraction and individually assayed. The data on B₆ distribution in the urine under a normal condition are tabulated in the first line of Table 1. The average values (expressed as μg/20 hr urine) of five extracts prepared from urines of adult male rats for a 20 hr fast were as follows: PLP, 2.91; PMP, 0.19; PL, 2.80; PN, 0.16; PM, 1.42.

Changes in urine B₆ vitamer levels in rats after an injection of DL-PeA

After feeding the rats on Oriental diet for several days, 200 mg/kg of DL-PeA (subconvulsive dose) was injected intraperitoneally into two rats at 2 p.m. and thereafter urine was collected during a fast of 20 hr. B₆ vitamers in the urine collected were extracted, separated and determined according to the procedure described above except for the minor modifications in the volume of eluant for the column chromatography.

| Days after injection of DL-PeA (200mg/kg) | PLP (μg/rat urine) | PMP (μg/rat urine) | PL-T (μg/rat urine) | PL (μg/rat urine) | PN (μg/rat urine) | PM (μg/rat urine) |
|-----------------------------------------|---------------------|-------------------|--------------------|-------------------|-------------------|------------------|
| Control                                 | 2.96±1.43           | 0.19±0.06         | 0                  | 2.80±2.01         | 0.16±0.07         | 1.42±0.40        |
| 1                                       | 1.91±0.16           | 0.07±0.01         | 14.14±3.81         | 41.00±12.07       | 0.20±0.09         | 0.99±0.20        |
| 2                                       | 0.85±0.23           | 0.33±0.04         | 1.09±0.11          | 2.82±0.64         | 0.25±0.14         | 0.11±0.06        |
| 3                                       | 1.49±0.79           | 0.24±0.02         | 1.01±0.22          | 2.65±1.17         | 0.22±0.12         | 0.15±0.02        |
| 4                                       | 1.97±0.79           | 0.19±0.11         | 0.85±0.55          | 3.11±0.86         | 0.34±0.13         | 0.16±0.07        |
| 5                                       | 2.36±0.54           | 0.20±0.12         | 0.75±0.42          | 2.46±0.97         | 0.29±0.11         | 0.27±0.17        |

Each value represents the mean ± S.D. of five experiments.

The results shown in Fig. 2 show the most striking change in B₆ distribution brought out by the DL-PeA treatment. The specific features of the chromatogram are a markedly increased PL and the appearance of a new peak, which is believed to be PL-T because authentic PL-T was eluted at the same buffer concentration as the new peak did (Fig. 2) and possessed B₆ activity equivalent to an equimolar amount of PL when assayed with S. carlsbergensis 4228 (15). The data on B₆ distribution in the urine of DL-PeA treated rats are shown in the second line of Table 1. The outstanding points about this data are the gross excretion of PL...
as well as PL-T. To know the course of excretion of PL and PL-T after one injection of DL-PeA, an experiment was carried out as follows; after the injection of DL-PeA (200 mg/kg) at 2 p.m. as described above, the animals were fasted for 20 hr from this time to next 10 a.m. During the period the urine sample was collected in 1 N perchloric acid, and then the animals were given the rat diet for 4 hr from 10 a.m. to 2 p.m. This procedure, except for the injection of DL-PeA, was repeated five times and each 20 hr urine was used for analysis of B₆ vitamers. As shown in Table 1, the most part of PL and PL-T was excreted in the urine of the first day, and the total amounts of urinary PL and PL-T for five days amounted to about 50 µg and 20 µg, respectively.

These observations on the urinary B₆ excretion are in harmony with the idea that the administration of DL-PeA leads to a rapid loss of B₆, especially PLP from the tissues (15). As suggested previously (15), L-PeA may bring about these effects by combining with PLP to form a thiazolidine structure (PLP-T), and the formed PLP-T may be rapidly released from the tissues into blood. Furthermore, the marked urinary excretion of PL-T, as well as PL, seems to be due to dephosphorylation of the released PLP-T to PL-T, followed by hydrolysis of PL-T to PL. This conversion from PL-T to PL seems to occur in the rat tissues, since it has been
known that the PL-T possesses $B_6$ activity almost equivalent to PL not only for the *S. carlsbergensis* 4228 (15) but also for $B_6$-deficient animals (M. MATSUDA et al. unpublished work).

**Changes in urine $B_6$ vitamer levels in rats after an injection of $D$-PeA**

The experiment was carried out by the same procedure as that of DL-PeA. When $D$-PeA (100 and 200 mg/kg) was administered intraperitoneally, the $B_6$ vitamer elution pattern of the urine was similar to that obtained with administration of DL-PeA (Fig. 3). The data on $B_6$ distribution in the urine (Table 2) also had a strong resemblance to those of DL-PeA (Table 1), though the excreted amounts of PL and PL-T were somewhat less in the rats treated with $D$-PeA than in that treated with DL-PeA.

| Table 2. Effect of d-penicillamine (100 mg and 200 mg/kg) administration on distribution of $B_6$ vitamers in rat urine. Values are expressed as $\mu g/20$ hr urine/animal. |
|---------------------------------------------------------------|
|                 | PLP | PMP | PL-T ($\mu g$/rat urine) | PL | PN | PM |
| Control         | 2.91±1.43 | 0.19±0.06 | 0 | 2.80±2.01 | 0.16±0.07 | 1.42±0.40 |
| $D$-PeA (100 mg/kg) | 2.23±0.95 | 0.17±0.09 | 7.90±0.75 | 15.95±3.92 | 0.33±0.03 | 1.02±0.20 |
| $D$-PeA (200 mg/kg) | 1.29±0.30 | 0.19±0.09 | 9.05±2.28 | 13.85±3.39 | 0.26±0.06 | 1.19±0.31 |

Control values are the same as in Table 1. Each value represents the mean ± S.D. of five experiments.

From these experimental results it is evident that following the injection of $D$-PeA there is a considerable increase in urine excretion of $B_6$ activity. The abnormal excretion of the $B_6$ activity is expected to cause a depletion of $B_6$ in animal tissues. JAFFE et al. (14) also showed previously that the administration of $D$-PeA to patients with rheumatoid arthritis resulted in an increase in urinary excretion of xanthurenic acid and kynurenine, which have been known as a parameter of $B_6$ deficiency. It is therefore concluded that $D$-PeA, like L-isomer, caused a depletion of PLP which can be attributed to the gross excretions of PL-T and PL in the urine. Since PeA is generally given to patients for prolonged periods without interruption, PN supplementation should be part of the therapeutic regimen, even when the $D$-isomer is used.

Moreover, it is presumed that the administration of PeA may affect urinary excretion of pyridoxic acid which is a final metabolite of vitamin $B_6$, but further investigations of this problem will be conducted in the future.

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