Identification of the Diketopiperazine of Histidylproline in Human Urine*

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During surveys of amines in the urine of children, Perry et al. (1) and Perry and Schroeder (2) found among the unidentified free bases present a substance which produced an orange-red color when chromatograms were sprayed with diazotized sulfanilic acid. This substance was designated "Compound 9." Subsequently Compound 9 has been detected in all amine concentrates of unhydrolyzed urines of a large number of children and adults. More recently this substance was found to be present in greatly increased amounts in the urines of patients with phenylketonuria who were being fed a low phenylalanine diet.

In this paper we present experiments which have led to the identification of Compound 9 as the diketopiperazine of histidylproline.

![Diketopiperazine structure](image)

The markedly increased amounts of the diketopiperazine of histidylproline in the urines of phenylketonuric patients who were receiving a commercial low phenylalanine preparation (Lofenalac, Mead Johnson) as the chief component of their diet. An aliquot of the pooled urines containing 7220 mg of creatinine was treated with Amberlite CG-50, type 2 (H+), essentially as described by Perry et al. (1). The concentrate of free urinary amines and other bases thus prepared was then subjected to ion exchange column chromatography, with the use of the method of Perry and Schroeder (2), each substance isolated from urine being subjected to high voltage electrophoresis on Whatman No. 1 paper at pH 6.0 in ammonium acetate buffer (0.5 M in acetate) at 26 volts per cm for 3 hours. The dipeptide, His-Pro, however, showed different paper chromatographic and electrophoretic mobilities. These results are listed in Table I.

When authentic HP-diketopiperazine and the compound isolated from urine moved at the same rate when subjected to high voltage electrophoresis on Whatman No. 1 paper at pH 6.0 in ammonium acetate buffer (0.5 M in acetate) at 26 volts per cm for 3 hours. The dipeptide, His-Pro, however, showed different paper chromatographic and electrophoretic mobilities. These results are listed in Table I.

Dinitrophenyl derivatives of His-Pro, of authentic HP-diketopiperazine, and of the putative HP-diketopiperazino isolated from urine were prepared by causing these compounds to react with 1-fluoro-2,4-dinitrobenzene in 1% aqueous triethylamine, by a modification of the method of Levy (4). The dinitrophenyl derivatives were then hydrolyzed in 5.7 N HCl for 8 hours at 105°, and the resulting amino acid and DNP-amino acids were identified by chromatographing them on paper together with appropriate standards (5). His-Pro yielded di-DNP-histidine and proline, while both authentic HP-diketopiperazine and the compound isolated from urine yielded imidazole-DNP-histidine and proline.

* The abbreviations used are: HP-diketopiperazine, diketopiperazine of histidylproline; DNP-, 2,4-dinitrophenyl.

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The infrared spectra of authentic HP-diketopiperazine and of the compound isolated from urine were virtually identical (Fig. 1), and the spectrum was interpreted as being consistent with the diketopiperazine structure.

Having identified Compound 9 isolated from urine as HP-diketopiperazine on the basis of its chromatographic properties on paper and on ion exchange columns, its electrophoretic behavior, its infrared spectrum, and its hydrolysis products, it seemed important to determine whether the diketopiperazine itself had been present in the original human urine, or whether it was merely an artifact of the procedures used in preparing the concentrate of urinary bases. It was conceivable that His-Pro might have been present in urine, and that it had then been cyclized to the diketopiperazine during elution of bases from Amberlite CC-50 with 4 M ammonium hydroxide-water (8:1:1).

The infrared spectra and of authentic His-Pro were subjected to the same procedures used for separating amines and other bases from urine (1), the final concentrate was found to contain only His-Pro and none of the diketopiperazine.

Semi-quantitative estimates of the urinary excretion of HP-diketopiperazine in normal subjects and phenylketonuric patients were made by visual comparison of chromatograms, sprayed with diazotized sulfanilic acid, prepared both from urinary base concentrate and from appropriate amounts of HP-diketopiperazine. Normal children and adults were found to excrete from 7 to 36 µg/100 mg of creatinine, while phenylketonuric patients on a low phenylalanine diet excreted from 125 to 2400 µg/100 mg of creatinine.

That the greatly increased urinary excretion of HP-diketopiperazine by phenylketonurics under dietary treatment is not related to a metabolic defect in phenylketonuria was demonstrated by a return to normal values for HP-diketopiperazine in urine when the same patients were permitted to consume a general diet. In addition, a patient with mongolism who excreted normal amounts of HP-diketopiperazine exhibited a definite 50-fold increase in urinary HP-diketopiperazine when placed on a low phenylalanine diet. The source of the greatly increased urinary HP-diketopiperazine in patients on this diet was traced to the Lofenalac which forms the main item of the diet. HP-diketopiperazine was recovered from Lofenalac when it was extracted with methanol, or when bases were adsorbed onto Amberlite CG-50 from an aqueous suspension of the powdered Lofenalac.

**DISCUSSION**

Stein (6) has shown that most of the proline in human urine exists in conjugated form, and he showed that acid hydrolysis of urine also liberates histidine, as well as many other amino acids. Meilman, Urvetsky, and Rapoport (7) have found prolylhydroxyproline to be one of the major prolyl peptides in human urine. These investigators found small amounts of the diketopiperazine of prolylhydroxyproline in urine but concluded that it arose from the dipeptide during their separation procedures. Plaquet, Biserte, and Boulanger (8) had earlier reported finding the diketopiperazines of prolylhydroxyproline and of prolylglycine in the urine of children and had likewise concluded that they resulted from cyclization of dipeptides during fractionation procedures on urine. More recently, Kibbick, Hashiro, Walters, and Milhorat (9) have reported that the diketopiperazine of prolylhydroxyproline is excreted as such in human urine.

The present demonstration of HP-diketopiperazine in urine suggests that other members of this class of compounds may also be excreted. Whether the small amount of HP-diketopiperazine normally present in urine is derived from the diet or represents a metabolic end product is not clear. Usual amounts of this compound were detected in the urines of two healthy adults who had fasted for 5 days, suggesting that HP-diketopiperazine may be an endogenous metabolite. The much greater quantities of the compound present in the urines of phenylketonurics receiving a low phenylalanine diet apparently result from absorption of HP-diketopiperazine from the intestinal tract and its excretion unchanged by the kidneys. It does not seem likely that this compound is related to the nutritional failure sometimes en-
countered in phenylketonuric infants under treatment with a low phenylalanine diet.

SUMMARY

A previously unidentified urinary base is excreted in large amounts by phenylketonuric patients receiving a low phenylalanine diet. This compound has been isolated and identified as the diketopiperazine of histidylproline. It is present in small amounts in all normal human urines.

REFERENCES

Identification of the Diketopiperazine of Histidylproline in Human Urine
Thomas L. Perry, Keith St. Clair Richardson, Shirley Hansen and Abram J. D. Friesen


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