Studies on the Unknown Amino Acids in Normal Human Urine

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Many of the ninhydrin-positive substances excreted in human urine still remain unknown. The present author carried out the isolation of some unknown ninhydrin-positive substances from the neutral amino acid fraction of human adult male urine. In this paper, the isolation and characterization of four amino acids were described.

INTRODUCTION

Although a number of studies have been made on amino acids in urine, many ninhydrin-positive substances excreted in human urine remain unidentified and have not been studied enough. For several years, studies on unknown ninhydrin-positive substances in normal human urine have been carried out in our laboratory and two of them have been isolated and identified as β-hydroxyasparagine\(^{15}\) and O-xilosyl-serine\(^{18}\). Recently, other three amino acids were isolated and identified as O-mannosyl-serine, O-mannosyl-threonine, and isoserine by us.\(^{16}\), \(^{19}\) MAEKAWA\(^{6}\) investigated on the basic amino acid fraction of human urine from normal adult male subjects. The isolation and characterization of four unknown ninhydrin-positive substances in urine are the subjects of this paper.

EXPERIMENTS AND RESULTS

Collection and desalting of urine and fractionation of the desalted ampholytes were carried out by the same methods described by TOMINAGA et al.\(^{15}\), \(^{18}\)

1) Materials

Urine excreted between 10 a.m. and 3 p.m. was collected daily from normal male subjects. After desalted, the ampholytes were pooled
and kept in a refrigerator. Every time when the stored ampholytes reached the amount obtained from 100 l of urine, they were passed through columns of weak basic and weak acidic resins successively, and the resulting effluent was fractionated on the column of Amberlite CG-120. This procedure was carried out once or twice a month all the year round, and the unknown ninhydrin-positive substances which appear through all seasons are the subjects of the present study.

2) Desalting

The urine was decolorized with charcoal (5g per liter) and filtered. A portion of each filtrate (1 l) was placed separately on the column (4.5 x 30cm) of Amberlite CG-120 (100-200 mesh) in the H+ form. Each column was washed with 2 l of water and eluted with 800 ml of 2N ammonia. The eluates were evaporated to dryness at 50°C under reduced pressure. The residue obtained from 10 l of urine was dissolved in 1.5 l of water and subjected to the column (4.5 x 30cm) of Amberlite CG-400 (100-200 mesh) in the OH- form. The column was washed with 2 l of water and eluted with 800 ml of 2N acetic acid. The eluate was evaporated to dryness at 50°C in vacuo.

3) Primary fractionation

The desalted ampholytes obtained from 50 l of urine were passed through columns (4.5 x 30cm) of Amberlite CG-45 (Type I) in the OH- form and CG-50 (Type I) in the H+ form, respectively. Most of the acidic and all of the basic amino acids present were adsorbed by these

![Fig. 1](image-url) The paper chromatograms of the neutral amino acid fractions eluted from Amberlite CG-120 column with 0.2N ammonia.

XS = O-xylosyl-serine
MCS = S-methylcysteine sulfoxide
Hyasp = β-hydroxyasparatic acid
Hyasp.NH2 = β-hydroxyasparagine
resins and removed. The effluent containing the neutral amino acids which was obtained from 100 ℓ of urine was next placed on the column (2.8×40cm) of Amberlite CG–120 (200–400 mesh) in the H\(^+\) form. The adsorbed amino acids were eluted with 0.2N ammonia and the effluent was collected into 20 mℓ-fractions. The amino acids in each fraction were examined by one-dimensional paper chromatography. Toyo Roshi No. 51 paper (40×40cm) and two solvent systems (I) n-butanol, acetic acid, water (4:1:1) and (II) 80% aqueous phenol were used for the paper chromatography.

Fig. 1 shows the result of the primary fractionation.

As shown in Fig. 1, the eluted amino acid fraction was divided into six subfractions by the order of elution, and the subfractions which contained little unknown ampholytes were discarded, except subfraction I and V.

4) Isolation and identification of sarcosine

The mother liquor prepared by removing O-xylosyl-serine (XS) and S-methylcysteine sulfoxide (MCS) from the subfraction I contains several unknown ninhydrin-positive substances, one of which moves to a position close to that occupied by methionine sulfoxide on the two-dimensional paper chromatogram was isolated and identified as sarcosine.

Sarcosine-containing fractions were collected from more than 1000 ℓ of urine, refractionated on the column (2.8×37cm) of Amberlite CG–

![Fig. 2 The infrared absorption spectra of sarcosine (authentic sample) (I) and of the substance isolated from human urine.](image)
400 (200–400 mesh) in the OH⁻ form, and eluted into 20 mℓ fractions with 0.2N acetic acid. Sarcosine was contained in the first three tubes and the tube No. 1 was free from any other ninhydrin-positive substance. The solution in the tube No. 1 was evaporated in vacuo and the resulting residue (320 mg) was dissolved in 2 mℓ water, and to this was added 18 mℓ of ethanol. A small amount of brown precipitate was yielded, but the crystallization was failed. After concentrated again, it was applied to 12 sheets of Toyo Roshi No. 51 paper and developed with the solvent (I). The position of the aiming substance (Rf = 0.28) was cut out and extracted with water. 64 mg of powder was obtained. This material was crystallized twice from 80–90% ethanol. The resulting hygroscopic crystals (20 mg) did not show the definite melting point (203–218°C with decomposition). The crystals were chromatographically identical with the authentic sarcosine (Rf values, 0.28 with solvent (I) and 0.73 with solvent (II). The values of the elementary analysis were C; 39.96%, H; 7.82%, and N; 15.43%. The calculated values for C₃H₇O₂N (sarcosine) were C; 40.44%, H; 7.92%, and N; 15.72%. Fig. 2 shows the infrared absorption spectra of the isolated and authentic sarcosines.

5) The unknown ampholytes in the subfracion V

Subfraction V collected from 100 ℓ of urine was applied to the column (1.3 x 20 cm) of Amberlite CG-400 (200–400 mesh) and eluted into 8 mℓ of fractions with 0.2N acetic acid. Fig. 3 and Fig. 4 show the paper chromatograms of them. In these figures, the spot except the seven known amino acids (glycine, isoleucine, leucine, methionine, phenylalanine, tyrosine, and histidine) indicate the unidentified substances.

![Image of chromatogram](image-url)

Fig. 3 The paper chromatogram of subfraction V eluted from Amberlite CG-400 column with 0.2N acetic acid.
(a) 'A' and 'B'  
Both substances were contained in the earliest portion of the eluate when the subfraction V was eluted from either Amberlites CG-120 or CG-400 column. But the substance 'A' was eluted a little faster than 'B' from the strong acidic resin (Amb. CG-120) and it was in the reverse order from the strong basic resin (Amb. CG-400). They were not decomposed by boiling in 6N HCl for 20 hours. The Rf values of 'A' and 'B' with the solvents (I) and (II) were 0.15, 0.68 and 0.18, 0.81, respectively. Only 'B' could be isolated.

(b) Isolation of 'B'  
The pooled 'B' fraction (80 ml) was applied to a small column (1.3 x 11 cm) of Amberlite CG-400 (200-400 mesh). A portion of ampholytes was flowed out into the effluent (10 ml). The column was eluted with 0.2N acetic acid, and 10 ml of fractions were collected. First three amino acid fractions mainly contained the substance 'B'. They were combined, concentrated, decolorized with charcoal, and filtered. The filtrate (2-3 ml) was added four volumes of ethanol, and amorphous precipitate was yielded. Further, 10 ml of ethanol was added to the solution, and the precipitate was filtered and dried (260 mg). Into 2 ml of water, 200 mg of this powder was dissolved and to this was added 8 ml of ethanol. A small quantity of the amorphous precipitate yielded was removed by filtration, and the filtrate was again added 10 ml of ethanol. A large quantity of the amorphous precipitate (102 mg) was obtained. It decomposed with bubbling at 190-191°C. The isolated 'B' was free from other ninhydrin-reacting substance on the two-dimensional paper chromatogram. The values of the elementary analysis were C; 42.69%, H; 8.00%, and N; 20.04%. Fig. 5 shows the infrared spectrum of the isolated 'B'.
Fig. 5 The infrared absorption spectrum of 'B'

(c) Isolation of 'PC'  An unknown sulfur-containing amino acid which moved to a position near cystine on the two-dimensional paper chromatograms of the subfraction V was frequently found. The substance was referred to as 'pseud cystine' (abbreviated as 'PC'). The selected fractions (20 ml) containing 'PC' were evaporated to a small volume, and 3 volumes of ethanol was added to this. The sparing soluble precipitate was obtained. The precipitate (140 mg) did not dissolve completely in 10 ml of boiling water, however, became soluble by the addition of 1 ml of 2N ammonia. After the filtration, it was added about four volumes of ethanol. The resulting precipitate was filtered, dried, and analyzed subsequently. On the paper chromatograms it revealed one ninhydrin-positive spot close to a position that was occupied by cystine. The Rf values of the isolated 'PC' and the authentic cystine were 0.02 and 0.02 with solvent (I), and 0.21 and 0.29 with solvent (II), respectively. It gave a positive reaction with platinum chloride potassium iodate test (proof of S-). The values of the elementary analysis were C; 34.83%, H; 6.11%, and N; 12.79%. \([\alpha]^3_{D} = +0.16\) (30 mg in 6 ml of 0.1N HCl), \([\alpha]^3_{D} = +0.76\) (30 mg in 6 ml of 1N HCl). The infrared spectrum is shown in Fig. 6.

Fig. 6 The infrared absorption spectrum of 'PC'
The substance 'O'  An unknown amino acid which showed
an orange color with ninhydrin on a paper chromatogram of the sub-
fraction V was isolated and identified as isoserine. The detail of the
isolation and the identification of the substance 'O' will be reported
in another paper.

DISCUSSION

With the introduction of new techniques, many new amino acids
or their derivatives have been discovered in the last twenty years. At
present, more than 170 of natural amino acids are known and during
the last few years new amino acids have been discovered on an average
of ten a year.

Concerning the amino acids in urine, β-amino isobutyric acid, 3-methyl-histidine, tyrosine-O-sulfate, β-hydroxyasparagine, O-
xylosyl-serine, O-mannosyl-serine, and O-mannosyl-threonine, and
isoserine in normal human urine, and arginosuccinic acid, cysta-
thionine, homocystine, 3-methoxy-4-hydroxyphenyl-alanine, iso-
valrthine, 5-amino-4-imidazol-carboxamide-S-5'-homocysteinyl-ribo-
side, homolanthionine, in abnormal human urine have been dete-
cted. In cat urine, special amino acids such as 1-N-methylhistidine,
and feline have been discovered. WESTALL detected 38 unknown
ninhydrin-positive substances besides 27 known amino acids from a
healthy adult male urine. Of these, 28 were labile to acid hydrolysis,
and of the remaining ten, five were those detected after hydrolysis.

According to the methods similar to WESTALL's, we have detected
many of unknown ninhydrin-positive substances whose amounts of the
daily excretion were presumed to be below 10 mg. Of the four subs-
tances described in the present paper, sarcosine only seems to corres-
pond to one of WESTALL's unknown substances. The remaining three referred to as 'A', 'B', and 'PC' were
all stable to acid hydrolysis, and from the results of the experiments
they are believed to be unknown new amino acids.

'PC' is a sulfur-containing amino acid and has close values to cy-
stathionine or lanthionine in the elementary analysis. However, its
infrared spectrum is different from that of cystathionine or of its iso-
mer, methyl lanthionine.

EVERED reported that the amount of daily excretion of sarcosine
in the urine of healthy adult male subjects was 5–9 mg. In the present
study, arather small quantity of sarcosine was isolated from the subfra-
tion I collected more than 1000 L of urine, but there is the possibility
that it may occur in larger amount in subfraction II or III which were
not studied this time.

'Y1', 'Y2' and 'Y3' in the Figs. 3 and 4 refer to proline or hydro-
xyproline-peptides. TOMINAGA et al. have preliminarily reported on
them. On the paper chromatogram, they give yellow color with ninhydrin or isatin. The hydrolysis of each of them yielded glycine and proline from ‘Y’; glycine and hydroxyproline from ‘Y’; and glycine, alanine, proline, and hydroxyproline from ‘Y’. The occurrence of these peptides in urine has not been reported in any other report.

CONCLUSION

The present paper described on the unknown ninhydrin-positive substances in the neutral amino acid fractions of the urine from normal human adults. These substances could be detected by paper chromatography on every 100 ml of column-chromatographed urine collected through all seasons of several years.

One of these unknown substances was elucidated to be sarcosine. The remaining three, ‘A’, ‘B’, and ‘PC’ are all acid stable, and ‘PC’ is a sulfur-containing amino acid. These substances neither correspond to the known amino acids nor were known their properties.

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