FREE AND BOUND AMINO ACIDS IN HUMAN URINE FROM NORMAL SUBJECTS AND PATIENTS WITH TROPICAL SPRUE

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SUMMARY

A comparative study has been made of the urinary excretion of amino acids in free and bound forms in normal South Indian subjects and subjects with sprue.

Chromatographic examination showed a quantitative decrease of urinary amino acids in the sprue group, but no qualitative differences between the two groups. A wide range of ninhydrin-positive compounds was noticed in both groups. A number of these compounds remain unidentified.

Free α-amino acid excretion was markedly reduced in male sprue patients as compared with normal controls. No such decrease was found in the female subjects. The excretion of bound hydroxyproline was increased in both male and female patients with sprue.

A significant lowering in the mean creatinine excretion was noticed in the male sprue patients.

INTRODUCTION

Although extensive work has been done on the excretion of free amino acid in urine, knowledge concerning the excretion of bound amino acids is limited. However, Stein⁴ has made a systematic quantitative study of both free and bound urinary amino acids. The data on the urinary excretion of amino acids in Indian subjects is mostly concerned with nitrogen partition in urine.⁴⁺ The present study was undertaken to investigate the excretion of free and bound urinary amino acids in normal Indian subjects, and in patients with tropical sprue. A brief report of this work has appeared⁴⁺.

MATERIALS AND METHODS

Chemicals

All chemicals were of the analytical grade (E. Merck). The solvents, phenol and n-butanol, were of the chromatography grade; p-dimethylanobenzaldehyde was

recrystallized once from aqueous ethanol; ninhydrin was obtained from Nutritional Biochemicals Corporation.

**Subjects**

Nineteen apparently healthy South Indian subjects (12 males; 7 females; age, 20-40 years) of the lower socio-economic class, who lived largely on vegetarian food and who had no clinical or laboratory evidence of gastrointestinal or metabolic disease were studied as suitable controls. Fifteen patients (9 males; 6 females; age, 20-40 years) with clinical and laboratory features of tropical sprue of similar socio-economic class and dietary habits as the controls, were also studied. Subjects in both the groups were hospitalized and were on a standard vegetarian diet.

**Collection of urine**

A 24-hour collection was made beginning at 8 a.m. one day till 8 a.m. the next day. Chloroform (10 ml) was added to the empty bottle, and toluene (10 ml) was added after the first voiding. The urine was stored in a cold room (0-5°C) and it was processed for analysis immediately or within a few days after collection.

**Preparation of samples for analysis and for paper chromatography**

Urine samples were desalted by Dowex 50 ion exchange fractionation. The break-through capacity of the column used was equal to twice the milliequivalence of the salt in urine, which was seldom more than 0.5 mequiv/ml even in the most concentrated urine. An aliquot (50 ml) of the 24-h urine sample was adjusted to pH 2-3 with hydrochloric acid. Since undiluted urine retarded the flow rate, the aliquot was diluted to 100 ml with water, and one third of this was applied separately to each of three Dowex 50X8, H⁺ form, columns (50 x 10 mm). The columns were then washed with water (usually 20 ml) until the pH of the washings was neutral. The breakthrough fluid plus washings were designated "urine break-through" fraction. The amino acids were then eluted from the column by 1 N ammonium hydroxide, and the eluate collected in fractions (20 ml). Fractions containing amino acids as determined by spot tests with ninhydrin were pooled, concentrated by lyophilising (VirTis mechanical freeze dryer), made up to 2.0 ml with isopropanol (100%, 0.1 N HCl) and stored at 4°C. This fraction was designated "urea ammonia eluate". Little hydrolysis of peptides, glutamine or asparagine was detected on storage under these conditions. The ammonia eluates used for chromatography contained free amino acids and peptides originally present in the urine, except phenylacetylglutamine, taurine, hippuric and cystic acids which were present in the break-through fraction.

**Paper chromatography**

The procedure of Subramanian and Rao was found to give the best resolution for a single two-dimensional run. The first solvent was buffered phenol (7 ml of 0.2 M KCl-HCl buffer, pH 10.0, in 50 ml of phenol) in the longer direction (I) of the paper and in the second direction (II) the solvent was butanol-acetic acid-water (4:1:1:1). Descending technique was employed, at room temperature (27-29°C). Ten µl of the ammonia eluate (corresponding to 0.25 ml of original urine) was applied on Whatman No. 1 papers (381 x 224 mm), which were previously treated with 0.2 M KCl-HCl buffer, pH 10.0, and dried. After the phenol run (20-24 h), the papers were dried at room

temperature overnight, and the edge (ca. 2.5 cm) trimmed off before the butanol run.
Using a standard amino acid mixture under these conditions, the leucines were not
separated, methionine was not quantitatively converted to the sulphoxide and the
separation of basic amino acids was found to be very much dependent on temperature,
the separation being poor at higher temperatures.

Special tests were made for arginine, histidine, tyrosine, proline, hydroxypro-
line, citrulline and tryptophan using the multiple sector technique of circular chro-
matography.

**Hydrolysis**

Urine ammonia eluates (0.2 ml) were hydrolyzed in sealed tubes at 110°, and
whole urine (50 ml) under reflux, with an equal amount of conc. HCl for 24 h. After
removal of the acid on a water bath, the hydrolyzates were dissolved in the original
volume of isopropanol (10% in 0.1 N HCl).

**Total amino acids**

The total amino acids in the ammonia eluates and in the hydrolyzates were
determined by the ninhydrin procedure of Rosen. The excretion of amino acids, as
mg α-amino acid N per day, was calculated from the leucine equivalents. The dif-
ference between the values of ammonia eluates before and after acid hydrolysis, was
considered to be due to amino acids in bound form. The values were corrected, where
necessary, for contribution from urea, but in the majority of cases this was negligible.

**Hydroxyproline**

Hydroxyproline was measured according to the method of Neuman and Logan with slight modifications.

**Urea**

Urea was determined by the method of Archibald.

**Creatinine**

Creatinine was determined by the Jaffe reaction using recrystallized picric acid.

**RESULTS**

**Qualitative analysis of amino acids**

Free amino acids (control subjects). By examination of paper chromatograms
the identity of several amino acids was established. Alanine, glycine, glutamine,
histidine and serine were the most prominent amino acids. Asparagine, cystine, lysine,
threonine and methionine sulfoxide (probably an artifact of methionine during phenol
chromatography) were easily detectable. Aspartic acid and the fast running amino
acids—leucines, valine, phenylalanine and tyrosine—were barely detectable. Arginine
could be detected in a few cases only by the sensitive Sakaguchi test.

In addition to the amino acids that were identified, the two-dimensional chro-
atograms (Fig. 1a) showed a number of ninhydrin-positive acid-stable compounds
(a-l) whose identity has not yet been established. The two spots “A” and “B”, which
were seen in a number of chromatograms, gave a brownish-orange colour with nin-
hydrin and were acid-labile.

Fig. 1. Diagrams of typical chromatograms showing the ninhydrin-stained spots in normal subjects. (a) urine ammonia eluate; (b) urine ammonia eluate hydrolyzate.

1. L-alanine; 2. arginine; 3. asparagine; 4. aspartic acid; 5. cystine; 6. glutamic acid; 7. glutamine; 8. glycine; 9. histidine; 10. hydroxyproline; 11. isoleucine; 12. leucine; 13. lysine; 14. methionine sulfoxide; 15. phenylalanine; 16. proline; 17. serine; 18. threonine; 19. tyrosine; 20. valine. Unidentified spots: A (above cystine) and B (below aspartic acid)—orange coloured spots, acid-labile; acid-stable purple spots; a (near cystine), b (near arginine) and c, d, e and f (around methionine sulfoxide), g and h (above hydroxyproline), i and j (left of proline), k (left of proline).

Amino acids in hydrolysates (control subjects). Chromatography of ammonia eluates before and after acid hydrolysis showed (Fig. 1 a and b) that a number of amino acid spots increased in intensity. Aspartic acid and arginine, which were barely detectable before hydrolysis, gave prominent spots. Striking increases were also observed in the leucine–isoleucine, valine and phenylalanine spots. Unidentified

Fig. 2. Diagrams of chromatograms of urine from sprue patient with very low amino acid excr. (a) urine ammonia eluate; (b) urine ammonia eluate hydrolyzate. For explanation refer to legend in Fig. 1.

spot "g" which was occasionally noticed in ammonia eluates was present in hydrolysates of all samples. Proline and hydroxyproline were identified by special tests.

**Free amino acids (sprue patients).** The most striking feature was that although the general chromatographic pattern of amino acids was comparable to that of normal urine, the intensity of a number of spots was greatly reduced. The spots due to the unidentified compounds found in the control urines were also noticed. In a few cases only 3 spots could be detected and the number increased on hydrolysis (Fig. 2 a and b).

**Amino acids in hydrolysates (sprue patients).** The results were generally similar to those of the control subjects. Aspartic acid, arginine, proline and hydroxyproline were detected only in the hydrolysates.

**Quantitative analysis**

**Creatinine.** The mean 24-h excretion in the group of normal males was 1191 mg, (Table I A) and the excretion in the group of male patients with sprue was 651 mg (Table I B). The difference between these two means is significant at the 1% level

**TABLE I A**

<table>
<thead>
<tr>
<th>Age</th>
<th>24-hour excretion, mg</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Creatinine</td>
<td>a-Amino acid</td>
<td>N</td>
<td>Free</td>
<td>Free-bound</td>
<td>Bound</td>
<td>Hydroxyproline (Bound)</td>
</tr>
<tr>
<td>Range</td>
<td>20-40</td>
<td>428-1625</td>
<td>10100</td>
<td>51-791</td>
<td>35-631</td>
<td>5.1-15.8</td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>30</td>
<td>1191</td>
<td>84</td>
<td>381</td>
<td>297</td>
<td>11.9</td>
<td></td>
</tr>
<tr>
<td>Std. error of the mean</td>
<td>1.96</td>
<td>87.8</td>
<td>11.12</td>
<td>69.66</td>
<td>63.10</td>
<td>1.0</td>
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**TABLE I B**

<table>
<thead>
<tr>
<th>Age</th>
<th>24-hour excretion, mg</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
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<tbody>
<tr>
<td></td>
<td>Creatinine</td>
<td>a-Amino acid</td>
<td>N</td>
<td>Free</td>
<td>Free-bound</td>
<td>Bound</td>
<td>Hydroxyproline (Bound)</td>
</tr>
<tr>
<td>Range</td>
<td>23-37</td>
<td>63-1104</td>
<td>30-101</td>
<td>42-868</td>
<td>8-767</td>
<td>6.0-38.2</td>
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<tr>
<td>Mean</td>
<td>28</td>
<td>631</td>
<td>52</td>
<td>191</td>
<td>139</td>
<td>21.5</td>
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</tr>
<tr>
<td>Std. error of the mean</td>
<td>1.73</td>
<td>38.5</td>
<td>7.39</td>
<td>90.16</td>
<td>81.8</td>
<td>4.15</td>
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</table>

(\( t = 5.63 \)). The mean 24-h excretion in the group of normal females was 554 mg (Table II A) and the excretion in the group of female patients 467 (Table II B). This difference is not significant.

**a-Amino acid nitrogen.** The results of a quantitative determination of free and bound amino acid nitrogen in urine, from control subjects and sprue patients are presented in Tables I and II. The bound amino acid N in both normal subjects and in sprue patients represented a large proportion of the total a-amino acid N, the

**TABLE II A**

FEMALE SUBJECTS (CONTROLS): DAILY EXCRETION OF CREATININE AND AMINO ACIDS

(No. of cases: 7)

<table>
<thead>
<tr>
<th>Age</th>
<th>24-hour excretion, mg</th>
<th>Creatinine</th>
<th>α-Amino acid N</th>
<th>Hydroxyproline (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Free</td>
<td>Free+Bound</td>
<td>Bound</td>
</tr>
<tr>
<td>Range</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>St. error of the mean</td>
<td>1.29</td>
<td>91.8</td>
<td>13.77</td>
<td>57.60</td>
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</table>

**TABLE II B**

FEMALE SUBJECTS (SPRUE): DAILY EXCRETION OF CREATININE AND AMINO ACIDS

(No. of cases: 6)

<table>
<thead>
<tr>
<th>Age</th>
<th>24-hour excretion, mg</th>
<th>Creatinine</th>
<th>α-Amino acid N</th>
<th>Hydroxyproline (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Free</td>
<td>Free+Bound</td>
<td>Bound</td>
</tr>
<tr>
<td>Range</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>St. error of the mean</td>
<td>1.73</td>
<td>51.1</td>
<td>24.98</td>
<td>73.98</td>
</tr>
</tbody>
</table>

average value being 2–3 times that of the free α-amino acid N. The daily excretion of free α-amino acid N in male subjects with sprue (52 mg/day) was markedly reduced as compared with that in the controls (84 mg/day). This difference is significant at the 5% level (t = 2.4). The mean 24-h excretion of bound amino acid N in the male controls was 207 mg and in the male sprue subjects 139 mg. Although the results are not statistically significant (P between 0.1 and 0.2) it is to be noted that there were 2 very high values in the sprue group and 3 unusually low values in the control group. In the males with sprue the excretion of bound amino acids, therefore, tends to be lower than in the controls. The excretion of bound amino acid N in the female controls was 128 mg and in the female sprue patients, 137 mg. The difference is not significant.

Hydroxyproline. The excretion of bound hydroxyproline in normal subjects and sprue patients is given in Tables I and II. The mean excretion in the sprue subjects (males, 21.3 mg; females, 19.6 mg/day) was higher than in the control subjects (males, 11.6 mg; females 10.5 mg/day). The difference is significant at the 5% level (t = 2.65 (females); 2.27 (males)).

**Comparison of total urine hydrolyzate and ammonia eluates**

In the desalting procedure employed in this study, some of the ninhydrin-positive compounds were not retained on the column and were present in the breakthrough fluid. In order to determine the nature of the materials in this fraction, the α-amino acid N in a total urine hydrolyzate of one sample from a sprue patient was analyzed and a comparison made with ammonia eluates obtained from the same urine.

TABLE III
COMPARISON OF TOTAL URINE HYDROLYZATE AND URINE AMMONIA ELUATE IN A SPREE PATIENT

<table>
<thead>
<tr>
<th>No.</th>
<th>Fraction*</th>
<th>24-h excretion, mg</th>
<th>z-Amino acid N</th>
<th>Hydroxyproline</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Urine hydrolyzate</td>
<td>103.2</td>
<td>42.5</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Urine hydrolyzate ammonia eluate</td>
<td>96.2</td>
<td>40.7</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Urine ammonia eluate</td>
<td>48.4</td>
<td>0.0</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Urine ammonia eluate hydrolyzate</td>
<td>99.7</td>
<td>22.0</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>Urine break-through</td>
<td>6.6</td>
<td>0.0</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>Urine break-through hydrolyzate</td>
<td>10.6</td>
<td>21.2</td>
<td></td>
</tr>
</tbody>
</table>

* Fraction 1: Acid hydrolyzate of whole urine; Fn. 2: whole urine hydrolyzate passed through Dowex 50 resin column and then eluted with ammonia; Fn. 3: whole urine passed through resin column and then eluted with ammonia and eluate analyzed; Fn. 4: hydrolyzate of Fn. 3; Fn. 5: Break-through fluid from whole urine after passage through resin column; Fn. 6: hydrolyzate of Fn. 5. For conditions of hydrolysis see text.

sample. The results are shown in Table III. It can be readily seen that the sum of the quantity of z-amino acid N in urine ammonia eluate-hydrolyzate and urine break-through fluid-hydrolyzates (116.3 mg; sum of 4 and 6) is close to the value (103.2 mg) obtained in the case of whole urine hydrolyzate. Under the conditions of hydrolysis, urea present in whole urine is not completely destroyed (cf. Stein9) and so, the corrected ninhydrin values have been given. The urine break-through fluid hydrolyzate contained a few amino acids, two of which correspond in N values to cysteic acid and taurine. The other amino acids: alanine, glutamic acid and glycine have been estimated by the procedure of Giri et al.10 in the urine break-through fluid (alanine 1.0; glutamic acid 3.2; glycine 5.0 mg/day) and in urine break-through fluid hydrolyzate (alanine 4.0; glutamic acid 36.0; glycine 20.0 mg/day). Acid hydrolysis of an ethyl acetate extract of the urine break-through fluid gave glycine and glutamic acid, suggesting that these amino acids were derived from hippuric acid and phenylacetyl-glutamine, respectively. The small amounts of glutamic acid and glycine in the unhydrolyzed break-through fluid probably arise from the conjugates during concentration of the fraction at acidic pH. Hydroxyproline is present in the hydrolyzates of both the urine ammonia eluate and in the break-through fractions.

DISCUSSION

The "spectra" of urinary amino acids in the normal adult human are very complex. King11 has reported recently the existence of at least 81 ninhydrin-positive materials in the urine and has been able to identify about 49 of them. Thus, a large proportion of them require further characterization. The quantity of processed urine employed in the present study is at least 10 times the quantity generally employed in urine chromatography thus enabling detection of amino acids present only in traces. A characteristic pattern noticed on the chromatograms of all urine samples was the location of a group of unidentified ninhydrin-positive compounds (c, d, e and f) in an area which is usually clear in two-dimensional chromatograms of many biological materials.

Chromatograms of hydrolyzates of urine ammonia eluates showed increases in the intensity of a number of spots with concomitant disappearance of some of them.

Aspartic acid, arginine, proline and hydroxyproline generally showed up only on hydrolysis. The presence of asparagine and of bound hydroxyproline is now well known.

In the sprue cases the pattern was quite similar to the normals, provided adequate quantities were employed for chromatography. However, using processed urine equivalent to 0.25 ml of original urine there is a considerable reduction in the intensity of the spots, and in some cases only three amino acid spots were noticed.

A notable exception to this generalized reduction in urinary amino acids was the excretion of bound hydroxyproline which was almost doubled in the sprue patients. In one case in which it was studied, hydroxyproline was also present in a bound form in the break-through fluid. The new hydroxyproline-containing fraction was purified by DEAE-Sephadex fractionation and was found to contain proline, hydroxyproline and a number of other amino acids, anthrone-positive materials, hexosamine and uronic acid (M. G. Cherian and A. N. Radhakrishnan, unpublished results). Westfall and Jagenburg reported the presence in urine of a dipeptide of proline and hydroxyproline. The imino acid hydroxyproline is rarely present in the free form in urine of adults, although in our studies it was encountered in one case (subject, Kan). We have also detected it in the free state in a case of Fanconi syndrome, while Jagenburg reported its absence in a case he studied. The significance of increased excretion of hydroxyproline in sprue cases is not known.

Quantitative data on the excretion of z-amino acid N indicated that a significant portion of the total z-amino acid N was present in bound form, presumably as peptides or as derivatives of amino acids like tyrosine-O-sulphate. In the present study the quantity of bound form of z-amino acid N excreted per day was 2–3 times that of free z-amino acid N both in sprue and in normals. This data is similar to the values of free and bound amino acids reported by Stein in whole urine and to the values of free amino acids obtained by Khachadurian et al. The occurrence of a variety of peptides in urine has been reported by Buchanan et al. However, since the presence of peptides in normal urine is still considered a matter of debate the results presented in this paper are significant.

Since ammonia eluates used in the present study do not include hippuric acid and other amino acid conjugates which are not retained on the Dowex 50 resin, the quantity of total bound forms of amino acids will be higher if the conjugates are also included. Stein has stated that only a small portion of the bound forms of the amino acids in urine can be accounted for as hippuric acid, glutamine and asparagine.

A comparison of male subjects with sprue showed that there was a significant reduction in the excretion of bound forms of amino acids. In the female subjects there was no significant difference between the normal and sprue groups. It is also to be noted that even in the population of normal female subjects selected for this study the amino acid excretion was low compared to that of male subjects. The difference is much larger than could be accounted for on the basis of weight difference between the two sexes. Further work on a larger sample of subjects is needed to substantiate this finding. Jagenburg and Soupart found no significant difference in the amino acid excretion between the two sexes.

Santini et al. have studied the excretion of glycine in patients with sprue. Although the 24-h excretion of glycine was similar in the sprue patients and in normal subjects, the glycine N as a percentage of total z-amino acid N was significantly higher.
in the patients. They have also noted lowered excretion of total α-amino acid N in patients with untreated tropical sprue.

The mean excretion of creatinine in the male subjects with sprue (651 mg/day) was lower than that of the control male subjects (1191 mg/day) but no such difference was found in female subjects. Santini et al. noted a similar lowering in the excretion of creatinine in sprue patients in Puerto Rico but the sex of their patients is not specified. The reason for the sex difference in creatinine excretion is at present not clear.

Variations in the levels of excretion of individual amino acids in subjects with sprue would, therefore, be of considerable interest, especially with regard to possible defects of enzyme systems involved in their metabolism.

ACKNOWLEDGEMENT

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REFERENCES
