Micromethods for Measuring Phenylalanine and Tyrosine in Serum

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Spectrophotofluorometric micromethods for the determination of phenylalanine and tyrosine on 25 μl of serum are described. These methods were applied to detect phenylketonuria among homozygotes and heterozygotes and in the newborn population. The data presented agree with those previously reported. (1) Phenylketonurics have markedly elevated serum phenylalanine and lower serum tyrosine than the controls. (2) The heterozygotes have a higher fasting serum phenylalanine and, after a standard oral phenylalanine test, show a higher and more prolonged rise of serum phenylalanine than the controls. There was a lesser increase of serum tyrosine after phenylalanine loading in the heterozygotes than the controls. (3) Premature infants of low birth weight have higher serum phenylalanine and tyrosine levels than normal birth weight infants, presumably due to enzyme immaturity. Simultaneous determinations of serum phenylalanine and tyrosine will differentiate newborn infants who are suspected to be phenylketonurics from the homozygotes (phenylketonurics).

Phenylketonuria is an hereditary condition characterized by mental retardation and the presence of phenylpyruvic acid in the urine. The condition is caused by a deficiency of activity of hepatic phenylalanine hydroxylase; the concentration of phenylalanine in the serum is greater and that of tyrosine lower than normal. The purpose of the present paper is to describe micromethods for the determination of serum phenylalanine and tyrosine and to illustrate their use in detecting phenylketonuria.

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Serum Phenylalanine

The procedure for the determination of phenylalanine is a modification of the spectrophotofluorometric method of McCaman and Robins (1) based on the principle that the fluorescence of the phenylalanine-ninhydrin-copper complex is enhanced by L-leucyl-L-alanine.

Reagents

(a) Buffered ninhydrin-peptide reagent is prepared daily by mixing 5 volumes of 600 mM succinate buffer (sodium salt) at pH 5.88 with 2 volumes of 30 mM solution of ninhydrin (5.34 gm./L.) and 1 volume of 5 mM L-leucyl-L-alanine (1.01 gm./L.). (b) Copper reagent is prepared daily by mixing 3 volumes of a 25 mM solution of sodium carbonate containing 0.40 mM Rochelle salt (2.66 gm. Na₂CO₃ and 113 mg. KNaC₄H₄O₆·4H₂O in 1 L. aqueous solution) with 2 volumes of 0.8 mM copper sulfate (200 mg. CuSO₄·5H₂O in 1 L. aqueous solution). (c) 0.6N trichloroacetic acid (98.0 gm./L.). (d) Phenylalanine standards are prepared by dissolving 5, 10, and 20 mg. of L-phenylalanine in 100 ml. of solution containing 7.5 gm. of bovine serum albumin. (e) The blank contains 7.5 gm. of bovine serum albumin in 100 ml. of aqueous solution.

Components of reagents (a) are refrigerated at 0–4°C; those of reagents (b) and (c) are stable at room temperature. Small aliquots of reagents (d) and (e) are kept frozen to prevent bacterial and fungal growth and thawed prior to use.

Procedure

(a) Add 25 μl. of trichloroacetic acid to 25 μl. of serum or heparinized plasma, blank, and standard; mix. Allow to stand for 10 min. and centrifuge at 5000 g for 5 min. (b) To 20 μl. of clear supernatant, add 300 μl. of buffered ninhydrin-peptide reagent, mix, and incubate at 60° for 2 hr. (c) Cool the mixture by immersion in cold tap water (about 20°) and add 2.0 ml. of copper reagent and mix. (d) Within 1 hr., determine the relative fluorescence at 515 mμ with an Aminco-Bowman* spectrophotofluorometer using an activating wavelength of 365 mμ.

Evaluation

Figure 1 shows the relation of pH to relative fluorescence in the determination of phenylalanine. An increase of 21.4% in relative fluorescence resulted from an increase of 0.10 in pH. Therefore, in addi-

*American Instrument Company, Inc., Silver Spring, Md.
tion to increasing the molarity of the buffer to stabilize the pH, bovine serum albumin in concentrations of 7.5 gms./100 ml. was added to the standard solution of phenylalanine to make the pH of the reaction mixture for the serum the same as that for the standard and equal to 5.80.

For the determination of phenylalanine in urine, bovine serum albumin was omitted from the standards and the blank. The relative fluorescence was proportional to the concentration of phenylalanine after incubating for 2 hr. at an optimal temperature of 60°. With the incubation mixture at pH 5.80, equimolar concentrations of other compounds produced fluorescence relative to phenylalanine as follows: tyrosine 5%, tryptophan 1.2%, leucine 1.1%, tyramine 0.6%, phenylactic acid 0.3%, arginine 0.1%, phenylacetic acid 0%, phenylpyruvic acid 0%. The recovery of phenylalanine added to serum averaged 102 ± 3.6%. The concentrations of phenylalanine in 100 samples of frozen serums determined on another day were the same as those before freezing. The correlation coefficient was +0.99, indicating that freezing the serums did not affect the level of phenylalanine. The phenyl-

**Fig. 1.** Effect of varying pH of incubation upon relative fluorescence in the phenylalanine method.
alanine concentration of ten replicates of a single serum sample averaged 15.2 mg./100 ml. with a standard deviation of ±0.3.

**Serum Tyrosine**

The procedure for the determination of tyrosine is a modification of the method described by Udenfriend (2) which is based upon the principle that tyrosine reacts with α-nitroso-β-naphthol to form a fluorescent product.

**Reagents**

(a) α-Nitroso-β-naphthol reagent: before use, mix 2 volumes of α-nitroso-β-naphthol solution, prepared by dissolving 200 mg./100 ml. of 95% ethyl alcohol and removing any sediment by filtration, with 3 volumes of 3.0N nitric acid (18.9% (v/v) solution) and 3 volumes of 0.10N sodium nitrite (6.90 gm./L.). (b) Ethylene dichloride. (c) 0.6N trichloroacetic acid (98.0 gm./L.). (d) Tyrosine standards by dissolving 2.5, 5, and 10 mg. in 100 ml. of water. (e) Blank of distilled water.

Components of reagents (a) and (d) are refrigerated at 0–4°.

**Procedure**

(a) Add 25 µl. of trichloroacetic acid to 25 µl. of serum or heparinized plasma, blank, and standard. Mix, allow to stand for 10 min., and centrifuge at 5000 g for 5 min. (b) To 20 µl. of clear supernatant add 200 µl. of α-nitroso-β-naphthol reagent; mix and incubate at 33° for 20 min. (c) Add 1 ml. of water and 3 ml. of ethylene dichloride, mix, and centrifuge. Transfer the aqueous phase (top) to another test tube and incubate at room temperature (about 25°) for 40 min. (d) Within 30 min. determine the relative fluorescence at 570 mµ with an Aminco-Bowman spectrophotofluorometer using an activating wavelength of 460 mµ.

**Evaluation**

Figure 2 shows the relation of relative fluorescence to incubation temperature. The relative fluorescence was the same over a range of incubation temperatures from 31° to 35°. At the midpoint (33°) of the plateau, maximum fluorescence was attained with 20 min. of incubation when followed by standing at room temperature (about 25°) for 40 to 70 min. Compared with tyrosine, equimolar concentration of other related compounds gave the following relative fluorescence:
tyramine 150%, 5-hydroxytryptophan 3.2%, 5-hydroxyindoleacetic acid 2.4%, tryptophan 1.3%, phenylalanine 0.1%, and tryptamine 0.1%. Recovery of tyrosine added to serum averaged 99.2 ± 7.9%. The concentrations of tyrosine in 100 samples of frozen serums determined on another day were the same as those before freezing; the correlation coefficient was +0.98. The tyrosine concentration of ten replicates of a single serum sample averaged 9.92 mg./100 ml. with a standard deviation of ±0.30.

**Results**

The mean and standard deviation for adults are given in Table 1. Fasting phenylalanine and tyrosine levels are shown for controls (normal adults), heterozygotes (parents of phenylketonurics), and homozygotes (phenylketonurics). The controls and heterozygotes were also given a standard oral phenylalanine test of 0.1 gm./kg. body weight of L-phenylalanine dissolved in fruit juice and serum samples were obtained 1 and 2 hr. after the load (3).
Table 1. Mean and Standard Deviation of Serum Phenylalanine and Tyrosine Level among Controls, Heterozygotes, and Homozygotes for Phenylketonuria

<table>
<thead>
<tr>
<th>Sample from</th>
<th>Phenylalanine (mg./100 ml.)</th>
<th>Tyrosine (mg./100 ml.)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Fasting</td>
<td>1 hr.</td>
</tr>
<tr>
<td>Controls</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No.</td>
<td>Mean ± S.D.</td>
<td>No.</td>
</tr>
<tr>
<td>90</td>
<td>1.55 ± 0.34</td>
<td>13.26 ± 3.97</td>
</tr>
<tr>
<td>Heterozygotes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No.</td>
<td>1.93 ± 0.53</td>
<td>16.85 ± 4.10</td>
</tr>
<tr>
<td>Homozygotes</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 2. Mean and Standard Deviation of Serum Phenylalanine and Tyrosine for Infants of Normal and Low Birth Weight as Compared with Adult Controls

<table>
<thead>
<tr>
<th>Sample from</th>
<th>Phenylalanine (mg./100 ml.)</th>
<th>Tyrosine (mg./100 ml.)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No.</td>
<td>Mean ± S.D.</td>
</tr>
<tr>
<td>Controls</td>
<td>90</td>
<td>1.55 ± 0.34</td>
</tr>
<tr>
<td>Normal birth weight</td>
<td>100</td>
<td>2.16 ± 0.49</td>
</tr>
<tr>
<td>newborns</td>
<td>50</td>
<td>4.29 ± 0.54</td>
</tr>
</tbody>
</table>

The mean and standard deviation for infants of normal and low birth weights are given in Table 2. These were similar to previous data published from this laboratory (4).

Finally, the phenylalanine method was used to screen 4000 newborn infants for phenylketonuria (5). The mean and standard deviation for this group were 2.1 ± 0.5 mg./100 ml. The values were not significantly influenced by maternal age or gravidity, nor by the sex, color, birth weight, or age of the infants. The lowest recorded serum phenylalanine on a phenylketonuric infant was about 7 standard deviations above the mean for the normal newborn population.

References