

## Microbiological Determination of Free Amino Acids in Human Plasma

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Microbiological determinations of free amino acids have been carried out in deproteinized plasma of a number of species<sup>1-5</sup>. The assay procedure is particularly well suited for routine analyses of material containing small amounts of amino acid. However, such materials may contain minimal amount of peptides<sup>6</sup> and the lactic acid bacteria may also utilize an essential amino acid in combined form<sup>7, 8</sup>. The use of these methods without preliminary hydrolysis may accordingly be criticized. Nevertheless, such methods are useful when comparative results are desired and they might conceivably become a useful tool for investigations of pathological conditions.

Hier and Bergeim<sup>4</sup> used 16 ml of human plasma from 40-50 ml of blood for the analysis of the amino acids in tungstic acid filtrates. The volume of the final medium was 2 ml per tube. By diminishing the final volume of the medium to a tenth of this amount, it seemed probable that a determination of the 18 amino acids, (which are analyzed) by microbiological methods could be carried out on 4 ml of plasma from about 10 ml of blood. However, the repeated withdrawal of 10 ml of blood from the organism may be a serious draw-back, especially when working with small animals. A micro adaptation of the microbiological procedure was accordingly<sup>9</sup> found where the final developed volume of basal medium corresponded to a drop, 45  $\mu$ l. Assays of 16 amino acids in a casein hydrolysate indicated that values could be obtained which closely agreed with those obtained in macroprocedures. In the studies reported here this method has been employed for the determination of free\* amino acids in human plasma deproteinized with tungstic acid, trichloroacetic acid, ferric sulfate, or ethanol.

\* Free as used throughout implies microbiologically available amino acids, and may include combined forms as well as free amino acids.

## MATERIAL AND METHODS

## Preparation of plasma filtrates

The human subjects were volunteer medical students who had not fasted previous to removal of the 10 ml sample used for test. Clotting was prevented by the use of heparin. The blood from 20 students was pooled and centrifuged for 20 minutes. The plasma was divided in four parts. The first was precipitated with sulfuric acid and sodium tungstate as described by Hier and Bergeim<sup>3</sup>. The precipitate was removed by centrifugation. The pH was adjusted with 2 N NaOH to pH 6.8. A few drops of sodium hydroxide were usually adequate, thus having a negligible effect on the volume. Each ml filtrate represented 0.33 ml of original plasma. The filtrate was concentrated *in vacuo* to a third of the original volume. Each ml thus represented 1 ml of original plasma. The manometric  $\alpha$ -amino nitrogen value was 0.061 mg per ml.

A second part of the pooled plasma was diluted with an equal volume of distilled water and a half volume of a fresh 50 per cent solution of trichloroacetic acid was slowly added with mixing. The final concentration of trichloroacetic acid was 10 per cent. The reagent was mainly removed by four extractions with ethyl ether in a separating funnel as previously described<sup>10</sup>. There was no trace of amino acids in the ether washings. The pH of the water phase was pH 5 and was brought to pH 7 with a few drops of 0.1 N NaOH. The water solution was concentrated *in vacuo*. 1 ml of the concentrated filtrate corresponded to 1 ml of plasma. The manometric  $\alpha$ -amino nitrogen value was 0.069 mg per ml.

A third part of plasma, 20 ml, was deproteinized with ferric sulfate and sodium hydroxide according to Somogyi<sup>11</sup>. By this procedure the plasma filtrate contains a comparatively large amount of sodium sulfate which was practically removed as previously described<sup>10</sup> by concentrating the filtrate to 10 ml, followed by addition of 10 ml of absolute ethanol. The precipitate of sodium sulfate was redissolved in a minimal volume of water and precipitated twice at an ethanol concentration of 50 per cent. The combined ethanol centrifugates were concentrated to dryness and dissolved in distilled water. Each ml of solution corresponded to 1 ml of plasma. The manometric  $\alpha$ -amino nitrogen value was 0.061 mg per ml.

A fourth part of the plasma was precipitated with four volumes of absolute ethanol. The precipitate was centrifuged and washed with 80 per cent ethanol. The combined centrifugate were concentrated *in vacuo* to a small volume and finally to dryness in a desiccator over P<sub>2</sub>O<sub>5</sub>. The dried material was extracted twice with dry ethyl ether and dissolved in distilled water. Each ml of solution represented 1 ml of original plasma. The manometric  $\alpha$ -amino nitrogen value was 0.034 mg per ml. The four concentrated filtrates were preserved in a frozen state under toluene until analyzed.

## Assay procedure

The general procedure has previously been described<sup>9</sup>. The sterilized concentrated filtrates were aseptically measured into the micro tubes either directly or in dilutions where 1 ml represented 0.66 or 0.33 ml of original plasma. The measured volume ranged from 25 to 3  $\mu$ l and water was added to bring the volume to 25  $\mu$ l in all tubes. 25  $\mu$ l of the double strength, sterilized and inoculated basal medium were aseptically added to each tube. Microorganisms, basal medium, ranges of standard curves, and incubation

Table 1. *Experimental conditions for the microbiological analyses.*

Amino acid	Medium	Microorganism	Standard curve $\mu\text{g}$ per 50 $\mu\text{l}$	Incubation time, hours
Alanine	Dunn <i>et al.</i> <sup>12</sup>	<i>L. citrovorum</i> (8081)	125–1000	48
Aspartic acid	Henderson and Snell <sup>13</sup>	<i>L. mesenteroides</i> P–60	62–500	48
Cystine	» » »	<i>L. arabinosus</i> 17–5	15–125	24
Glutamic acid	» » » *	» » »	125–1000	»
Glycine	Dunn <i>et al.</i> <sup>12</sup>	<i>L. citrovorum</i> (8081)	62–500	48
Leucine	Henderson and Snell	<i>L. arabinosus</i> 17–5	31–250	48
Proline	» » » *	<i>L. mesenteroides</i> P–60	62–250	48
Serine	» » » **	<i>L. casei</i> (7469)	125–1000	48
Threonine	» » » ***	<i>L. fermenti</i> 36	125–750	48
Valine	» » »	<i>L. arabinosus</i> 17–5	62–500	48

\* pH of basal medium 6.0.

\*\* Single-strength medium diluted with an equal volume of water<sup>9,14</sup>.

\*\*\* Citrate exchanged for acetate<sup>21</sup>.

times are given in Table 1. The procedures followed for the culture and inoculum have been described in previous papers<sup>9, 14, 15</sup>. A casein hydrolysate was always included as an extra control in the determinations of the amino acids. For each amino acid several separate assays were carried out with an average deviation of approximately  $\pm 10$  per cent for all amino acids. In each series, five assay levels were used. The amino acids used as standards were dried *in vacuo*, at room temperature, and kept *in vacuo* in a desiccator containing silica gel. DL-forms of threonine and valine were employed. The natural isomers of the others were used.

## RESULTS AND DISCUSSION

Three of the investigated amino acids, leucine, threonine, and valine were analyzed to facilitate a comparison with data obtained by Hier and Bergeim<sup>4</sup> on free amino acids in human plasma. The remaining seven compounds have not been determined in human plasma with microbiological methods, but alanine and glycine have been analyzed by Gutman and Alexander<sup>16</sup> by means of chemical methods. It was accordingly possible to control the reliability of several of the results obtained with the micro method used in the present investigation.

It was also possible to determine whether the use of tungstic acid filtrates, three times as concentrated as those used by Hier and Bergeim<sup>3</sup>, had any effect on the microbiological data relative to that obtained with plasma

filtrates prepared by trichloroacetic acid, ferric, sulfate, and alcohol precipitation, where the precipitating agents had been removed from the filtrate. Such a comparison would be of interest since it could be supposed that some of the amino acids to be analyzed, aspartic acid, cystine and serine were present in such small amounts that it was necessary to use concentrated tungstic acid filtrates. The results obtained are summarized in Table 2.

Table 2. Amino acid content of normal human plasma precipitated in four different ways.

Reported as microgram of amino acid per ml of plasma.

Amino acid	Tungstic acid filtrate (I)	Trichloroacetic acid filtrate (II)	Ferric hydroxide filtrate (III)	Filtrate from ethanol precipitation (IV)	Filtrate * dilution
Alanine	36.0	42.0	44.0	16.2	1.0
Aspartic acid	6.2	4.0	7.2	2.4	1.0
Cystine	7.0	6.6	0.8	4.2	0.33
Glutamic acid	36.4	35.4	40.0	7.5	0.33
Glycine	22.0	18.0	18.0	6.2	0.66
Leucine	17.2	11.8	19.5	6.5	0.33
Proline	41.0	38.2	36.5	13.5	0.66
Serine	5.6	9.8	8.2	2.8	1.0
Threonine	14.0	—	13.5	5.4	1.0
Valine	29.4	28.0	26.8	7.5	0.33

\* The figures 0.33, 0.66 and 1.0 means that each ml of filtrate represents the corresponding volumes of human plasma.

The data indicate several interesting points. With tungstic acid precipitation, and in the filtrate dilution, 0.33, used by Hier and Bergeim<sup>4</sup> the remaining part of this acid in the filtrate does not inhibit the growth of the tested microorganism. The leucine and valine values obtained with filtrates I—III agree fairly well and also with those obtained by Hier and Bergeim. Cystine has not previously been determined in human plasma filtrate. Hier<sup>18</sup> using a microbiological method reports values ranging from 5 to 15  $\mu\text{g}$  per ml plasma in dogs. The low value obtained in the ferric hydroxide filtrate may depend upon an oxidation of cystine to a microbiologically inactive form. The glutamic acid values may include a part of the glutamine fraction present in our filtrates. The conversion of biologically active glutamine to inactive pyrrolidonecarboxylic acid during the sterilization of the neutralized filtrates may only be partial<sup>17</sup>.

Two amino acids have been determined in a filtrate dilution of 0.66, glycine and proline. The glycine values are in good agreement with those obtained by Gutman and Alexander with a chemical method<sup>16</sup>. Proline has not previously been determined in human plasma filtrate\*, Henderson *et al.*<sup>5</sup> using a microbiological method obtained a value of 43  $\mu\text{g}$  per ml of plasma in rats, more in accordance with our values.

Finally, four amino acids have been determined directly in the concentrated filtrates. Agreeing values were obtained practically throughout with filtrates I—III. The mean value for threonine given by Hier and Bergeim<sup>4</sup> is 20.8  $\mu\text{g}$   $\pm$  4.9 with several values around 12  $\mu\text{g}$  in the series in good agreement with the values reported here. With a chemical method Gutman and Alexander obtained 37  $\mu\text{g}$  of alanine per ml human plasma from non-fasting subjects. This value has been confirmed in these studies. According to the previous investigators<sup>5, 19</sup>, the amounts of aspartic acid in human and dog plasma should be negligible. Our experience is that a small aspartic acid spot is a rather constant phenomenon on two-dimensional chromatograms of human plasma<sup>20</sup>. As shown in that paper human plasma also seems to contain a small amount of asparagine. The microbiological aspartic acid values reported in this paper may accordingly include some asparagine. Serine has previously not been determined in human plasma filtrate.

In accordance with the low  $\alpha$ -amino-nitrogen value in the ethanol filtrate the microbiological determinations of the different amino acids carried out with this filtrate gave low results. Generally, the yield was about a third of that obtained with filtrates I—III. This result may be stressed with regard to the numerous attempts made to quantitatively evaluate the ninhydrin spots on two-dimensional chromatograms of ethanol filtrates from plasma and extracts of different organs.

#### SUMMARY

1. The micro adaptation of the usual microbiological procedure for the determination of amino acids previously reported has been used for analysis of amino acids in human plasma filtrate.

2. A comparison has been made of the amino acid content in filtrates from precipitation with tungstic acid, trichloroacetic acid, ferric hydroxide, and ethanol. Agreeing result were obtained with the three first mentioned filtrates.

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\* Alexander<sup>19</sup> quotes from a personal communication by S. W. Hier that the proline concentration in plasma should be negligible.

3. The presence of tungstic acid in the filtrates did not markedly influence the growth of the microorganism even when concentrated filtrates were analyzed.

4. Values are reported for cystine, proline and serine in human plasma filtrates. These amino acids have not previously been determined.

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