A MICROBIOLOGICAL PROCEDURE FOR THE ASSAY OF AMINO ACIDS WITH CLOSTRIDIUM PERFRINGENS (WELCHII) BP6K*

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In the previous paper on the growth requirements of Clostridium perfringens BP6K it was shown that thirteen amino acids, i.e. arginine, leucine, histidine, isoleucine, methionine, threonine, phenylalanine, tryptophan, valine, glutamic acid, serine, cystine, and tyrosine, are essential for the growth of the organism. The omission of any one of these amino acids from an otherwise complete medium resulted in either very little or no growth. The possibility of using this rapidly growing organism for amino acid assay has been investigated and the results indicate that Clostridium perfringens may be used satisfactorily for the assay of the above amino acids.

This procedure has a number of advantages over the present microbial assay methods for determining amino acids: (a) The method does not require aseptic technique. (b) The assay is rapid. The induction period is so short that growth is visible in 2 to 3 hours after inoculation and the assay is completed in 16 hours. (c) The same seed culture may be used for 1 week as a source of the inoculum. (d) The response of the organism to increments of the amino acids essential for the rat is more nearly linear than with other organisms. (e) The culture medium is not autoclaved, and possible destruction of any of the constituents is thereby avoided.

EXPERIMENTAL

The procedures for the preparation of the "stock culture" and "seed culture" media, transfer, and growth of Clostridium perfringens on these media, and the preparation of the double strength basal medium for assay purposes have been given in the previous paper.

Inoculum—1 cc. of the seed culture obtained aseptically from the stock seed culture is centrifuged and the supernatant discarded. The packed cells are resuspended in 10 cc. of distilled water and centrifuged a second time. These washed cells\(^1\) are resuspended in 1 cc. of water and a dilution

\(^*\) The work described in this paper was assisted by the United States Army.

\(^1\) An unwashed cell suspension of 1:200 was used satisfactorily for the assay of histidine, leucine, isoleucine, arginine, phenylalanine, and tyrosine.
Procedure for Assay—The procedure for assaying an amino acid in this chemically defined medium is as follows:

A set of eight to ten Pyrex culture tubes (15 X 150 mm.) is used to obtain a standard growth curve. 5 cc. of double strength basal medium (deficient in the amino acid being assayed) are pipetted into each tube. Graded amounts of the amino acid under test are added to each tube. The final volume of each tube is adjusted to 10 cc. with distilled water. The amount of the amino acid to be added per tube to obtain a complete growth curve varies with each particular amino acid to be assayed. For instance, the standard curve for tryptophan requires a range of 0 to 80 \( \gamma \) per tube; therefore, eight different levels of the amino acid, i.e. 0, 10, 20, etc., up to 80 \( \gamma \), are added per tube. However, the range of the standard curve for each amino acid is different, as is shown in Figs. 1 to 6.

To assay the sample for an amino acid several different, accurately measured quantities of the diluted hydrolysate, adjusted to pH 7.1 to 7.2, are added in duplicate to 5 cc. quantities of the basal medium (deficient in the amino acid to be assayed). At least two of the dilutions should correspond to points near the middle of the standard growth curve. The final volume is made to 10 cc. with distilled water.

0.1 cc. of sodium azide solution containing 2 mg. per cc. is added to all tubes. The contents of each tube are mixed by inversion. Glass caps are used to cover the tubes. The tubes are placed in a boiling water bath for 20 minutes, cooled, and inoculated with 1 drop of the diluted inoculum, after which they are incubated in a constant temperature water bath at 45\(^\circ\) for a period of 16 hours.

After incubation the contents of the tubes are mixed by inversion and the density of growth determined with a turbidimeter (1). The optical density may be determined with an Evelyn photoelectric colorimeter equipped with a 660 ma filter.

Preparation of Sample for Analysis—The procedure of Stokes et al. (2) is used for preparing a sample of protein for analysis. 1 gm. of protein is introduced into a small Pyrex tube, 10 cc. of 3.5 \( \times \) HCl are added, and the tube is sealed. The sealed tube is autoclaved 10 hours at 15 pounds pressure, cooled, and the contents of the tube are carefully washed into a graduated cylinder. NaOH is added until the point of neutralization is reached, and the final volume is adjusted to 300 cc.

Tryptophan analysis is made on an alkaline hydrolysate of the protein. 50 mg. of the dried protein are hydrolyzed with 4 cc. of 5 \( \times \) NaOH in sealed tubes for 10 hours at 15 pounds pressure. The precipitate that forms as a result of the hydrolysis is filtered off and washed with distilled water.
The combined washings are added to the filtrate. The pH of the filtrate is adjusted to 7.2 and the final volume made to 50 cc. Complete racemization of the tryptophan is assumed to occur when the sample has been hydrolyzed under these conditions. Since n-tryptophan is inactive for Clostridium perfringens, the final results of the assay must be multiplied by a factor of 2 to obtain the correct tryptophan value.

**RESULTS AND DISCUSSION**

Figs. 1 to 6 show the standard curves for l-methionine, l-tryptophan, dl-threonine, dl-phenylalanine, l-histidine, l-tyrosine, l-leucine, dl-valine, l-arginine, dl-isoleucine, l-glutamic acid, dl-serine, and l-cystine. The curves show a linear response to increments of the amino acids essential for the rat, while serine, cystine, and tyrosine give sigmoid growth curves. All the indispensable amino acids with the exception of lysine may be assayed. However, lysine may be assayed provided the sample tested contains neither pyridoxamine nor pyridoxal. Fig. 7 shows a standard growth curve for lysine in the presence of 60 γ of pyridoxine per tube.
The correlation of cell counts, turbidities, and optical densities is shown in Fig. 8. The cell counts were made according to the United States Army method (3). Turbidities were determined with a photoelectric turbidimeter and represent galvanometer readings; i.e., microamperes. Optical
densities were calculated from the transmission values obtained with an Evelyn photoelectric colorimeter (optical density $= 2 - \log G$) with a 660

![Graph of standard growth curves of DL-valine and DL-isoleucine](Image)

**Fig. 4.** Standard growth curves of DL-valine and DL-isoleucine

![Graph of standard growth curves of DL-methionine and DL-phenylalanine](Image)

**Fig. 5.** Standard growth curves of DL-methionine and DL-phenylalanine

The measurements with the Evelyn photoelectric colorimeter were made on the undiluted culture tubes. The results obtained indicate that turbidity and optical density are proportional to the cell count.
Fig. 6. Standard growth curves of L-glutamic acid and D-serine.

Fig. 7. Response of Clostridium perfringens to pyridoxamine and pyridoxal on a medium without lysine, and the growth response for L-lysine in the presence of pyridoxine.
Table I shows the amino acid analyses of β-lactoglobulin, egg albumin, and silk fibroin as determined with Clostridium perfringens in this laboratory, and, for comparison, the results of Stokes et al., determined with Streptococcus faecalis on samples of the same preparation of protein. The protein samples were dried and hydrolyzed under conditions described by Stokes et al. (2). The analytical results obtained by these two different organisms are essentially in agreement, except that the phenylalanine content of β-lactoglobulin was found to be 3.2 instead of 4.3 per cent.

However, our result is in close agreement with that of Brand et al., who have reported the phenylalanine content of β-lactoglobulin to be 3.5 per cent.

The use of non-aseptic technique in making the assay is an advantageous departure from the usual microbiological assay procedure. Sterilization of the medium in a boiling water bath instead of autoclaving reduces the possibility of destruction of vitamins and amino acids and the formation of inhibitors. Snell (5) has shown that autoclaving pyridoxal with tryptophan or histidine destroys the growth-promoting activity of this vitamin.
MICROBIOLOGICAL ASSAY OF AMINO ACIDS

Table I

Amino Acid Content of Purified Proteins

The proteins were dried at 105° for 3 hours. The results were calculated as percent of dry weight.

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Clostridium perfringens</th>
<th>Streptococcus faecalis*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Histidine</td>
<td>1.6</td>
<td>1.5</td>
</tr>
<tr>
<td>Arginine</td>
<td>15.1</td>
<td>2.8</td>
</tr>
<tr>
<td>Leucine</td>
<td>7.3</td>
<td>15.3</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>5.5</td>
<td>7.0</td>
</tr>
<tr>
<td>Valine</td>
<td>2.6</td>
<td>5.5</td>
</tr>
<tr>
<td>Methionine</td>
<td>4.8</td>
<td>2.5</td>
</tr>
<tr>
<td>Threonine</td>
<td></td>
<td>4.6</td>
</tr>
<tr>
<td>Tryptophan</td>
<td></td>
<td>2.1</td>
</tr>
<tr>
<td>Phenyldalanine</td>
<td></td>
<td>4.3</td>
</tr>
</tbody>
</table>

* Stokes, Gunness, Dwyer, and Caswell analyzed samples of the same preparation of protein, using Streptococcus faecalis.

Fig. 9. Standard growth curves of L-tryptophan. Broken lines, growth obtained under aseptic conditions; solid lines, growth obtained under non-aseptic conditions with sodium azide present in the medium.

for certain organisms. Not only is there a considerable saving of time in performing the assay, but the assay may be made by semiskilled personnel. Standard growth curves obtained by the non-aseptic technique have been checked against growth curves obtained under aseptic conditions.
Fig. 9 shows the similarity of standard growth curves for tryptophan obtained by using aseptic and non-aseptic conditions. Under aseptic conditions the tubes of medium were sterilized for 10 minutes at 15 pounds pressure, 0.4 cc. of sterile 25 per cent glucose was added, and the tubes were inoculated with a washed diluted cell suspension. Under non-aseptic conditions the tubes of medium containing glucose and sodium azide were boiled 20 minutes, cooled, and inoculated with a diluted cell suspension which was previously washed under non-aseptic conditions.

Non-aseptic technique in making assays may be employed without running the risk of contamination for the following reasons: (a) Heating the assay tubes in a boiling water bath for 20 minutes destroys most of the vegetative bacteria; (b) the use of sodium azide inhibits the growth of most aerobes; (c) the short induction period reduces the possibility of contamination; and (d) the high incubation temperature of 45° inhibits the growth of most air-borne organisms.

The authors are indebted to Alice Griner Tytell for making the cell counts; to Dr. W. H. Stein of the Rockefeller Institute for β-lactoglobulin, egg albumin, and silk fibroin; and to Dr. J. S. Fruton for the D and L isomers of methionine.

**SUMMARY**

A microbiological assay procedure has been devised for the determination of thirteen amino acids, nine of which are essential for the rat. The advantages of this procedure are as follows:
1. The assay may be done under non-aseptic conditions.
2. The organism grows so rapidly that the assay is completed in 16 hours.
3. One seed culture may be used for at least 1 week.
4. The culture medium need not be sterilized by autoclaving.
5. The response of the organism to increments of the amino acids essential for the rat is very nearly linear.

**BIBLIOGRAPHY**

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