A UNIFORM MEDIUM FOR DETERMINATION OF AMINO ACIDS WITH VARIOUS MICROORGANISMS*

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The use of lactic acid bacteria for the determination of amino acids which they require is now widespread. The basic techniques used and the various individual methods proposed are very similar, and have been reviewed elsewhere (1-3). These similarities have led some investigators (4, 5) to propose assays of a large number of amino acids under more or less uniform conditions with a single organism. Media used with such general methods have not been optimal for other organisms also useful for assay work. Thus far, however, no single organism has proved suitable for the determination of more than about eight of the amino acids. Lack of an absolute requirement (6, 7), variation in relative activity of optical isomers (8), the now known interrelationships between certain vitamins and some amino acids (9-12), and disturbances resulting from unknown causes combine to make use of a variety of organisms advisable or even necessary when analyses for several different amino acids are required. Use of more than one test organism for the determination of a single amino acid is also valuable as added evidence for or against validity of an assay (1, 2).

An obvious advance in methodology, therefore, would be the development of a single medium which could be made deficient as desired in any single amino acid, and which could be used successfully with any one of a variety of test organisms. This has been the object of the present study. The medium herein proposed has been extensively investigated with respect to its suitability for Lactobacillus arabinosus 17-5, Streptococcus faecalis R, and Leuconostoc mesenteroides P-60. For these and four additional species of lactic acid bacteria, it supports acid production equal to or greater than had been previously reported with specialized media for each organism. The single medium with the appropriate amino acid omitted has been employed successfully to determine fourteen different amino acids.

EXPERIMENTAL

Cultures and Inocula—Organisms used were Lactobacillus arabinosus 17-5, Streptococcus faecalis R, Leuconostoc mesenteroides P-60, Lactobacillus casei,

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Lactobacillus delbrueckii 5, Lactobacillus delbrueckii 3, and Lactobacillus fermenti 36. These were carried by biweekly transfer as stab cultures in an enriched basal medium. This was prepared by reducing the concentrations of glucose and sodium citrate shown in the basal medium (Table I) by one-half and adding 0.5 per cent Bacto-tryptone, 0.5 per cent Bacto-yeast extract, and 2 per cent Bacto-agar. All cultures were incubated at 37° until good growth occurred in the stab culture (24 to 48 hours) and were then refrigerated until the next transfer.

For growing inocula, this same medium with agar omitted was employed. This liquid medium was put in tubes in 5 ml. lots, sterilized by autoclaving for 20 minutes at 15 pounds pressure, and kept in the refrigerator. Inocula were prepared by transfer from the stock culture and grown for 8 to 12 hours at 37°. The cells were centrifuged and resuspended in a volume of sterile 0.9 per cent sodium chloride solution 5 to 25 times greater than that of the medium in which they were grown. 1 drop of suspension was used for each assay tube. Lyophylized cultures, prepared as described by Nymon, Gunsalus, and Gortner (15), were also employed successfully in the later phases of the work. Instead of being used directly as inocula, the resuspended dried cells were transferred to the inoculum medium, incubated for 8 to 12 hours, and used as described above.

Procedure—Assays were carried out in 18 X 150 mm. Pyrex culture tubes, and in a total volume of 2 ml. The samples or standards were added in volumes from 0.2 to 1 ml., water being added to make 1 ml.; 1 ml. of the basal medium, prepared at twice the concentration shown in Table I, was then added. Additions of both water and samples were made with the rapid automatic dispenser of Cannon.4 Sterilization was accomplished by autoclaving the tubes for 10 minutes at 10 to 12 pounds pressure. A metal cover approximately 3 inches deep and lined with a heavy padding of cotton was placed over each rack of 60 tubes. This cover was removed for approximately 1 minute in a small room previously freed from dust with steam, while 1 drop of inoculum was added to each tube from a sterile pipette.

1 Dunn et al. (6), because of identical nutritive requirements, have concluded that Lactobacillus casei and L. delbrueckii 5 are the same organism. Although the nutritive requirements of our culture of L. casei correspond to those reported by Dunn, those of L. delbrueckii 5 do not. It is possible, therefore, that two different cultures are being used under the latter name.

2 Inocula grown for 18 to 24 hours may be used at somewhat greater dilution without noticeably affecting the results.

3 Strepococcus faecalis suspensions were diluted approximately 25-fold; suspensions of Lactobacillus arabinosus, Leuconostoc mesenteroides, and other organisms were diluted from 5- to 10-fold, depending upon the amount of growth. The amount of inoculum used is not a critical factor in determining success of the assay.

4 Cannon, M. D., in press.
After incubation in a forced air incubator for 60 to 72 hours at 37°, the lactic acid produced was titrated directly in the assay tubes with 0.04 N NaOH. The end-point was determined electrometrically by an adaptation of the technique employed by Pennington et al. (16). During titration,
the contents of each tube were stirred vigorously with an air stream. The
details of this procedure, as applied to volumes of 0.2 ml., are described in
the accompanying paper (17). The same technique with correspondingly
larger equipment was used in the present studies. Thymol blue and other
indicators tried gave unsatisfactory end-points in the presence of the
multiple buffers in the medium (Table I), even though the end-point
selected corresponded to the inflection point in the titration curve of the
buffer mixture. The curve, together with that obtained by electrometric
titration of the same buffer mixture, is shown in Fig. 1.

![Fig. 1. Curves relating galvanometer deflection to pH of buffered solution](image)

**Basal Medium**—The composition of the basal medium finally adopted is
shown in Table I. In preliminary trials, this medium supported as good or
better growth of each of the three assay organisms as did the specialized
media then being used in this laboratory (18, 19). These latter media,
though essentially alike, differed in minor respects and were representative
of improved media used for these organisms by many investigators. The
adequacy of the new medium for the various organisms was further tested
by running assay curves for leucine in the unchanged medium, and in
similar media in which the concentration of single ingredients or a group of
related ingredients was varied from a fraction to several times that present
in the initial medium. Eleven levels of L-leucine, ranging from 0 to 60 γ
per 2 ml., with all points in duplicate, were run to establish each standard
curve, and curves were obtained simultaneously with all three test organisms (*Lactobacillus arabinosus*, *Streptococcus faecalis*, and *Leuconostoc mesenteroides*). A superior medium was adjudged to be that variation in which (a) the greatest acid production resulted from addition of excess leucine, (b) the greatest slope of the standard curve was secured over the assay range, and (c) the standard curve was most nearly linear in the assay range. A summary of the results of these investigations is given below.

![Graph showing the effect of sodium citrate concentration on the response of *Streptococcus faecalis* to leucine.](http://www.jbc.org/figure/2)

**Fig. 2.** Effect of sodium citrate concentration on the response of *Streptococcus faecalis* to leucine.

**Citrate and Acetate Buffers**

The superiority of citrate over acetate buffers in promoting growth and acid production of *Streptococcus faecalis* has been adequately demonstrated (20, 21). Attempts to use citrate buffer for other organisms led to the observations (22) that 2 per cent sodium citrate was toxic, but that the toxicity could be overcome by increasing the concentrations of magnesium and manganese ions in the medium. When such increases in the metallic ion concentrations were made, citrate was an effective buffer for several other lactic acid organisms as well. For *Lactobacillus arabinosus* and *Leuconostoc mesenteroides*, 2 per cent of sodium citrate was superior to 1 or 3 per cent, while for *Streptococcus faecalis* (Fig. 2) 3 per cent gave greater acid production at high concentrations of leucine. Even with this organ-
ism, however, the usable portion of the standard curve was not altered by increasing the citrate concentration above 2 per cent. Sodium citrate could be replaced by potassium citrate in equivalent concentrations without altering these results.

Sodium acetate was included in this medium because of its demonstrated growth stimulating activity for several lactic acid bacteria (23); varying the concentration from 0.05 to 0.3 per cent had no effect on acid production, shapes, or slopes of the standard curves when other buffers were present at the concentrations indicated in Table I.

**Ammonium Chloride and Sodium Chloride Concentrations**

To eliminate possible disturbing effects of ammonium salts present in protein hydrolysates, ammonium chloride was added to the medium. Variations in concentration from 0 to 1.5 per cent had no effect on the response of *Streptococcus faecalis* or *Leuconostoc mesenteroides* to leucine. At levels of 0.75 per cent and higher, acid production by *Lactobacillus arabinosus* was depressed at all levels of leucine. Ammonium chloride has therefore been added at a lower concentration, 0.3 per cent.

With ammonium chloride at this level, 1 per cent of added sodium chloride was tolerated by *Streptococcus faecalis*. This concentration of salt was slightly inhibitory to the other two organisms but 0.5 per cent was harmless. 10 to 20 mg. of sodium chloride thus represent the maximum allowable amount of this salt which should be added with samples for assay.

**Phosphate Concentration**

An increase of the concentration of dipotassium phosphate up to 1 per cent resulted in increased acid production by *Streptococcus faecalis* (Fig. 3). *Leuconostoc mesenteroides* P-60 gave smaller increases, while acid production by *Lactobacillus arabinosus* was independent of phosphate concentration between 0.1 and 1 per cent. The assay range for leucine, however, is not lengthened by the addition of amounts of phosphate above 0.5 per cent. This concentration was therefore selected for use in the medium.

**Other Inorganic Constituents**

Previous results (22) have shown that Salts B at the level usually incorporated do not provide adequate manganese or magnesium to permit optimal growth of assay organisms other than *Streptococcus faecalis* in a medium high in citrate. Salts C (13), which differ from Salts B in that they contain a 4-fold greater concentration of manganese sulfate, have been used. Comparisons of 0.5 per cent Salts B with 0.5 to 4 per cent Salts C were made. Except for *Streptococcus faecalis*, where there was no difference, 0.5 per cent Salts C was superior to 0.5 per cent Salts B. Increasing concentra-
tions of Salts C were beneficial up to 2 per cent; higher levels gave no further improvement (Fig. 4). Salts C, at 2 per cent level, were therefore used in the medium. In separate experiments, addition of a mixture of trace elements (Cr++, I−, B4O7−, Zn++, Cu++, and Co++) to provide 2 γ of each element per 2 ml. had no effect on the response to leucine.

_Amino Acid Concentrations_

The concentrations of amino acids chosen were considered high, representing in most cases 10 to 20 times the requirement for each in the presence of an excess of the others. The total concentration of the naturally occurring isomers in the medium is 0.38 per cent. Eighteen amino acids were used; hydroxyproline, norleucine, and norvaline were not included, since they have not been reported to affect growth of these organisms in any way at low concentrations. The concentration of the entire mixture of amino acids was first varied from one-half to twice the level indicated in Table I. Slightly diminished acid production by *Streptococcus faecalis* at the lower level was the only effect noted. Glutamic acid, aspartic acid, alanine, arginine, and lysine were varied individually over similar ranges of concentration without effect on the response to leucine of any organism.

![Graph](http://www.jbc.org/)

**Fig. 3.** Effect of phosphate concentration on the response of *Streptococcus faecalis* to leucine.
The originally selected concentrations of amino acids were thus considered adequate.

**Glucose Concentration**

Glucose concentrations of 1, 2, 3, and 4 per cent were compared. 2 per cent glucose supported acid production equal to that obtained with 3 and 4 per cent for *Streptococcus faecalis* and *Leuconostoc mesenteroides*, but *Lactobacillus arabinosus* showed increased acid production in the upper part of the curve (Fig. 5) with 3 and 4 per cent glucose. While increasing glucose concentration from 1 to 2 per cent greatly increased the usable assay range, no similar increase resulted from higher concentrations of sugar; 2 per cent was therefore selected for use in the medium.

It should be noted that the curves obtained with 1 or with 2 per cent of sugar are superimposable over the lower part of the assay curve. If one wishes to work within this range, there is no evidence that any advantage (i.e., greater accuracy or higher precision) would result from the use of sugar concentrations above 1 per cent.
Vitamin Concentrations

The vitamins were added at relatively high concentrations. Pyridoxal, the form of vitamin B₆ available to each of these microorganisms, was used. Concentrations of the entire vitamin mixture equal to one-eighth and one-fourth that shown in Table I were inferior, especially for *Leuconostoc mesenteroides* and *Streptococcus faecalis*. Levels from one-half to twice that adopted gave identical standard curves.

![Diagram](http://www.jbc.org/)

**Fig. 5.** Effect of glucose concentration on the response of *Lactobacillus arabinosus* to leucine.

Purine and Pyrimidine Bases

Omission of the purine and pyrimidine bases from the medium resulted in an induction period in the leucine response curve obtained with *Leuconostoc mesenteroides*. Other organisms were unaffected. No improvement in response curves with any organism resulted from varying the concentration of this supplement from one-half to twice that adopted.

Use of Medium with Other Test Organisms

The adequacy of the assay medium for *Lactobacillus arabinosus*, *Streptococcus faecalis*, and *Leuconostoc mesenteroides* having been proved, it was next compared with previously described media for its ability to support
growth and acid production of several other lactic acid bacteria. The results of a comparison for Lactobacillus delbrueckii 5 with the medium of Stokes et al. (4) are shown in Fig. 6. The maximum acid production and the shape and slope of the standard curves differ markedly. Similar data for Lactobacillus casei grown on the medium of McMahan and Snell (24) are shown in Fig. 7. Here the curves were identical in the lower portion, but the new medium gave a greatly extended assay range when the titrimetric method was used, in part because of the increased amounts of sugar and buffer present. L. fermenti gave acid production on this medium comparable to that recently reported with a specialized medium by Dunn et al. (6). L. delbrueckii 3 likewise grew well, giving approximately 90 per cent of the theoretical acid production (considered to be 11 ml. of 0.04 N acid per 2 ml. of medium) in the complete medium. It was concluded that the medium can be employed unchanged for most of the lactic acid bacteria hitherto used for assay of amino acids.

Analyses of Protein Hydrolysates—Four purified proteins were analyzed a number of times for fourteen different amino acids, this medium and a number of test organisms being employed. Hydrolysis was accomplished by autoclaving 0.5 gm. of the protein in sealed Pyrex tubes with 20 ml. of 3 N hydrochloric acid for 5 hours at 15 pounds pressure. Variation of the
hydrolysis procedure for casein from 2 N hydrochloric acid for 5 hours to 3 N hydrochloric acid for 10 hours did not alter the results significantly. For tryptophan analyses, both enzymatic and alkaline hydrolysates were employed. The alkaline hydrolysates were prepared by autoclaving 0.5 gm. of protein with 10 ml. of 5 N sodium hydroxide in a sealed Pyrex tube for 15 hours at 15 pounds pressure. Complete racemization was assumed (25). Enzyme digestion (26) was accomplished by shaking 50 ml. of an unbuffered suspension containing 230 mg. of protein, 20 mg. of Merck's pancreatin, 4 mg. of Wilson's hog intestinal mucosa, and a little toluene for 3 days.

![Graph](http://www.jbc.org/)

**Fig. 7. Comparative response of Lactobacillus casei to leucine in two assay media**

Each determination was made with three to six tubes at each of six levels of the amino acid standard and with three tubes at each of five levels of the samples (0.2, 0.4, 0.6, 0.8, and 1 ml.). In most cases, analyses were conducted simultaneously for at least six amino acids.

Such extensive assays were made primarily to test the applicability of the new medium to the assay of each of the several amino acids for which test organisms are available. The results, though apparently comparable in precision to those reported by previous workers, are not felt to represent the maximum precision attainable in microbiological assays with this or other media.

The results of these analyses are shown in Table II and are compared with the range of values from the literature found by microbiological assay
### Table II

**Amino Acid Composition of Purified Proteins**

<table>
<thead>
<tr>
<th>Amino acid†</th>
<th>Casein</th>
<th>Bovine serum albumin</th>
<th>Edestin</th>
<th>Colostrum pseudoglobulin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Gm. per 100 gm.</td>
<td>Literature values</td>
<td>Gm. per 100 gm.</td>
<td>Literature values</td>
</tr>
<tr>
<td>Arginine</td>
<td>3.78 ± 0.65‡</td>
<td>3.8 ± 0.4‖</td>
<td>6.2 ± 0.5§</td>
<td>6.1 (27)</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>7.4 ± 0.03³</td>
<td>6.9 ± 0.8³</td>
<td>11.1 ± 0.16²</td>
<td>10.6 (28)</td>
</tr>
<tr>
<td>Glutamine</td>
<td>21.7 ± 1.7²</td>
<td>21.6 ± 0.8³</td>
<td>16.8 ± 0.9³</td>
<td>16.9 (28)</td>
</tr>
<tr>
<td>Histidine</td>
<td>2.82 ± 0.1¹</td>
<td></td>
<td>3.62 ± 0.2³</td>
<td>3.80 (28)</td>
</tr>
<tr>
<td>S. faecalis</td>
<td>3.09 ± 0.24⁴</td>
<td>3.06 ± 1.0⁷</td>
<td>3.7 ± 0.17²</td>
<td>4.1 (27)</td>
</tr>
<tr>
<td>L. mesenteroides</td>
<td>7.6 ± 0.35⁵</td>
<td>6.4 ± 1.6⁶</td>
<td>2.97 ± 0.2²</td>
<td>3.3 (27)</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>Leucine</td>
<td>10.25 ± 0.3⁸</td>
<td>9.6 ± 2.4³</td>
<td>11.8 ± 0.6²</td>
</tr>
<tr>
<td></td>
<td>Lysine</td>
<td>8.07 ± 0.3³</td>
<td>7.76 ± 1.1³</td>
<td>10.3 ± 1.1¹</td>
</tr>
<tr>
<td>Methionine</td>
<td>2.69 ± 0.26⁴</td>
<td>2.85 ± 0.21¹</td>
<td>0.86 ± 0.04⁴</td>
<td>0.81, 0.77 (33)</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>5.45 ± 0.34⁶</td>
<td>5.36 ± 0.5⁶</td>
<td>6.48 ± 0.22²</td>
<td>6.4 (27)</td>
</tr>
<tr>
<td>Proline</td>
<td>11.6 ± 0.7⁶</td>
<td>11.2¹</td>
<td>5.1 ± 0.3²</td>
<td>5.6 (28)</td>
</tr>
<tr>
<td>Threonine</td>
<td>4.28 ± 0.28¹</td>
<td>4.2 ± 0.4⁴</td>
<td>6.2 ± 0.2²</td>
<td>6.5 (28)</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>6.2 ± 0.7⁶</td>
<td>5.8 ± 0.7⁴</td>
<td>4.3 ± 0.4²</td>
<td>5.2 (27)</td>
</tr>
<tr>
<td></td>
<td>Valine</td>
<td>Tryptophan Hydrolyzed by enzymes</td>
<td>Tryptophan Hydrolyzed by NaOH</td>
<td></td>
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<tr>
<td></td>
<td>7.15 ± 0.41\textsuperscript{1}</td>
<td>6.67 ± 1.1\textsuperscript{1}</td>
<td>6.6 ± 0.04\textsuperscript{1}</td>
<td></td>
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<tr>
<td></td>
<td>5.4 (27)</td>
<td>6.5 (28)</td>
<td></td>
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<tr>
<td></td>
<td>6.6 ± 0.1\textsuperscript{1}</td>
<td>6.63 (32)</td>
<td>9.3 ± 1.0\textsuperscript{1}</td>
<td></td>
</tr>
<tr>
<td></td>
<td>8.7 (30)</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>6.6 ± 0.12</td>
<td>1.02\textsuperscript{1}</td>
<td>2.2\textsuperscript{1}</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.86\textsuperscript{1}</td>
<td>1.24 (32)</td>
<td>2.27 (36)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3.1\textsuperscript{1}</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>3.2 (30)</td>
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</tbody>
</table>

* All values are expressed as gm. of amino acid per 100 gm. of dried ash-free protein. The casein was prepared from skim milk according to the procedure of Cohn and Hendry (38). It was washed by resuspending the protein twice in 95 per cent ethanol, once in absolute ethanol, and three times in ether; N = 15.2 per cent, ash = 0.5 per cent (dry basis). Bovine serum albumin was kindly supplied by Dr. L. L. Lachat of the Armour Laboratories, Chicago; N = 15.9 per cent, ash = 0.2 per cent (dry basis). Edosin was prepared from hemp seed by the method of Osborne (39), and was recrystallized four times from sodium chloride solution; N = 18.7 per cent, ash = 0.55 per cent (dry basis). Colostrum pseudoglobulin was kindly supplied by Mr. R. G. Hansen of this department; N = 16.0 per cent, ash negligible (30). The amino acids used as standards were dried in vacuo at 30° and kept in vacuo in a desiccator containing anhydrous calcium chloride. dl forms of isoleucine, valine, methionine, threonine, and phenylalanine were employed; the natural isomers of the others were used. Products obtained from three to five sources were compared for microbiological activity. With one exception (isoleucine), the activity of the various samples checked within the limits of experimental error. Merck's dl-isoleucine was used as the standard in this instance. dl-Methionine was employed throughout these studies as a standard, though unpublished data from this laboratory indicate that d-methionine has measurable activity for Streptococcus faecalis in the presence of L-methionine. Hence the values reported here may be slightly low.

† Lactobacillus arabinosus was used for glutamic acid, leucine, phenylalalanine, valine, and tryptophan, Streptococcus faecalis for arginine, histidine, methionine, and threonine, Leuconosic mesenteroides for aspartic acid, histidine, lysine, proline, tyrosine, and isoleucine. Severe drift in assay values was encountered when L. arabinosus was employed for isoleucine determination; Leuconosic mesenteroides and four other organisms gave values for isoleucine which agreed and showed no noticeable drift. Recent results (Sirny et al., unpublished) indicate that L. delbrueckii is more suitable for arginine assay than is Streptococcus faecalis.

†† Superscripts indicate the number of determinations made or the number of values cited as averages from literature. In all columns the variation indicates the extreme values found.
of these same proteins. In view of the small number of analyses made in some cases, the range of values is given, and the data are not treated statistically.

The data represent the results of separate analyses of a number of different acid hydrolysates of each protein (e.g., eight for casein). A solution of amino acids, compounded to contain roughly the relative concentration of each amino acid required by the various test organisms, was analyzed each time. With very few exceptions, the amount of each amino acid found by assay of this mixture agreed to within ±5 per cent with the amount added, and the agreement was usually much better than this.

SUMMARY

1. A uniform medium has been developed for the microbiological determination of amino acids with a number of the most widely used lactic acid bacteria. High acid production, low blanks, and satisfactory standard curves for each of fourteen different amino acids were obtained.

2. The concentrations of single or closely related components of the medium were varied over wide ranges and approximately optimal concentrations for each selected for use in the medium.

3. Hydrolysates of purified casein, edestin, bovine serum albumin, and colostrum pseudoglobulin were analyzed for fourteen amino acids. Reproducible values, in most cases comparable in magnitude and variation with those reported from other laboratories by microbiological methods, were obtained.

Addendum—Since the submission of this paper, the above medium and method have been found suitable for assay of glycine. Leuconostoc mesenteroides is used as the test organism. For this purpose, it is ordinarily necessary to recrystallize the D,L-alanine used in the medium, since this amino acid, as obtained commercially, is sometimes contaminated with glycine. A sample of Merck's D,L-alanine, for example, was found to contain 0.6 per cent of glycine by microbiological assay. This was eliminated by one recrystallization.

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