AMINO ACID UTILIZATION BY *LEPTOSPIRA CANICOLA*^{1, 2}

MARY R. GERHARDT AND MERIDIAN GREENE BALL

Department of Bacteriology, University of California, Los Angeles, California

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Several carefully conducted studies by other workers, which were designed to demonstrate utilization of amino acids by leptospirae, have been reported previously (Ono, 1938; Ward and Starbuck, 1941; Savino and Rennella, 1944; Greene, 1945). Ono (1938), Savino and Rennella (1944), and Greene (1945) agree that asparagine stimulates growth, but there is disagreement among the authors concerning stimulation by glutamic acid, alanine, arginine, glycine, tryptophan, and leucine. These conflicting results may have been caused by differences in quantities of amino acids used, by the combinations employed, or by the basic medium to which the amino acids were added.

Since the leptospirae grow best in a medium such as Schüffner's, which is composed of animal serum and small quantities of a suitable peptone, it is possible that the medium supplies the proper balance of all components which are essential to the organism and many which are stimulatory. Thus, it seemed likely that an analysis of Schüffner's medium at various intervals during an incubation period would reveal more clearly which, if any, amino acids were being utilized by the organism. Since the mean generation time of these organisms was found to be approximately 48 hr (Chang, 1947), samples were taken at intervals during a 48-hr incubation period.

The first attempts to demonstrate utilization of amino acids were done by paper chromatography under the assumption that an amino acid which was being used would disappear from chromatograms prepared with inoculated Schüffner's medium. A chromatogram prepared with a sample of fresh, sterile Schüffner's medium would reveal which amino acids were available to the organism. Several studies based upon this prin-

ciple were conducted before Fulton and Spooner (1956) pointed to the fact that the non-protein-nitrogen value of sterile Korthof's medium increased slightly upon incubation at 30 C. Since we then found this to be true with Schüffner's medium also, it seemed apparent that an accurate study of the utilization of amino acids by leptospirae could be done only quantitatively. Also, it seemed obvious that incubated samples of sterile Schüffner's medium would have to be analyzed as a control measure. Therefore in this study, amino acid utilization by *Leptospira canicola* was demonstrated by quantitative microbiological assays on sterile and inoculated Schüffner's medium incubated for 48 hr. The assay results are compared with the less sensitive qualitative paper chromatographic technique.

MATERIALS AND METHODS

Organism and cultivation. *L. canicola* (Hond Utrecht IV), obtained from the departmental culture collection was used as the test organism throughout this study. The organism was maintained in 3-ml quantities of Schüffner's medium, and transferred in duplicate every 3 weeks with a 10 per cent inoculum for each subculture. These cultures were incubated at 30 C.

Schüffner's medium (Schüffner and Mochtar, 1927) was prepared by adding 1.5 g of Witte's peptone, 300 ml of Ringer's solution, and 150 ml of Sørensen's double phosphate buffer, pH 7.2 (72 ml 0.066 M Na₂HPO₄ + 100 ml 0.066 M KH₂PO₄) to 1500 ml of boiling distilled water. This mixture was boiled for 30 min, refrigerated overnight, and filtered. The pH was adjusted to 7.4 to 7.6. The medium was bottled and autoclaved for 15 min at 121.5 C. When transfers were to be made, 2.7 ml of this medium were pipetted aseptically into sterile tubes and 0.3 ml of pooled, Seitz-filtered rabbit serum, which had been inactivated for 30 min at 56 C, was added. When the medium was employed in experimental studies, the desired quantity of freshly prepared, filtered, adjusted medium was placed in a flask of suitable size, and autoclaved as previously

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described. Ten per cent sterile, pooled rabbit serum was added just before inoculation.

Analysis of amino acids in Schüffner's medium by paper chromatography. Qualitative analysis for amino acids was done according to the two-dimensional ascending technique of Williams and Kirby (1948) using phenol-ethanol-water (Iyer, 1954) as the first dimensional solvent and 70 per cent *n*-propanol (Bentley and Whitehead, 1950) as the second dimensional solvent.

Clean, dry, gallon jars were used as the vessels for preparing the paper chromatograms. A chemically clean petri dish lid was placed in each jar and 10 ml of the proper solvent were pipetted into the lid. An additional 10 ml of solvent were placed around the lid. The jar was closed, sealed with masking tape, and equilibrated overnight at room temperature. The solution to be analyzed was adsorbed onto a spot in the lower left corner of a 9 in square of Whatman no. 1 filter paper by repetitive spotting. A finely drawn capillary tube was used for this purpose and a hair drier was used to dry the spot after each application. The starting spot was $\frac{1}{2}$ in from both edges of the paper.

After the first dimensional run, the chromatograms were dried overnight at room temperature and then steamed in the autoclave for 10 min without pressure. This was followed by a 15-min drying period in the autoclave. Subsequent to the second dimensional development, the chromatograms were dried at room temperature and sprayed with 0.2 per cent ninhydrin in ethanol. The identity of all ninhydrin-reacting spots was established by comparing R_f values with those previously determined for known amino acids in each solvent. Tyrosine was not identified chromatographically, because the amino acid produced a long, pink streak on one-dimensional paper chromatograms developed with 70 per cent *n*-propanol.

A quantity (270 ml) of Schüffner's medium in a 500-ml Erlenmeyer flask was inoculated with 30 ml of a 5-day-old culture of *L. canicola*. Immediately after inoculation and at 4, 6, 8, 24, and 48 hr incubation, 15-ml samples were removed aseptically. Directly after the first sample had been taken from the culture flask, a 15-ml sample was drawn from a flask of sterile Schüffner's medium. Another sterile control sample was removed after 48 hr incubation at 30 C. A tube of heart infusion broth was inoculated with 1 ml of

each sample as a control for contaminants and the remaining 14 ml of each sample were Seitz-filtered. Five ml of a filtrate were added drop by drop to 10 ml of acetone in order to precipitate proteins and proteose. Refrigeration overnight enhanced the process of precipitation. After centrifugation, the supernatant fluid was decanted and placed in a 30-ml Erlenmeyer flask. This material was evaporated to dryness at room temperature. The residue was extracted with 0.1 N HCl according to Iyer (1954) and neutralized with 0.1 N NaOH. The neutralized, concentrated solution was brought up to a volume of 1 ml with distilled water. This was added to an equal volume of acetone to insure complete separation from protein and proteose. This mixture was centrifuged and the supernatant fluid was added to an equal volume of chloroform. After centrifuging, 0.5 ml of the top layer was decanted and evaporated to approximately 0.2 ml in an oven at 65 C. An attempt was made to prepare chromatograms with approximately 0.1 ml of this concentrated material which would contain the amino acids in approximately 1 ml of Schüffner's medium.

Analysis of amino acids in Schüffner's medium by microbiological assay. A quantity (210 ml) of Schüffner's medium was prepared in each of 8 L flasks. Six of the 8 flasks were inoculated with 21 ml of a suspension of *L. canicola*. The organisms had been sedimented from a 5-day-old culture by centrifugation, and suspended in an amount of Schüffner's salt solution equal to the original volume of medium. Immediately after inoculation, 1 ml was removed from 1 flask and cultured for purity. The remainder was Seitz-filtered. This filtrate was deproteinized with 4 per cent sodium tungstate in 6 N H₂SO₄ and evaporated to dryness under reduced pressure according to the method of Dunn *et al.* (1956). This residual material was taken up with distilled water equal in amount to the original volume of filtrate. The contents of the 5 other inoculated flasks were treated similarly after 4, 6, 8, 24, and 48 hr of incubation at 30 C, respectively. The contents of the 2 sterile flasks of medium were treated as described above, one immediately after inoculation of the test flasks, and the other after 48 hr of incubation at 30 C. A microbiological assay for 17 amino acids was done on these medium extracts according to Dunn *et al.* (1949). Since this method is not suitable for analysis of alanine, the quantities of

that amino acid were not investigated. In this study, the mean deviation was never over ± 10 per cent.

This assay could be done only once, therefore as a control measure, a 1 per cent Casamino acids (Casamino acids, Difco) solution was tested simultaneously. The values obtained compared favorably with those given for Casamino acids by Hawk *et al.* (1947).

RESULTS

According to the assay values obtained on sterile Schüffner's medium, a general increase in arginine, glutamic acid, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tryptophan, and tyrosine occurred during the 48-hr incubation period. An increase was also observed in values obtained for samples of inoculated Schüffner's medium with the exception of glycine; however, the final quantity of several of these amino acids in the control samples was significantly greater than quantities found in the culture medium

samples. These results, which are presented graphically in figure 1, indicate utilization of glycine, lysine, phenylalanine, serine, and tryptophan by *L. canicola* over a 48-hr incubation period.

The quantities of amino acids present in samples of inoculated Schüffner's medium incubated for 0, 4, 6, 8, 24, and 48 hr are presented in figure 2. According to these results, utilization of arginine, methionine, phenylalanine, and tryptophan occurred within the first 4 hr of incubation. Utilization of serine occurred between the 4th and 6th hr of incubation, and aspartic acid and glycine appeared to be utilized after 6 hr of incubation. Glycine appeared to be used continually thereafter throughout the 48 hr experimental period.

The results obtained with paper chromatography using phenol-ethanol-water and 70 per cent *n*-propanol indicate that in some cases, as with glycine, histidine, and serine, the amount of an amino acid present in 1 ml of Schüffner's medium is not large enough to produce a visible ninhydrin-

Figure 1. Increase in amino acids in sterile Schüffner's medium and in Schüffner's medium inoculated with *Leptospira canicola*. Mean deviation ± 10 per cent. Microbiological assay: black bar = inoculated, Seitz-filtered Schüffner's medium; white bar = sterile, Seitz-filtered Schüffner's medium.

* PC = paper chromatography result is indicated by + for amino acid present and - for amino acid not demonstrable.

† AA = amino acids; 1, arginine; 2, aspartic acid; 3, glutamic acid; 4, glycine; 5, histidine; 6, isoleucine; 7, leucine; 8, lysine; 9, methionine; 10, phenylalanine; 11, proline; 12, serine; 13, threonine; 14, tryptophan; 15, tyrosine; 16, valine.

X = not determined.

Figure 2. Analysis of amino acids in Schüffner's medium inoculated with *Leptospira canicola*; samples drawn at 0, 4, 6, 8, 24, and 48 hr after inoculation. Mean deviation ± 10 per cent. Microbiological assay values are indicated by bars.

* PC = paper chromatography result is indicated by + for amino acid present and - for amino acid not demonstrable.

† AA = amino acids: 1, arginine; 2, aspartic acid; 3, glutamic acid; 4, glycine; 5, histidine; 6, isoleucine; 7, leucine; 8, lysine; 9, methionine; 10, phenylalanine; 11, proline; 12, serine; 13, threonine; 14, tryptophan; 15, tyrosine; 16, valine.

X = not determined.

reacting spot. This can also be seen with lysine and proline (figure 2), which were not demonstrable chromatographically until the medium contained over $15 \mu\text{g}$ per ml of the amino acids.

We cannot explain the absence of arginine in the 48-hr samples according to paper chromatog-

raphy since the microbiological assay value for these samples is close to $15 \mu\text{g}$ per ml, whereas a sample drawn at 8 hr incubation containing $10 \mu\text{g}$ per ml of arginine was positive chromatographically. However, these results emphasize the fact that the paper chromatographic tech-

nique is not reliable when used as a tool to detect very small quantities of amino acids.

The amino acid cystine is not present in Schüffner's medium according either to microbiological assay or to paper chromatography. Therefore, cystine is not mentioned in the figures.

DISCUSSION

When *L. canicola* is cultured in unmodified Schüffner's medium over a 48-hr incubation period, the organism utilizes arginine, aspartic acid, glycine, lysine, methionine, phenylalanine, serine, and tryptophan as determined by microbiological assay.

These results agree with those of Ono (1938) who found that arginine, glycine, glutamic acid, lysine, and tryptophan accelerated growth when added to Schüffner's medium. They also agree with the findings of Savino and Rennella (1944) who demonstrated stimulation of the test organism by addition of aspartic acid to experimental media. Furthermore, in her studies, Greene (1945) was able to replace the Witte's peptone in Schüffner's medium with aspartic acid, although growth was never as good as in the unmodified medium. Contrary to this study and to Ono's results, Ward and Starbuck (1941), Greene (1945), and Fulton and Spooner (1956) found that growth was not stimulated, or was reduced, by addition of arginine, glycine, and methionine to experimental media, or to 20 per cent serum in which the test organism was being cultivated. Fulton and Spooner (1956) also found that addition of serine to 20 per cent serum inhibited respiration of *Leptospira icterohemorrhagiae*, and Greene (1945) reported reduction of growth upon addition of phenylalanine to experimental media for culturing *L. canicola*. However, since the present investigation is a quantitative analysis of amino acids in a medium which supports the optimal growth of leptospirae, it may demonstrate more accurately the true picture of amino acid utilization by the organisms. Furthermore, since the amino acid content of the sterile medium increased upon aging in the incubator, in the inoculated medium it was possible to determine utilization only of those amino acids which were being taken up by the organisms more rapidly than they were being formed in the medium. Therefore, we cannot eliminate the possibility that removal of other amino acids

from the medium by the leptospirae may be masked by the simultaneous formation of an equal or larger amount of those amino acids by the slow breakdown of proteins, proteoses, and peptones in the medium.

The increase in amino acids in both control and culture media incubated at 30 C for 48 hr confirms the findings of Fulton and Spooner (1956), who reported an increase in NPN and amino-N values in both control and culture medium in their studies on the nutrition of *L. icterohemorrhagiae*, which they attribute to the slow breakdown of serum proteins in Korthof's medium.

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SUMMARY

Utilization of glycine, lysine, tryptophan, arginine, aspartic acid, methionine, serine, and phenylalanine by *Leptospira canicola* cultivated in Schüffner's medium was demonstrated by microbiological assays done on samples of medium at various intervals during a 48-hr incubation period. The samples were also examined for amino acids by paper chromatography and the results of the two methods were compared.

There was a general increase in arginine, glutamic acid, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, tryptophan, and tyrosine in sterile Schüffner's medium which also had been incubated at 30 C for 48 hr. This would indicate the formation of free amino acids by the slow breakdown of proteins, proteoses, and peptones in the medium.

Cystine could not be demonstrated as a component of Schüffner's medium by paper chromatography or by the microbiological assay technique employed.

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