STUDIES ON THE AMINO-ACIDS IN THE HCl AND $Ba(OH)_2$ HYDROLYSATES OF THE ACID-FAST BACTERIA BY THE PAPER PARTITION CHROMATOGRAPHY

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With regard to amino acids of bacterial hydrolysates, using the paper partition chromatography, Polson[1], and Polson, Ralpha and Wyckoff[2] reported three kinds of unknown amino acids which they had found in bacterial hydrolysates of *E. Coli.*

Johnson and Brown[3], and Johnson and Coghill[4] studied the distribution of nitrogen in tubercle bacilli after removal of both lipins and tuberclinic acid. This fractionw as analysed by the method of Van Slyke. The finding of chief interest was a high content of hexone bases, comparable to that found by other observers in certain plant proteins; cystine was present but only in traces.

At about the same time Campbell[5] published amino acid analyses of bovine type tubercle bacilli; the Van Slyke method was used here also, except for the determination of histidine and tyrosine. The only previous extended study of amino acids of *Mycobacterium tuberculosis* was made by Tamura[6], who used the method of Kossel and Kutscher.

Acid-fast bacteria have many different aspects from other bacilli: having a good amount of lipins for their bacterial composition, they also resist acid, alcohol, disinfectant and various kinds of chemicals. It is known that the developing speed of pathogenic acid-fast bacteria and of non-pathogenic acid-fast bacteria differs greatly in the two types. Since the difference is supposed to be caused by a peculiar kind of component in bacterial protein, the writer made studies on amino acids of *M. tuberculosis var. hominis* (common name: Tubercle bacillus, human type), *M. tuberculosis var. avium* (common name: Tubercle bacillus, avian type), *M. ranae*, *M. smegmatis* (common name: Smegma bacillus), by means of paper partition chromatography.

The experimental results obtained by the writer are to be described as follows:

Methods

1. Preparation for getting Bacterial hydrolysate[7]

The acid-fast bacteria cultivated in the Oka-Katakura medium, were removed carefully by platinium loop so as not to touch the medium, and suspended on distilled water which was centrifuged for 15 minutes at 4,000 rev. per minute to get its deposit in the bottom of the vessel; this deposit was again centrifuged to get newer one, and this treatment was further repeated.

About 0.5 gr. of the newest deposit (bacteria) closed in a test-tube together with 5 c.c. of 8N-HCl was hydrolyzed by subjection to heat of 105°~110°C for the period of 15~20 hours. Then it was condensed on water bath in a watch glass to get hydrochloric acid hydrolysate.

About 0.5 gr. of another bacilli also closed in test-tube together with 5 c.c. of 10% $Ba(OH)_2$ was subjected to a 110°C heat for 24 hours: after this, the hydrolysate neutralized by acetic acid on a watch glass was condensed on the water bath and Barium salts resolved by acidifying with hydrochloric acid. This was barium hydroxide hydrolysate.

2. Method for chromatography.

On the corner "D" of a 40 cm square Toyo filter paper No. 2 (see Fig. 1), put the sample (0.5 mg for amino acid) soaking in a circle whose diameter is about 5~7 mm (+ in the figure). When the spot has dried, made a cylinder of the paper by clipping the A and B corners together, and held this cylindrical paper upright in a Petri dish containing phenol (with 10% water) until the liquid went up to the height of 35 cm by capillary motion in an airtight vessel. About 24 hours was needed to reach that height in the airtight vessel with a temperature of about 20°C.

When the phenol in the paper tissue completely dried up by being given heat, another cylindrical form was made by clipping the B and C corners of the square paper, and it was held in lutidine + aniline (9 : 1) solution (saturated with water up to 95%).

Through these two experiments, the amino acids were separated into two dimensions. When completely dried by subjection to heat, a 0.25% ninhydrin-butanol solution was lightly sprayed on the paper, and then by application of heat at 95°C, the amino acids on the paper showed various colors.

Consden, Gordon and Martin[8], and Polson[9] have shown that the chemically

related amino acids occupy certain definite regions on two dimensional paper chromatogram. Conclusions can be drawn regarding their probable identity. The acidic, glutamic and aspartic acids occupy a region close to the origin. The basic, histidine, lysine and arginine are the top middle, while the neutral aromatic amino acids, tyrosine and phenylalanine are along the bottom of the chromatogram. The neutral aliphatic amino acids are regularly distributed, whatever the pair of solvents employed, the straight-chain homologous of glycine, alanine, valine, and leucine always fall on a smooth curve. This is illustrated by Fig. 1, which is a tracing from a two-dimensional chromatogram of an artifical mixture. The writer has identified the kind of amino acids by the Rf value, the change of the color by applying ninhydrin, with reference to Fig. 1.

Results

The writer is also pleased to advise of the following results as to the hydrolysate of four other kinds of acid-fast bacteria which were hydrolyzed with hydrochloric acid and barium hydroxide.

(1) In the hydrochloric acid hydrolysate of *Mycobacterium tuberculosis var. hominis*, aspartic acid, glutamic acid, cystine, glycine, alanine, histidine, valine, leucine (+isoleucine ?), serine, probably β-hydroxy-glutamic acid (spot No. 13), and an unknown spot (No. 14) were found. This is illustrated by Fig. 2.

Amino acids which were similar to those in the hydrochloric acid hydrolysate were also found in the barium hydroxide hydrolysate, however, the spot (No. 14) was not observed.

This is illustrated by Fig. 3.

(2) In the hydrochloric acid hydrolysate of *Mycobacterium var. avium*, aspartic acid, glutamic acid, cystine, glycine, alanine, histidine, valine, leucine (+ isoleucine ?), serine, β-hydroxy-glutamic acid probably (spot No. 13) and an unknown spot (No. 14) were detected. This is illustrated by Fig. 4.

In the barium hydroxide hydrolysate, the amino acids which were similar to those in the hydrochloric acid hydrolysate were observed, but the spot (No. 14) was not observed. This is illustrated by Fig. 5.

(3) In the hydrochloric acid hydrolysate of *Mycobacterium ranae*, aspartic acid, glutamic acid, cystine, glycine. alanine, histidine, valine, leucine (+ isoleucine ?), serine, tyrosine, phenylalanine, threonine probably, β-hydroxy-glutamic acid presumably and an unknown spot (No. 14) were detected. This is illustrated by Fig. 6.

Fig. 1. Two-dimensional chromatogram of a mixture of artifical amino acids.

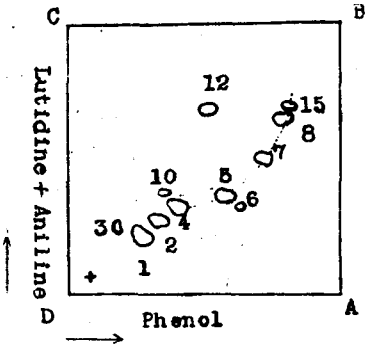


Fig. 2. Two-dimensional chromatogram of HCl hydrolysate of *M. tuberculosis var. hominis.*

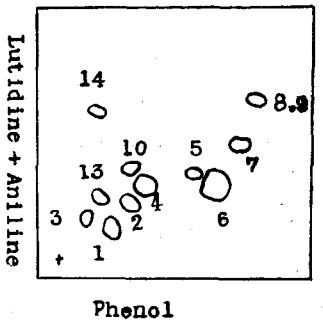


Fig. 3. Two-dimensional chromatogram of $Ba(OH)_2$ hydrolysate of *M. tuberculosis var. hominis.*

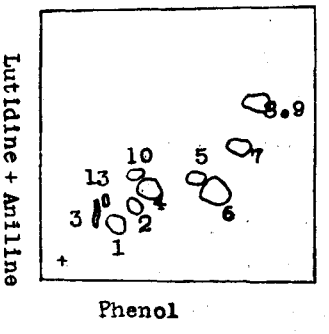


Fig. 4. Two-dimensional chromatogram of HCl hydrolysate of *M. tuberculosis var. avium.*

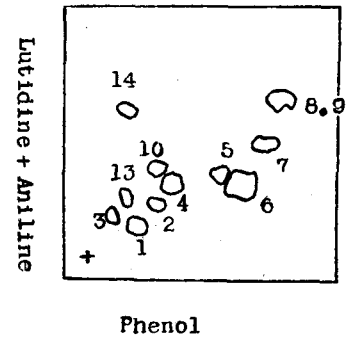


Fig 5. Two-dimensional chromatogram of $Ba(OH)_2$ hydrolysate of *M. tuberculosis var. avium.*

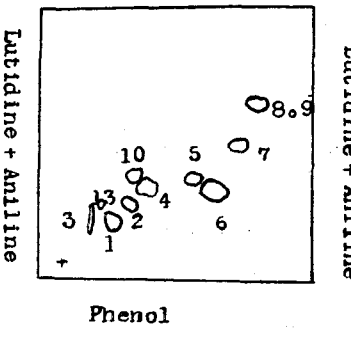


Fig. 6. Two-dimensional chromatogram of HCl hydrolysate of *M. ranae.*

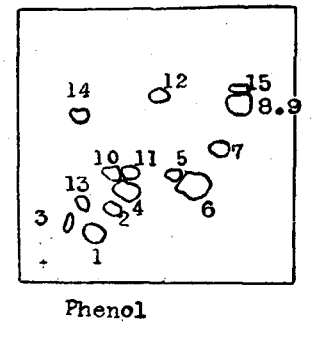


Fig. 7. Two-dimensional chromatogram of $Ba(OH)_2$ hydrolysate of *M. ranae.*

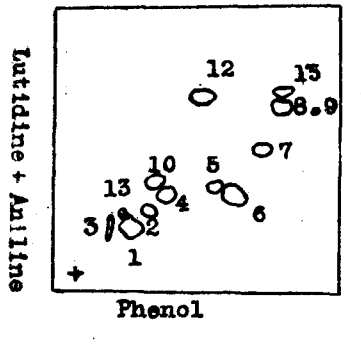


Fig. 8. Two-dimensional chromatogram of HCl hydrolysate of *M. smegmatis.*

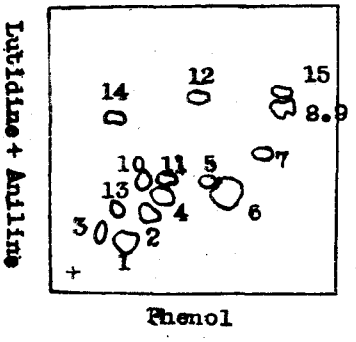
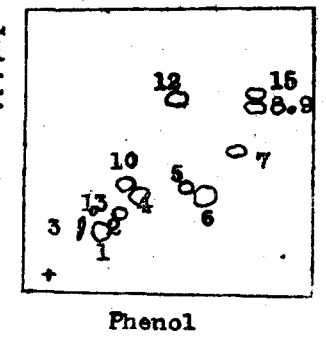


Fig. 9. Two-dimensional chromatogram of $Ba(CH)_2$ hydrolysate of *M. smegmatis.*



1. aspartic acid
2. glutamic acid
3. cystine
4. glycine
5. alanine
6. histidine
7. valine
8. leucine
9. isoleucine
10. serine
11. methionine (presumably)
12. tyrosine
13. β-hydroxy-glutamic acid (presumably)
14. unknown spot
15. phenylalanine

In the barium hydroxide hydrolysate, the amino acids which were similar to those in the hydrochloric acid hydrolysate were detected, however, a spot (No. 14) and another one seeming to be threonine were not observed. This is illustrated by Fig. 7.

(4) In the hydrochloric acid hydrolysate of *Mycobacterium smegmatis*, aspartic acid, glutamic acid, cystine, glycine, alanine, histidine, valine, leucine (+isoleucine ?) serine, a threonine probably, tyrosine, pheylalanine, a spot presumably β-hydroxyglutamic acid and a spot (No. 14) were detected. This is illustrated by Fig. 8.

In the barium hydroxide hydrolysate, the amino acids similar to those in the hydrochloric acid hydrolysate were found, but nothing as to No. 14 spot and another one (to be supposed as threonine) was detected. This is illustrated by Fig. 9.

Discussion.

Many a chemist has reported that a large quantity of hexone bases is contained among acid-fast bacteria. But in the present author's paper partition chromatography experiments, lysine and arginine that are counted as hexone bases were not detected, although a large quantity of histidine was seen.

Recently A. Pereira and J. A. Serra[10] have reported the method of quantitative microdetermination of amino acid after paper chromatography using a Lumetron colorimeter with a 550-mμ filter and a microcell 2 cm thick. They obtained the following results. The sensitivity of the ninhydrin reaction is different on the amino acids, histidine being the least sensitive.

In the present writer's experiments, histidine which had been derived from four kinds of bacteria was plentifully detected in its spots rather than the other. But since no trace of lysine and arginine was observed, writer proposes to make further study of the material, using different kinds of solvents.

Concluding this report, the writer wishes to express his heartfelt thanks to Prof. Tetsuo TOMIYAMA, Faculty of Agriculture, Kyushu University, Prof. Tsuneyuki SAITO, Hokkaido Gakugei University, (i. e. Univ. of Liberal Arts), and Dr. Yoshio UTIYAMA, Hakodate Quarantine Office who gave all possible assistance to promote the research, supplying valuable amino acids and the strains of acid-fast bacteria which were indispensable to the course of the study.

Summary

The writer studied amino acid of acid-fast bacteria by means of paper partition

chromatography with the two solvents 90% phenol and lutidine + Aniline (9 : 1) mixture (saturated with water up to 95%). The following are the results of the experiments.

In the hydrochloric acid hydrolysate of *M. tuberculosis var. hominis* and *M. tuberculosis var. avium*, aspartic acid, glutamic acid, cystine, glycine, alanine, histidine, valine, leucine (+isoleucine ?), serine, probably it may be β-hydroxy-glutamic acid and an unknown spot (No. 14) were found. Amino acids which were similar to those in the hydrochloric acid hydrolysate were also found in barium hydroxide hydrolysate; however, the unknown spot (No. 14) was not observed.

(2) In the hydrochloric acid hydrolysate of *M. ranae* and *M. smegmatis*, aspartic acid, glutamic acid, cystine, glycine, alanine, histidine, valine, leucine (+isoleucine ?), serine, tyrosine, phenylalanine, a threonine presumably, a β-hydroxy-glutamic acid presumably, and an unknown spot (No. 14) were detected. The amino acids which were similar to those in the hydrochloric acid hydrolysate were also found in barium hydroxide hydrolysate, but nothing as to a spot (No. 14) and another one (possibly threonine) were detected.

(3) Other chemists have reported that a large quantity of hexone bases is contained among acid-fast bacteria, but in the present paper partition chromatography experiments, lysine and arginine that are counted as hexone bases were not detected, although a large quantity of histidine was seen.

References.

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