STUDIES ON THE PROTEIN OF VARIOUS MYCOBACTERIA
III. ON THE CHEMICAL PROPERTIES OF THE SEVERAL
PEPTIDES ISOLATED FROM THE BACTERIAL CELLS
OF *MYCOBACTERIUM AVIUM*

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In a successive series of papers published from our laboratory by Kasuya *et al.* (1956) and Hirai (1956), extensive studies on the chemical properties of the proteins obtained both from the culture filtrate and from the bacterial cells of the human tubercle bacilli have been reported.

The technique of the investigation used throughout these works has been the same in principle; namely, that every protein should be separated into its single peptides, and their identification and classification should in turn define the chemical characteristics of the original protein as a starting material.

With respect to the methodology, it can be said that such an approach is considered to play an important role also in exploring chemical knowledge on the protein of the different species of Mycobacteria. Thus, in major part, this paper deals with an application of the same methods to the study of the constituting peptide of *Myco. avium*. However, as a minor difference, it is to be mentioned that only the protein extracted from dried and defatted bacterial cells by dilute sulfuric acid was used as a starting material because the yield of protein from the culture filtrate was, generally, very small. Also, as to the technique of the determination of the N-terminal residue, not only the DNP-technique but also the PTC-(phenylthiocarbamyl) method were utilized when necessary.

MATERIALS AND METHODS

Starting Material: *Myco. avium*-62 was cultured for six weeks in Lockemann's synthetic medium. Cells which were filtered from the unheated culture by filter paper were dried and defatted. From these defatted cells, the protein was extracted in quite the same way as was described by Hirai (1956).

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Homogeneity Test: This was done by Tiselius Electrophoresis (Fig. 1).

Fig. 1. Tiselius electrophoretic patterns of Sa. (Sadako Hirai, 1956) prepared from the culture filtrate of *Myco. avium*-62, in phosphate buffer pH 7.8, 65 V., 8 mA., 45 min.

Isolation of the DNP- and PTC-Peptide by the Counter Current Distribution (CCD): The technique used in the preparation of the DNP-protein, its partial degradation and the separation of the resultant DNP-peptides were quite the same as were described in a previous paper (Kasuya *et al.*, 1956).

However, as will be discussed later, the failure of the DNP-method in identifying the N-terminal residue of the peptide contained in the fast moving peak (e) has necessitated the introduction of the PTC-method in combination with the CCD. The PTC-derivative of protein was prepared by the method of E. O. P. Thompson (1954). One hundred mg of protein previously oxidized with performic acid was dissolved in 3 cc of dioxane-H₂O=1:1 (v/v), to which was then added 0.2 cc of phenyl-isothiocyanate under stirring. The pH had been adjusted to 8.6 throughout the reaction. After stirring for 40 minutes the reaction was completed, and the solvent was evaporated in reduced pressure, and the PTC-peptide thus obtained was subjected to CCD by the same solvent system as was used in the separation of DNP-peptides. In the case of PTC-peptide, the color was more faintly brownish, so the approximate site of the peak was detected by the measurement of optical density at 500 μ m.

The Identification of the DNP- and PTC-Peptides: The methods of the identification of the DNP-peptides which were separated by CCD was the same as described previously (Kasuya *et al.*, 1956). But the stepwise degradation of the PTC-peptide was carried out according to the method described by S. W. Fox (1951); that is, Peak (e) was drawn out, and after complete removal of the solvent *in vacuo*, it was incubated at 37°C for 6 hours with the addition of 3 cc of dioxane preliminarily saturated with dry hydrogen chloride gas at room temperature. Then the dioxane-HCl was evaporated in reduced pressure, and the residue was distributed between benzene-H₂O (1:1) with shaking. For the regeneration of the amino acid of thiohydantoin, the benzene phase, containing thiohydantoin of the N-terminal residue, was dried and hydrolyzed in a sealed tube at 120°C for 24 hours with a mixture of formic acid: acetic acid anhydride: perchloric acid 1.0:5.5:1.5 (v/v), after W. S. Reith *et al.* (1954).

The tube was then cut very carefully as it contained a high pressure of oxygen which was evolved during the hydrolysis. The acid was neutralized by N-KOH to pH 3.0 against BPB. After removal of the major part of the solvent in reduced pressure, it was left for 24 hours in a vacuum desiccator over sulfuric acid and sodium hydroxide. Then the regenerated amino acid was extracted with ethanol:H₂O=1:1, and was run in paper chromatography. For the development of the color not only 0.2% ninhydrin in butanol was used but also 0.2% isatin in acetone containing 4% acetic acid was employed (Oreskes *et al.*, 1955).

The molar ratio of the yielded peptides designated in terms of the area under the curve measured by paper method (Kasuya *et al.*, 1956).

RESULT

1. CCD patterns of the DNP-peptides are shown in Figs. 2 to 5. The molar ratio of the peptides yielded in each peak is as follows:

$$a:d:e=315:443:720=(\text{circa})1:1:2$$

Fig. 2. CCD-i, CCD $\left(\frac{n\text{-BuOH}}{5\%\text{-NH}_4\text{OH}}\right)$ of DNP-Sa. *Myco. avium*-62 after H·COOOH treatment.

Fig. 3. CCD-ii, CCD $\left(\frac{\text{AcOEt}}{\text{pH6.8-Phosph. buff.}}\right)$ of the peak b in Fig. 2.

Fig. 4. CCD-iii, Re-CCD $\left(\frac{\text{AcOEt}}{\text{pH6.8-Phosph. buff.}}\right)$ of the peak d in Fig 3.

Fig. 5. Paper-chromatogram of the N-terminal amino acids

2. Paper chromatograph of the DNP-amino acids, and of the amino acids regenerated from the PTC-derivatives, as the N-terminal residues. The results are summarized in Table 1,

Table 1. Catalogue of the constituting peptide isolated from DNP-peptide by CCD after preliminary oxidation with performic acid

Obtained from peak	N-terminal residue	K	Amino acid composition	Peptide No.
a	DNP-Asp	0.1	Asp-[Ser. Gly. MetSO ₂ . Try. Thr. Val.- α -NH ₂ -Bu. Glu. CySO ₃ H]	I
d	DNP-Ala	1.0	Ala-[Asp. Glu. α,ϵ -(NH ₂) ₂ -Pim. Tau. Gly.-Tyr. Leu. Lys. Arg. CySO ₃ H]	II
e	HO-Pro	1.8	HO-Pro-[Glu. Ala. His. Val. Tyr. CySO ₃ H]	III

DISCUSSION

First to be discussed is the method of identification of the N-terminal residue of the peptide contained in Peak (e). Here the DNP-method had failed to yield any DNP-amino acid except dinitroaniline, probably because of the destruction either of the imino ring of proline or of the imidazol ring of histidine, when they were the N-terminal residue in question. In this case, the use of the PTC method, however, gave a satisfactory result in proving the residue to be hydroxyproline: the color developed by ninhydrin was yellowish brown, by isatin blue green (Oreskes *et al.*, 1955); and its R_f value was 0.51 in 0.3%-NH₃-phenol which is more characteristic of hydroxyproline than of proline.

Next to be discussed is the significance of the results with respect to the chemical differences in the proteins of the different species of mycobacteria. The immunological difference, observed by skin test, between the tuberculin protein of *Myco. avium* and that of the mammalian type of tubercle bacilli, *viz.* *Myco. tbc. var. humanus* and *bovinus*, has been shown already by F. B. Seibert and Morley (1950). But from the chemical viewpoint, no appreciable contribution has appeared except the data of the overall amino acid analysis of the avian tubercle bacilli by Yamamura and Shimizu (1950). Now, according to our results, the chemical difference of the proteins obtained from the bacterial cells of the two different species of *Mycobacterium*, *viz.* *Myco. avium* and *Myco. tbc. var. humanus* has been clearly demonstrated.

The peptide obtained from Peak (a) was:

the aspartic acid peptide, which was quite similar to the one previously obtained from the human tubercle bacilli (Kasuya *et al.*, 1956), and so far said to be the species common.

Nevertheless, from Peak (d):

alanine peptide has been obtained; while in contrast to this, the peptide obtained from the corresponding peak of the human tubercle bacilli is, as is already known,

β -Ala peptide (Kasuya and Hagitani, 1955; Kasuya *et al.*, 1956). And it is interesting enough that the N-terminal residues of the similar peptides appositive in partition coefficient of the two species of *Mycobacteria*, pathogenic and non pathogenic, have been proven to be the structural isomers of the alanine reciprocally.

The fact that the N-terminal residue of the peptide from (e) has been proved to be a hydroxyproline was an unexpected evidence:

This is also interesting when we consider the peptide from the corresponding peak in the case of the human tubercle bacilli was also β -Ala peptide. This reminds us too of the fact that the only instance in which proline has been found to be the N-terminal residue of a peptide was the case in protamin (Ando *et al.*, 1952).

The original protein was shown to have single peak in electrophoretical curve visualized under the condition given in Fig. 1. However, in reference to the numbers of the variety of the peptides obtained, and the molar ratio mentioned in the experimental part, the original protein is also considered to be a mixture of at least two components as was in the case of the previous report (Kasuya *et al.*, 1956), which is supposed to be as follows:

Component A³⁾=Peptide(I) + Peptide(III)

Component B³⁾=Peptide(II) + Peptide(III)

SUMMARY

1. The constituting peptides of the protein obtained by dilute sulfuric acid extraction from dried and defatted bacterial cells of *Myco. avium*, have been studied.

2. The techniques used in this investigation were in major part the same as those used in the previous papers except that, for the identification of N-terminal residue, not only the DNP-technique was employed, but also the PTC (phenyl-thiocarbamyl) method was also introduced, by which the identification of the N-terminal residue of the hydroxy-proline-peptide was made possible.

3. As a result, the constituting peptides have been proved to be each one mol of aspartic acid peptide, and alanine peptide, and two mols of hydroxy-proline peptide.

4. The comparative biochemical difference of the constituting peptides of *Myco. avium* and of *Myco. tbc. var. humanus* has been discussed.

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³⁾ A and B are the two components of proteins supposed to have approximately the same mobility in electrophoresis,

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