BIKEN'S JOURNAL 4, 121-130 (1961)

Extracellular Proteins of Tubercle Bacilli
II Some Serological Properties of Purified Extracellular Proteins of a Virulent Strain (H37Rv) of *Mycobacterium tuberculosis*[1),2)]

MASAHIKO YONEDA AND YOSHIO FUKUI

Department of Tuberculosis, Research Institute for Microbial Diseases, Osaka University, Osaka
(Received for publication, May 1, 1961)

SUMMARY

By using a double agar gel diffusion technique, some of the serological properties of the purified protein fractions isolated from unheated culture filtrates of a virulent strain (H37Rv) were studied. The results were as follows:

(1) The purified protein fractions (mtp-1, mtp-3 and mtp-4) tested against anti 0-80 per cent fraction rabbit serum in Ouchterlony's plate formed a single precipitation line while no precipitation occurred with fraction mtp-2. These precipitation lines were lost completely by heating the proteins at 70°C for 10 minutes, showing the heat lability of these antigens.

(2) Formation of a coalescence of precipitation lines in double diffusion analysis indicated that fractions mtp-1 and mtp-3 were of identical serological specificity, and differed greatly from fraction mtp-4 since it forms a crossing precipitation line with the former antigens.

(3) Analyses of the antigenic relationship between the purified protein fractions and proteins A, B, C showed that the latter proteins contained a small amount of fraction mtp-1 (or-3) as a common antigen while the antigen in B was cross-reacting with mtp-1 (or -3). No antigen of identical specificity with fraction mtp-4 was detectable in proteins A, B and C.

(4) Addition of some tuberculin protein preparations obtained from heated culture filtrates and of a crystalline tuberculin peptide preparation to the anti 0-50 per cent fraction rabbit serum failed to inhibit the precipitation reactions between the antiserum and fractions mtp-1, mtp-3 and mtp-4.

INTRODUCTION

The previous paper (Yoneda and Fukui, 1961) of this series described the isolation of four protein fractions (mtp-1, mtp-2, mtp-3 and mtp-4) from unheated culture filtrates of an H37Rv strain of *Mycobacterium tuberculosis*. The isolation method employed a combination of 50 per cent ammonium sulfate fractionation and zone-electrophoresis. Various chemical and physico-chemical analyses indicated the considerable apparent purity of three final fractions (mtp-1, mtp-3

1) This was aided by a grant from the Ministry of Education, Japan

2) An outline of this work was reported at the 33th Meeting of the Japanese Bacteriological Association (July 18, 1960) and also at the 6th meeting for the Chemical Study of Tuberculosis (April 3, 1961).

and mtp-4). A preliminary study showed that these protein fractions were antigenic and, when tested in agar by the double diffusion method, they were found to give almost a single precipitation line against crude antisera such as the anti 0-80 per cent ammonium sulfate saturation fraction rabbit serum. The availability of purified preparations of some extracellular proteins of this organism has permitted us to carry out investigations on the role of these proteins in tuberculous infection and acquired immunity. Before attempting these investigations, however, we thought it of significance to elucidate some of the serological properties of the proteins.

The present report concerns agar gel precipitation analyses of these thr e protein fractions with reference to their mutual identification, heat stability and their relation to the fractions present in the proteins A, B and C prepared by Seibert's method. In addition, the failure of the addition of some tuberculin proteins, to inhibit precipitation reactions between our purified protein fractions and their respective antibodies is reported.

MATERIALS AND METHODS

1. *Protein preparations*

The preparation and chemical, and physico-chemical properties of the four purified extracellular proteins (mtp-1, mtp-2, mtp-3 and mtp-4) of *M. tuberculosis* H37Rv strain were described in the preceding paper (Yoneda and Fukui, 1961). Concentrated solutions of the proteins (1-2 per cent) in borate-phosphate buffer (pH 8.3, $\mu=0.1$) were stored at -20°C until needed, and, unless otherwise noted, dilutions in saline was made just before use.

2. *Tuberculin preparations*

Seven kinds of so-called tuberculin preparations were used in this study. The proteins A, B and C prepared from unheated culture filtrates of a virulent strain (Aoyama B) of *M. tuberculosis hominis* by Seibert's method (1949) were generously supplied by Dr. Aoki, the 5th Department of the Institute for Infectious Diseases, University of Tokyo. PPDS and π (Toda and Takeya, 1955) were kindly given by Dr. T. Toda, Department of Bacteriology, Medical School, University of Kyushu and a crystalline tuberculin peptide preparation prepared by the method of Yamamura *et al.* (1960) was kindly provided from Dr. Yamamura's laboratory. Old tuberculin used was obtained from a stock in this institute.

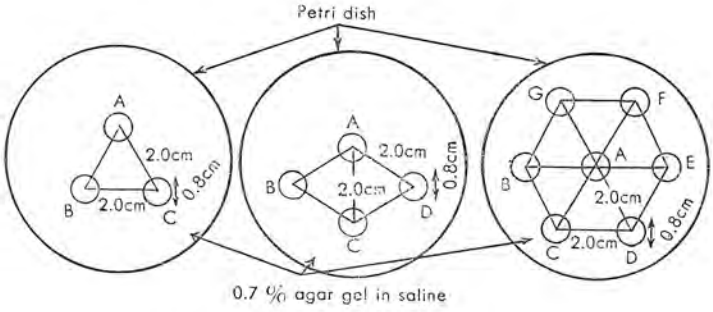
3. *Preparation of antisera*

The preparation of anti 0-80 per cent. 0-30 per cent and 30-50 per cent ammonium sulfate saturation fractions rabbit sera are described in the preceding paper (Yoneda and Fukui, 1961).

4. *Gel-diffusion precipitation technique*

The Petri dish method as described by Ouchterlony (1948) was used with the following slight modifications. 0.7 per cent Difco Bacto agar in physiological saline with added merthiolate (1: 10,000) was used. Antibiotic testing metal cups were first placed in Petri dishes in the pattern shown in Figure 1 and then the agar solution was poured into the dish. After the agar had solidified, the cups were removed gently so that wells were formed and a drop of melted agar was added to seal the bottom of the wells. Agar plugs and small amounts of water remaining in the wells were removed just before sealing the dishes. The wells were then filled with liquid antigen or antiserum and the

plates incubated at 37°C in a small chamber in which other Petri dishes of water are placed to prevent drying. Wells were never refilled.



A : Antiserum 0.3 ml
B, C, D, E, F and G : Various antigens

Fig. 1. Form and arrangement of reservoirs in the modified Ouchterlony's method

RESULTS

1. *Serological specificity of the purified antigens*

Double-diffusion precipitation reactions in tubes, using Oakley's method with a slight modification, showed that both mtp-1 and mtp-3 gave only a single

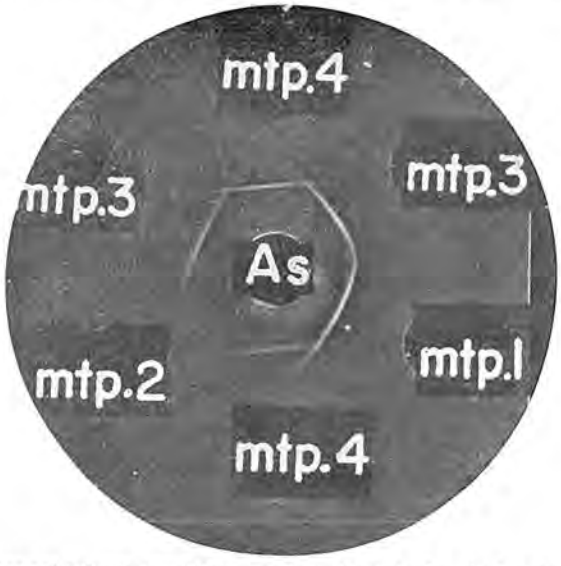


Fig. 2. Agar gel diffusion precipitation pattern demonstrating the interrelationship between the four purified protein fractions

As (center) : Anti 0-50 % fraction rabbit serum.
The protein concentration of the antigens was 250 μg/ml.

precipitation line and mtp-4, two lines, one of which was very faint (1961). A study was made of the serological interrelationship between these antigens using Ouchterlony's double-diffusion precipitation technique (1948) in plates.

In an Ouchterlony plate, undiluted anti 0-50 per cent fraction rabbit serum was added to the center well and antigen solutions containing 250 μg/ml of mtp-1, mtp-2, mtp-3 and mtp-4 were added to six peripheral wells. The plates were incubated at 37° C for 3 days and then photographed.

Figure 2 is an Ouchterlony plate showing the interrelationship of these antigens in terms of their serological specificity. As is seen in the photograph, a coalescent precipitation line is formed with mtp-1 and mtp-3 against the antiserum in the plate, while this coalescent line crosses a precipitation line formed with mtp-4. The failure to show another precipitation line due to the minor antigen component in mtp-4 shown in Oakley's tube, may be partly due to a lower resolution power in the plate than in the tube and partly to the lower concentration of antigen solution employed (250 μg/ml).

2. *Heat lability of the purified antigens*

From the data of chemical and UV absorption analyses described in our preceding paper (1961), in which the purified fractions employed as antigens in this study were shown to be of protein nature, the heat lability of the precipitation reactions of these antigens is to be expected.

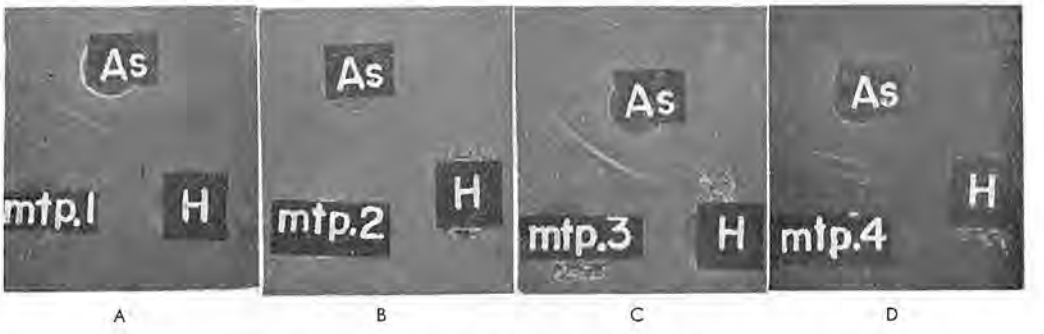


Fig. 3. Agar gel diffusion precipitation patterns produced by heated and unheated purified fractions

A : Between heated and unheated mtp-1
B : Between heated and unheated mtp-2
C : Between heated and uuheated mtp-3
D : Between heated and unheated mtp-4
As: Anti 0-50 % fraction rabbit serum.
H : Purified fraction heated at 70° C for 10 minutes.
The protein concentration of the antigens was 250 μg/ml.

Figure 3 illustrates the change in the precipitation lines formed with these antigens against antiserum before and after heating the antigens at 70° C for 10

minutes. In each plate, the top well was filled with 0.3 ml of undiluted anti 0-50 per cent fraction serum and the lower wells were filled with 0.3 ml of corresponding heated and unheated antigen solutions (250 μg/ml). The plates were incubated at 37° C for 3 days and then photographed.

As is seen in these photographs, whereas unheated mtp-1, mtp-3 and mtp-4 antigens tested against the antiserum develop a single precipitation line in the plates, no precipitation line was formed with the corresponding heated antigens. A detailed study of the effect of temperature on the precipitation reaction between the antigens and antiserum in Ouchterlony plates showed that the precipitation lines all disappeared after heating the antigens at 60° C for 10 minutes. No precipitation was seen with mtp-2 whether or not it was heated.

3. *Serological relationship between the purified proteins (mtp-1, mtp-3 and mtp-4) and the proteins A, B and C*

As described in the preceding section, the three purified fractions mtp-1, mtp-3 and mtp-4 appear to be heat labile antigens which form single precipitation lines against a crude antiserum when tested in Ouchterlony plates. Moreover mtp-1 (or mtp-3) was distinguishable from mtp-4 in serological specificity. Since the proteins A, B and C obtained from unheated culture filtrates of human tubercle bacilli by Seibert's method (1949), which are considered to be the most well defined tuberculo-proteins, still contained a variety of precipitating antigens (Seibert *et al.*, 1957), studies were made to see if there was any antigen in protein A, B and C corresponding to mtp-1 (or mtp-3) or mtp-4.

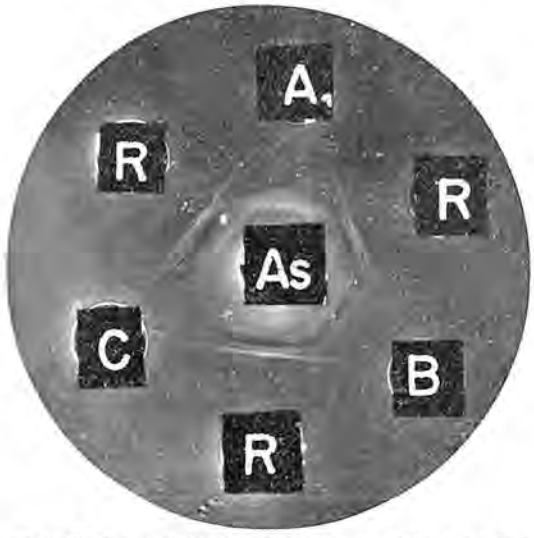


Fig. 4. Agar gel diffusion precipitation pattern demonstrating the relationship between the 0-80 per cent fraction and proteins A, B, C.

As (center) : Anti 0-80 % fraction rabbit serum:
A, B, C : The Prateins A, B and C (3 mg/ml) R : 0-80 % fraction (1 mg/ml)

Figure 4 shows the serological relation between a concentrated unheated culture filtrate of H37Rv strain obtained by 80 per cent ammonium sulfate saturation and the proteins A, B and C in an Ouchterlony plate. The center well was filled with 0.3 ml of undiluted anti 0-80 per cent fraction rabbit serum and the peripheral wells, with 0.3 ml of 1 mg/ml of the concentrated crude culture filtrate and of 3 mg/ml of the proteins A, B and C respectively. The plate was incubated at 37° C for 3 days and then photographed.

The photograph shows three precipitation lines between the crude antigen and the antiserum, one of which coalesces with a slightly diffuse line formed with the proteins A, B and C while the other two sharp lines do not.

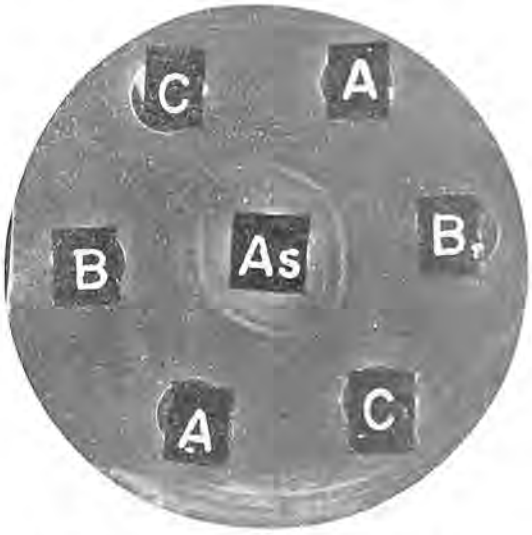


Fig. 5. Agar gel diffusion precipitation pattern demostrating the interrelationship between proteins A, B and C

As (center) : Anti 0-80 % fraction rabbit serum
A, B, C : Proteins A, B and C (3 mg/ml)

Figure 5 illustrates the pattern of precipitation reactions of the proteins A, B, C and the anti 0-80 per cent fraction rabbit serum in an Ouchterlony plate and shows the serological interrelation between these antigens. The concentrations of the proteins A, B and C solutions were the same as in the previous experiment. From the photograph at least two precipitation lines are seen to be formed with all of the antigens tested and these lines coalesce with each other, but the outer coalescent line forms a spur between A and B. This coalescent line was not formed when the antigens were heated at 70° C for 10 minutes, while the other diffusive line near the antiserum well still appeared.

The results indicate that there are at least two precipitating antigens in common in the proteins A, B and C. Presumably the heat labile antigen was the one which coalesced with one of the precipitation lines of our crude concentrated antigen in the plate as shown in Figure 4. Moreover, the heat labile antigen of B was found to be partially identical with that of A in terms of its specificity. Experiments were therefore made to see which of our purified antigens corresponded to this common antigen.

In Ouchterlony plates, the top well was filled with 0.3 ml of undiluted anti 0-50 per cent fraction rabbit serum and the lower wells with 0.3 ml of 3 mg/ml of the proteins A, B or C and 0.3 ml of 250 μg/ml of mtp-3 or mtp-4 respectively. Plates were incubated at 37° C for 3 days and then photographed. Figure 6

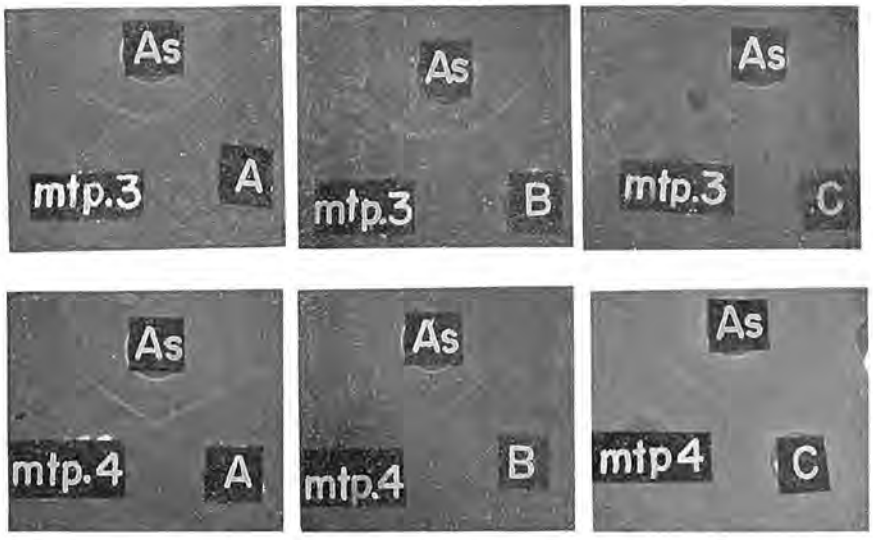


Fig. 6. Agar gel diffusion precipitation patterns demonstrating the relation between proteins A, B, C and two purified protein fractions

(1), (2), (3) : Between mtp-3 and proteins A, B, C
(4), (5), (6) : Between mtp-4 and proteins A, B, C
As : Auti 0-50 % fraction rabbit serum.
Protein concentration : mtp-3 and mtp-4 250 μg/ml. proteins A, B and C 3 mg/ml.

illustrates these photographs. As can be seen, the precipitation line developed between the mtp-3 well and the antiserum well fused completely with one of the lines formed with the proteins A and C, but fused with a spur in the case of B. The single precipitation line formed with mtp-4, on the other hand, crossed the line in both cases and no line was seen coalescing with the former line. When the protein A, B and C solutions were diluted to 250 μg/ml for use as antigens in similar experiments, no precipitation line was formed with the A, B and C antigens

and the line formed with mtp-3 showed no bending even in the parts closest to these antigen wells.

4. *Reaction between various tuberculin preparations and the anti 0-80 per cent fraction rabbit serum*

Using old tuberculin, PPDS, π, and Yamamura's crystalline tuberculin preparation, experiments were made to see if these tuberculins combined with the specific precipitating antibody for mtp-1 (or -3) or for mtp-4.

Ring tests showed that, with the exception of crystalline peptides, all the tuberculin preparations contained a small amount of heat stable antigen which was thought to be due to contaminating tuberculous polysaccharides. However, when tested in Ouchterlony plates at a concentration of 3 mg/ml of antigen, no visible precipitation line were formed against anti 0-80 per cent fraction rabbit serum. It was thought that some of the tuberculins or their derivatives might combine with the precipitating antibody in the antiserum resulting in a block of the precipitation reaction between mtp-1 (or -3) or mtp-4 and the antibody. To test this possibility 5 mg of PPDS, π, and of a crystalline peptide preparation provided by Dr. Yamamura were each dissolved in 1 ml of the antiserum and incubated at 37° C for 60 minutes. The precipitating activity of these sera were compared with that of untreated antiserum by the ring test and Ouchterlony's double diffusion precipitation technique in agar. In the Ouchterlony plates, the central well was filled with 0.3 ml of untreated or of tuberculin-treated antiserum and peripheral wells, with 0.3 ml of 250 μg/ml concentration of the purified antigens mtp-3 and mtp-4,

No change was seen of the precipitating capability of treated antisera in terms of antiserum dilution by the ring test. Figure 7 illustrates the pattern of the pre-

Fig. 7. Agar gel diffusion precipitation pattern produced by mtp-1, mtp-3 and mtp-4 against anti 0-80 per cent fraction rabbit serum mixed with 5 mg/ml of crystalline peptides

As+CP : Anti 0-80 % fraction rabbit serum mixed with 5 mg/ml of crystalline peptides.

The protein concentrations of mtp-1, mtp-3 and mtp-4 were 250 μg/ml.

cipitation reaction in an Ouchterlony plate with mtp-4 against the anti 0-80 per cent fraction rabbit serum treated with 5 mg of crystalline peptide preparation. As can be seen from the photographs, both mtp-3 and mtp-4 react resulting in the formation of sharp precipitation lines between the antigen and antiserum wells.

DISCUSSION

As discussed in the preceding paper (1961), mtp-1 and mtp-3 are very similar in terms of their chemical composition, particularly in their sedimentation constants and electrophoretic mobilities. This lead us to suspect that the two protein fractions might also have similar serological specificity. The data presented here clearly show that this is the case. In an Ouchterlony plate, both mtp-1 and mtp-3 gave a single precipitation line against crude antisera and the line coalesced without any spur formation. These results suggest that the determinant groups of these proteins responsible for combining precipitating antibody are essentially the same. Mtp-4, on the other hand, is definitely different from both mtp-1 and mtp-3 not only in its serological specificity but also in its physico-chemical properties. Therefore, from a serological point of view, one may consider that the soluble fraction precipitable by 50 per cent ammonium sulfate is comprised of two major serologically active protein antigens. Preliminary column-chromatographical analyses using DEAE-cellulose have also suggested that this is the case (unpublished data).

Seibert *et al.* (1957) stated that in a serological study of her highly purified proteins A, B and C by the agar gel diffusion technique in tubes, there were a variety of precipitating antigens. In this study it was further shown that there was at least one heat labile antigen in common between the proteins A, B and C. Moreover, it is of particular interest, that we found that the antigens in A and C were identical with mtp-1 (or -3) but the antigen in B, cross reacted with mtp-1 (or -3) in the agar gel diffusion precipitation in plates. Although exact values are not yet available, we found that about ten times more of proteins A, B and C than of mtp-3 (or -1) was needed to form precipitation lines in the same position. This could be accounted for by assuming that there is not more than 10 per cent of mtp-3 (or -1) or c.r.m. in proteins A, B and C. If this is generally the case with proteins prepared by precipitation at their isoelectric point and with alcohol, it may be very difficult to use them for serological investigations on tubercle bacilli. No detectable antigen forming a coalescent precipitation line with that of mtp-4 was found in the proteins A, B and C. However, since the proteins A, B and C used in this study were prepared not from the unheated culture filtrate of H37Rv but from that of a different strain (Aoyama B) of *M. tuberculosis hominis*, the results may merely be due to an absence of mtp-4 from the latter culture filtrate. To test this, the 0-80 per cent ammonium sulfate saturation fraction of unheated culture filtrates of Aoyama B strain were tested in Ouchterlony plates against the anti H37Rv

0-80 per cent fraction rabbit serum using mtp-1, mtp-3 and mtp-4 as indicators, the filtrate was found to contain both mtp-1 (or -3) and mtp-4. However, differences in the conditions used both for obtaining the culture filtrate and for preparing the proteins A, B and C might well cause the elimination or denaturation of their mtp-4.

Preliminary skin reaction experiments in H37Rv bacilli sensitized guinea pigs showed that the purified proteins mtp-1, mtp-2, mtp-3 and mtp-4 possesed a considerable tuberculin activity. Very recently Yamamura *et al.* (1961) succeeded in obtaining crystalline tuberculin peptides from the cell bodies of various human type tubercle bacilli. Therefore we tested whether these active peptides could inhibit the precipitation reactions between one of the purified protein antigens and the antiserum by specifically combining precipitating antibodies. However, they did not. Moreover, no other tuberculin derivatives tested inhibited the reaction. Since these tuberculin preparations were prepared either from heated culture filtrates or heat killed cells of tubercle bacilli, it may be that the specific combining group, if any, was destroyed by heating.

Although the ring test showed that purified proteins mtp-1, mtp-3 and mtp-4 still contained appreciable amounts of some heat resistant antigen which may presumably represent polysaccharides, available data suggest that these three fractions are valuable reagents for serological investigations. Thus attempts are now being made to purify these proteins further by column-chromatography in DEAE cellulose and to apply the purified antigens to a taxonomic study of *Mycobacteria*.

ACKNOWLEDGEMENTS

The authors are grateful to Drs. M. Hori and H. Shôji for their interest in this work and are also greatly indebted to Dr. Yamamura for supplying his valuable crystalline peptides, to Dr. Aoki, for the proteins A, B and C and to Drs. Toda and Takeya, for PPDS and π.

REFERENCES

Ouchterlony, Ö. (1948). Antigen-antibody reaction in gels. *Arkiv. Kemi. Mineral Geol.* **26 B,** 1-9.

Seibert, F. B. (1949). The isolation of three different proteins and two polysaccharides from tuberculin by alcohol fractionation. *Am. Rev. Tuberc.* **59,** 86.

Seibert, F. B. and soto-Figueroa, E. (1957). Study of tuberculin protein and polysaccharide antigens by gel-diffusion technique. *Am. Rev. Tuberc.* **75,** 601-607.

Toda, T. and Takeya, K. (1955). Studies on purified tuberculin protein. *In Recent Investigations on Purified Tuberculin.* (edited by Toda, T.), pp. 1-26.

Yamamura, Y., Morishima, S., Tanaka, A. and Shojima, K. (1960). Studies on tuberculin active peptide. I. The isolation, crystallization and properties of tuberculin active peptide from tubercle bacillus. *Biochim. et Biophys. Acta* **38,** 252-258.

Yoneda, M. and Fukui, Y. (1961). Extracellular proteins of tubercle bacilli. I. Zone-electrophoretic purification of the proteins of a virulent strain (H37Rv) of *Mycobacterium tuberculosis*. *Biken's J.* **4,** 25-40.