BIKEN JOURNAL Vol. 8, 201-223, 1965

EXTRACELLULAR PROTEINS OF TUBERCLE BACILLI
V. DISTRIBUTION OF α AND β ANTIGENS IN VARIOUS MYCOBACTERIA[1]

MASAHIKO YONEDA[2], YOSHIO FUKUI[2] and TAKAHISA YAMANOUCHI[3]

Department of Tuberculosis Research, Research Institute for Microbial Diseases, Osaka University, Osaka
(Received September 15, 1965)

SUMMARY By employing an immunodiffusion technique, the distribution of α and β antigens was studied in some detail in 120 test strains of mycobacteria including: *M. tuberculosis*, 9; *M. bovis*, 4; *M. avium*, 9; isoniazid-resistant strains of *M. tuberculosis* and *bovis*, 32; *M. tuberculosis* strains resistant to drugs other than isoniazid derivatives, 9; *M. microti* (Vole bacillus), 1; *M. ulcerans*, 1; *M. paratuberculosis*, 1; *M. balnei*, 1; *M. lepraemurium*, 2; saprophytic, 6; "unclassified mycobacteria" 45. From the distribution pattern of α, β and of the cross-reaction material with α, it was suggested that the strains tested may be divided into the following four general groups: I. *Strains in which both α and β are detectable.* With the sole exception of isoniazid-resistant strains, this group includes all the strains of *M. tuberculosis and bovis* and one strain of *M. microti* tested. II. *Strains in which no extracellular β but α is detectable.* These include all the isoniazid-resistant strains of *M. tuberculosis* and *bovis* tested. They can be divided further into the following two sub-groups: 1) β-negative strains and 2) intracellular β-positive strains. III. *Strains in which no β is detectable but cross-reaction material with antigenic determinants partially in common with those of α antigen is found.* In this group are included all the strains of *M. avium*, *M. paratuberculosis*, *M. balnei*, *M. ulcerans*, *M. lepraemurium* tested and 24 out of 45 strains of the unclassified mycobacteria used. The strains tested were further divisible on the basis of the antigenic specificity of their cross-reaction material. IV *Strains in which neither α nor β is detectable.* All saprophytic strains including *M. fortuitum*, *M. phlei*, *M. smegmatis*, three other unidentified strains (Takeo, Dencho and Jucho) and the 21 unclassified strains of mycobacteria are included in this group.

1. This investigation received financial support from the World Health Organization.
2. Department of Tuberculosis Research I.
3. Department of Tuberculosis Research II.

INTRODUCTION

Previous communications from this laboratory (YONEDA and FUKUI, 1961 a, b; FUKUI and YONEDA, 1961; YONEDA, 1963), reported the isolation and partial characterization of two proteins, α and β, from unheated culture filtrates of a virulent strain (H37Rv) of *Mycobacterium tuberculosis*. These proteins were both found to behave as complete antigens and to be immunochemically distinguishable. The preceding communication (FUKUI, HIRAI, UCHIDA and YONEDA, 1965) presented evidence to indicate further that the α and β proteins comprise nearly 70 per cent of the total protein fraction released by the organism into the medium and that they are also present on/in the cell, presumably as surface antigens. Thus, both α and β appear to constitute the antigenic structure of the particular mycobacterial culture employed, as very major protein components.

From this finding together with the preliminary observation that these antigens were not detectable in the culture filtrates of several strains of so-called saprophytic mycobacteria tested (unpublished data), it was thought that these antigens might possibly have some association with the biological types of mycobacteria. It was even thought that either α or β might be a type specific antigen, whose type specificity obviously is related to the significance of these antigens in the serological typing for mycobacterial classification. Our attention was thus focused on the question of the existence and the immunochemical status of α and β in various mycobacteria, and an investigation has been made of the distribution and characterization of these antigens in many strains of mycobacteria, on the firm recognition of which any consideration of the taxonomical significance of α and β must rest.

The present communication describes the results so far obtained from antigenic analyses of 120 strains of various mycobacteria. The analyses were mainly carried out by Ouchterlony's immunodiffusion technique and sometimes by quantitative precipitation tests employing highly purified preparations of α and β antigens and their respective specific antisera as indicators.

MATERIALS AND METHODS

1. *Mycobacterial strains*

Except for the strains of unclassified mycobacteria kindly provided by Dr. ERNEST RUNYON, all the strains employed were from the stock collection of the Departments of Tuberculosis Research and Leprology of this Institute. They were as follows: *M. tuberculosis*, 9 strains (H37Rv, H37Ra, H_2, Toda, Takagaki, Kurono, Frankfult, Kamiike and Aoyama B); *M. tuberculosis* (originating from strains H37Rv), 9 strains which are resistant to streptomycin, kanamycin, PAS, pillaromycin, rufomycin, viomycin, zygomycin, DDS and 1314TH respectively. *M. bovis*, 4 strains (Ravenel, TC50, BCG-1 and Miwa); *M. tuberculosis* and *bovis* resistant to isoniazid, 32 strains; *M. microti*, 1 strain; *M. avium*, 9 strains (Kirchberg, 4110, A71, Flamingo, A62, 4121, Demoiselle, E38686 and Jueki); *M. ulcerans*, 1 strain; *M. paratuberculosis*, 1 strain; *M. balnei*, 1 strain; *M. lepaemurium*, 2 strains (Hawaii and Douglas), the bacterial extracts of which were supplied by Dr. TATSUO MORI of the Department of Leprology of this Institute; saprophytic mycobacteria, 6 strains (*M. fortuitum*, *M. smegmatis*, *M. phlei*, Takeo, Dencho and Jucho); "unclassified mycobacteria" provided by Dr. RUNYON, 45 strains.

2. *Test antigens*

As test antigens, crude concentrates of unheated culture filtrates and/or mechanical cell disintegrates of the test cultures were used. The preparation procedures were as follows: 1) *Crude concentrates of unheated cluture filtrates* were prepared from the strains which could be grown perfectly on/in a modified Sauton's medium (YONEDA and FUKUI, 1961 a). One loopful pericle or a large drop of whole culture, pre-grown in this medium, was inoculated into 80 ml volumes of the medium in 300 ml Erlenmeyer flasks and incubated at 37°C for three to four weeks, depending on which test strains was used. After incubation, the bacilli were filtered off aseptically through Toyo No. 50 filter paper and then a Millipore filter (HA, 0.45 μ) in the cold without previous heating of the culture. The resulting

culture filtrates were then concentrated to 1/10 to 1/20 of their original volume with carbowax 6000 (polyethylene glycol). 2) *Cell disintegrates* of all the test strains were prepared. Except for *M. paratuberculosis* and *M. lepraemurium*, these strains were grown on a solid medium (Ogawa medium) for three weeks. After incubation, several loopfuls of the culture on the surface of the slant were mixed with roughly an equal volume of quartz powder (200 mesh) and then ground in a small mortar for about three minutes in the cold. The resulting muddy paste was finally mixed with roughly an equal volume of phosphate buffer, pH 7.8, (M/15). The mixture was stood for a few minutes, and then the still very turbid supernatant was employed as test antigen.

3. *Indicator antigens*

Highly purified preparations of α and β, which were previously described as mtp-1 (or mtp-3) and mtp-4 respectively (YONEDA and FUKUI, 1961 a, b), were employed as indicator antigens. The concentration of these antigens in M/15 phosphate buffer (pH 7.0) solution for agar gel diffusion analyses was 20 to 50 μg protein per ml. The α and β antigens were prepared from unheated culture filtrates of strain H37Rv by a combination of ammonium sulfate fractionation, zone-electrophoresis and chromatographic fractionations. The details of the preparation procedure and the immunochemical properties of these antigens were given in previous papers (YONEDA and FUKUI, 1961 a, b; FUKUI and YONEDA, 1961).

4. *Indicator antisera*

Anti-α and -β rabbit sera were used as indicator antisera. The amounts of specific precipitating antibodies were 0.14 and 0.15 mg antibody N per ml of serum respectively. They were prepared by immunizing rabbits with highly purified preparations of α and β antigens in incomplete Freund's adjuvants according to the schedule described previously (YONEDA and FUKUI, 1961 a; FUKUI and YONEDA, 1961; FUKUI, HIRAI, UCHIDA and YONEDA, 1965).

5. *Antisera against crude culture filtrates*

Antisera of strains, P8 (*M. kansasii*) and 4110 (*M. avium*), were prepared by immunizing rabbits with concentrated crude filtrates. The entire immunization procedure was as described previously (YONEDA amd FUKUI, 1961 a). Rabbit antisera were also prepared for the cross-reaction material with α in P8 culture, as described in a later section.

6. *Immunodiffusion test*

The double diffusion precipitation method in agar described by OUCHTERLONY (1948) was employed with a slight modification. The results were usually examined after standing the agar plate at room temperature for five to seven days.

7. *Quantitative precipitation*

The quantitative precipitation test was done as described by HEIDERBERGER and KENDALL (1955). As antigens, α and a partially purified preparation of the cross-reaction material with α in P8 strain were used, and as antibodies, specific anti-α rabbit serum and a rabbit antiserum for the cross-reaction material were used. The nitrogen content of the specific precipitates were determined by a modified micro-Kjeldahl-Nessler method (YOKOI and AKASI, 1955).

8. *Zone-electrophoresis*

Zone-electrophoresis using starch as the supporting medium was performed according to the method described by KUNKEL and SLATER (1952) with a slight modification (YONEDA and FUKUI, 1961 a).

RESULTS

Unless otherwise specified, the three wells in the Ouchterlony's plate employed for antigenic analyses of test strains were arranged in a regular triangular pattern and in there were put the test antigens, indicator antigen and antiserum respectively, so that the presence and immunochemical status of the common antigen with α or β in the test antigens could be examined.

The antigenic analyses of 120 strains of mycobacteria employing the Ouchterlony's technique suggested that the distribution of α and β in the test strains is apparently type specific rather than strain, species or genus specific. From the distribution pattern, these 120 mycobacterial strains could be roughly divided into the following four groups: 1. Strains in which both α and β are detectable. 2. Strains in which no extracellular β but only α is detectable. 3. Strains in which no β but a cross-reaction material with some antigenic determi-

nants in common with α is detectable. 4. Strains in which neither α nor β is detectable.

1. *Strains in which both α and β antigens are detectable*

With the sole exception of isoniazid-resistant strains, which will be described later, all strains of *M. tuberculosis*, *M. bovis* and *M. microti* (Vole bacillus) tested can be put into this group.

Fig. 1, 2 and 3 show typical immunodiffusion patterns in Ouchterlony's plates with test antigens prepared from representative strains (H37Ra, Ravenel) which belong to *M. tuberculosis* and *bovis* respectively and from a strain of *M. microti*.

It can be seen that the test antigen reacts with either anti-α or -β serum forming a precipitation line in each strain and the line fuses completely with a single line formed between the indicator antiserum and α or β antigen respectively. The results clearly indicate that these test antigens contain both α and β as precipitating antigens.

Similar analyses were made repeatedly of the test antigens prepared from 17 other strains of *M. tuberculosis* including H37Rv, H_2, Toda, Takagaki, Kurono, Frankfult, Kamiike and Aoyama B, 9 strains of H37Rv resistant to antituberculous drugs other than isoniazid derivatives and from 3 other strains (TC50, BCG-1 and Miwa) of *M. bovis*, and the results were exactly the same as these described above.

Whether or not the antigenic determinants of α or β of human type strains are absolutely identical with those of the corresponding antigen of bovine type is not known. However, it has sometimes been observed that, when antiserum against curde culture filtrates of *M. bovis* (Ravenel) was used instead of anti-α serum, the precipitation line formed with crude Ravenel antigen fuses, forming a spur-like line, with a single precipitation line of α antigen. Although it is still uncertain whether this was a true spur, it may be that some difference exists between α and the corresponding antigen of bovine type in terms of antigenic specificity. Purified bovine type α antigen and its specific antiserum is now being prepared in order to clarify this point.

2. *Strains in which no extracellular β but only α is detectable*

The 32 isoniazid (INH)-resistant strains of *M. tuberculosis* and *bovis* tested are included in this group. These strains are listed in Table 1. Thus, among these, 19 were stable, highly resistant strains and the other 13 were unstable strains with lower resistance.

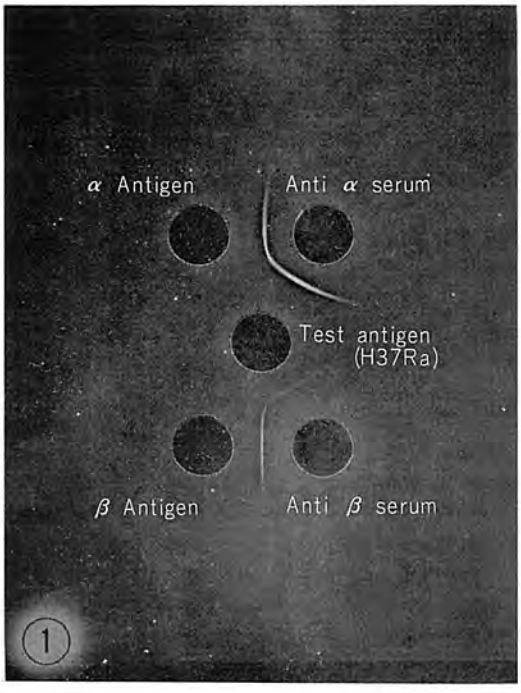
From immunodiffusion analysis of concentrated unheated culture filtrates and of heavy suspensions of intact cells, it was first concluded that none of the INH-resistant strains tested had any β but only α antigen. However, further analysis of the mechanical disintegrates of washed intact cells revealed that β antigen is detectable in the disintegrates of all the 13 strains with lower resistance. On the other hand, in neither culture filtrates nor disintegrates of the intact cells of 19 highly resistant strains could β antigen be found.

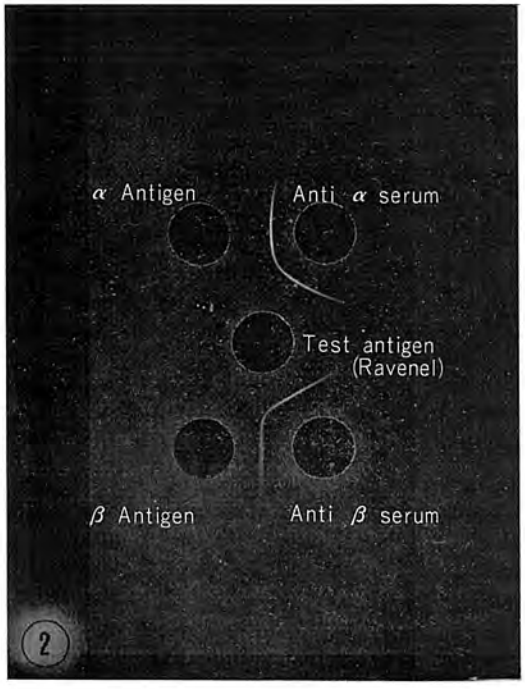
The result of a typical experiment with the Ravenel strains is illustrated in Fig. 4.

In this experiment, the following three strains of Ravenel (*M. bovis*) were employed: 1. original strain (INH-sensitive); 2. INH-low-resistant strain (0.1—0.2 μg/ml); 3. INH-high-resistant strain (10–20 μg/ml). Both concentrated culture filtrates and supernatant fractions of cellular disintegrates obtained by centrifugation at 105,000 $\times$ g for 120 minutes were prepared from each strain and these were used as extracellular and intracellular test antigens respectively.

As may be seen in this Figure, no precipitation line is formed between anti-β serum and the culture filtrate antigens prepared either from INH-high-resistant or -low-resistant strains. This indicates the absence of extracellular β antigen in these strains, irrespective of the degree of their INH resistancy.

On the other hand, as also seen in Fig. 4, in the case of supernatant fractions of cellular





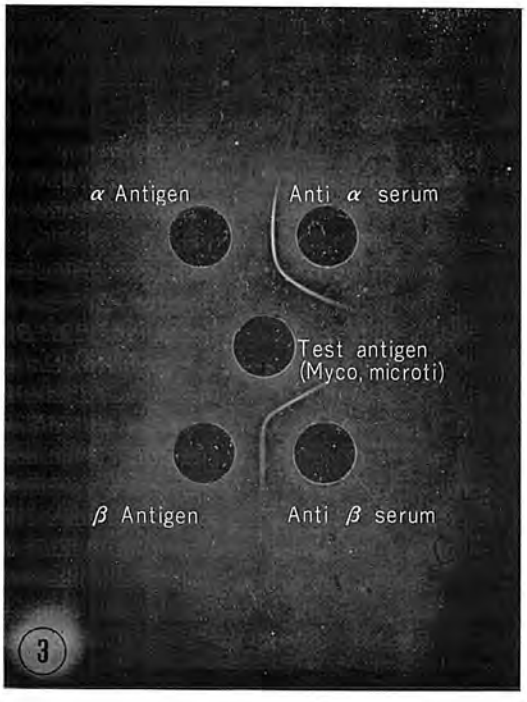


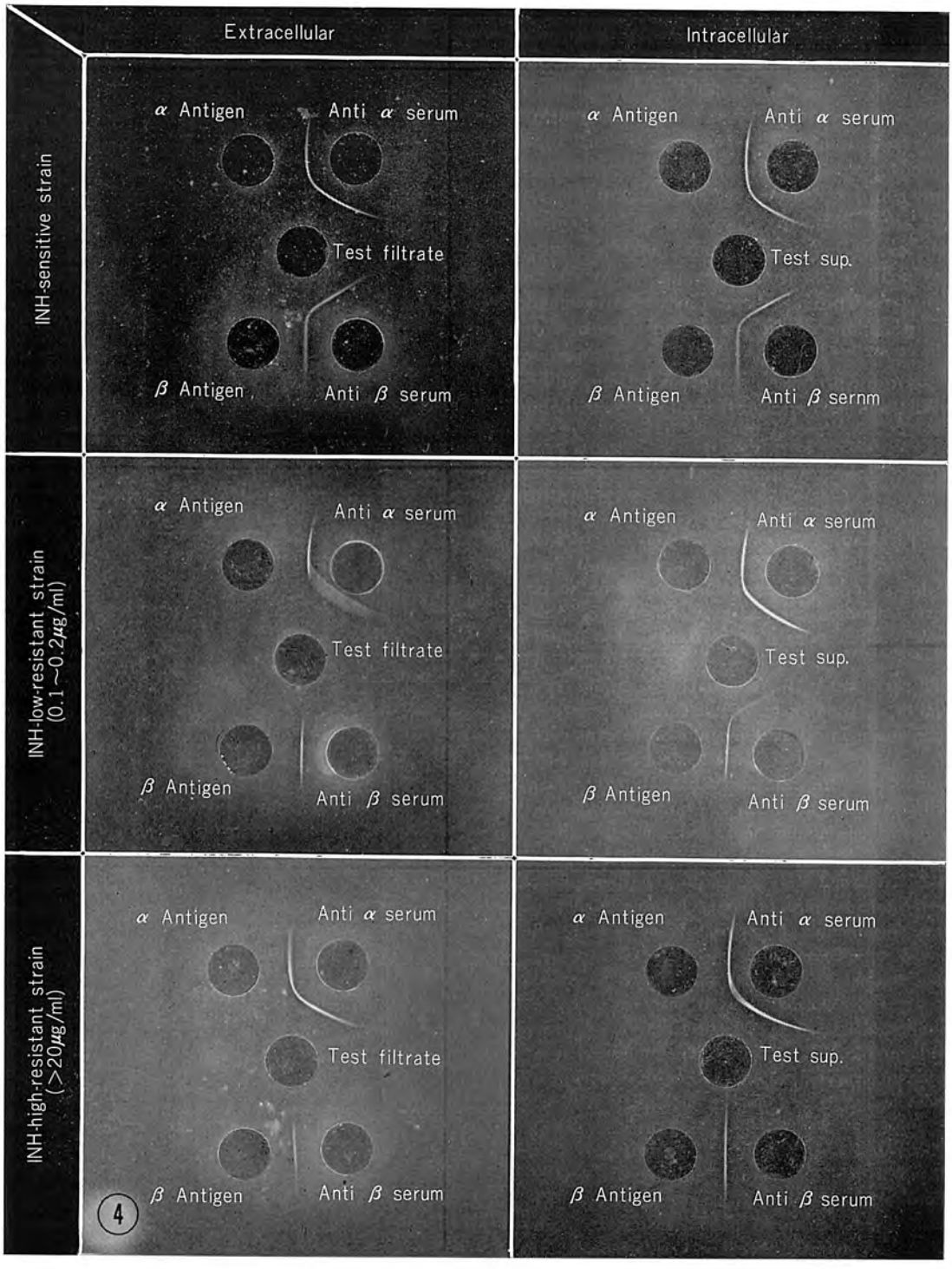
FIGURE 1 Immunodiffusion pattern demonstrating the presence of α and β antigens in strain H37Ra (*M. tuberculosis*).¶
Test antigen: Concentrated culture filtrate.
Indicator antigens: α and β antigens (each 50 μg/ml)
Antisera: Anti-α and -β rabbit sera

FIGURE 2 Immunodiffusion pattern demonstrating the presence of α and β antigens in strain Ravenel (*M. bovis*).
Test antigen: Concentrated culture filtrate
Indicator antigens: α and β antigens (each 50 μg/ml)
Antisera: Anti-α and -β rabbit sera

FIGURE 3 Immunodiffusion pattern demonstrating the presence of α and β antigens in Vole bacillus (*M. microti*).
Test antigen: Concentrated culture filtrate
Indicator antigens: α and β antigens (each 50 μg/ml)
Antisera: Anti-α and -β rabbit sera

FIGURE 4 Immunodiffusion analyses of culture filtrates and mechanical disintegrates of INH-sensitive and -resistant strains.

Test antigens: Concentrated culture filtrates or mechanical disintegrates
Indicator antigens: α and β antigens (each 20 μg/ml)
Indicator antisera: Anti-α and -β rabbit sera


Extracellular
Intracellular
INH-sensitive strain
INH-low-resistant strain (0.1 ~0.2μg/ml)
INH-high-resistant strain (>20μg/ml)
α Antigen
Anti α serum
Test filtrate
Test sup.
β Antigen
Anti β serum
Anti β sernm
4

disintegrates, a precipitation line is formed between the test antigen of the INH-low-resistant strain and anti-β serum and the line fuses completely with a single precipitation line with indicator β antigen, while no precipitation line corresponding to β is detectable with the test antigen prepared from the INH-high-resistant strain. These results clearly show that neither extracellular nor intracellular β is present in detectable amounts in the INH-high-resistant strain, but β antigen is present in the INH-low-resistant strain employed as an intracellular component. Similar experiments were made on the other INH-resistant strains listed in Table 1 and the results were similar.

The absence of β antigen in INH-high-resistant strains was also demonstrated by an absorption technique. The absorption of α and β antibodies from a mixture of anti-α and -β sera either with intact cells or with insoluble cellular debris of INH-resistant strains was according to the procedure described in a preceding communication (FUKUI, HIRAI, UCHIDA and YONEDA, 1965), and the possible absorption of antibodies against α or β in antiserum by these antigens was examined by immunodiffusion in Ouchterlony's plates which

TABLE 1 *List of isoniazid (INH)-resistant test strains of mycobacteria*

Strains		Species	Resistancy (INH μg/ml)	α	β	
					extra	intra
Stocked	H37Rv H37Ra Kurono Mukai Toda Mizuki	*M. tuberculosis*	>20	+	−	−
	Ravenel BCG-1 BCG-2 BCG-3	*M.* bovis				
	H37Ra Kurono H37Rv(E) H37Rv(M)	*M. tuberculosis*	0.2	+	−	+
	BCG-1 Ravenel	*M. bovis*				
Newly isolated from patients	H.H., T.S., S.M., T.S., O., F.K., T.Y., M.M., T.F.	Recognized as human type tubercle bacilli by Niacin Test	>10	+	−	−
	F., G., M., R., I., S., M2.		0.1	+	−	+

were arranged as shown in Fig. 5. The antigens for absorption was obtained from three kinds of INH-resistant strains of Ravenel as described previously.

It may be seen in this Figure that, in case of the INH-high-resistant strain, a precipitation line is formed between the β antigen well and the central well into which a mixture of anti-α and -β sera was put, while no corresponding line appears in the case of the INH-low-resistant strain. This clearly shows that the specific antibody against β in the mixed antiserum could be absorbed with the antigen prepared from the INH-low-resistant strain but not with that from the INH-high-resistant one, indicating the absence of β antigen in the latter strain.

Since β antigen, which was previously named mtp-4, was found to constitute one of two main protein peaks on the zone-electrophoretic diagram of an ammonium sulfate fraction (30–50% Fr.) of unheated culture filtrates of strain H37Rv (YONEDA and FUKUI, 1961 a), it was of interest to see if the protein peak corresponding to β (mtp-4) is absent in the same ammonium sulfate fraction of INH-resistant strains. To test this, unheated culture filtrates of a strain H37Rv resistant to INH (10 μg/ml) were first fractionated with ammonium sulfate and the fraction precipitating at 30–50 per cent saturation was subjected to zone-electrophoresis as described in a previous section.

The result is shown in Fig. 6. The dotted line in the diagram indicates the position of the protein peak when β antigen is subjected to zone-electrophoresis. It may be seen from the diagram that the test faction shows two protein peaks, one major and one minor peak, in the electrophoretic pattern, but no protein peak with the same mobility as β antigen.

The difference between the protein fraction appearing in the diagram as a minor peak and β in terms of antigenic specificity was proved by immunodiffusion. Thus, the protein fractions in the eluates from the 8th to the 20th segments of the starch block were each tested for their antigenic identity with α or β antigen

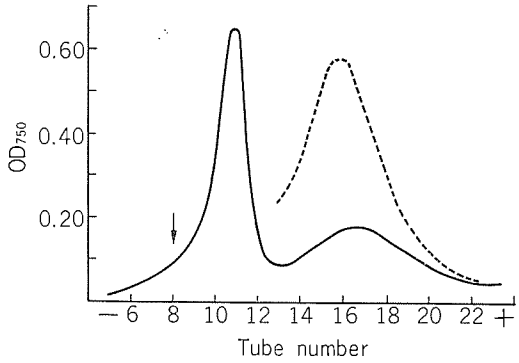


FIGURE 6 Zone-electrophoregram demonstrating the absence of β antigen in INH-resistant strain.
Conditions: borate-phosphate buffer pH8.3 $\mu=0.1$, 2 mA/cm^2, 16 hr run, protein (85.0 mg)
Elution: 2 times with 10 ml of buffered saline
------ Position of β antigen fraction

in Ouchterlony's plates. The results are illustrated in Fig. 7.

As can be seen in the Fig., the eluates from the 10th to 12th segments react with anti 30–50% Fr. rabbit serum forming a precipitation line which fuses with the line of α antigen, while those from the 14th to 20th segments do not form any precipitation line corresponding to β antigen. It was further observed that, even when the latter eluates were concentrated so as to make the protein contents quite high, no precipitation line was formed between anti-β serum and the concentrated eluates. It is thus evident that there is essentially no protein component corresponding to β antigen in the INH-resistant strain.

Thus, on the basis of the findings described above, the isoniazid-resistant strains tested may be tentatively divided into the following subgroups: A. β-negative strains; B. intracellular β-positive strains.

3. *Strains in which one can detect no β antigen but a cross-reaction material with some antigenic determinatnts in common with those of α antigen*

In this group are included all the strains of *M. avium*, *M. lepraemurium*, *M. paratuberculosis*, *M. balnei*, *M. ulcerans* tested and 24 of the 45 strains of "unclassified

FIGURE 5 Immunodiffusion patterns of test serum after absorption with cells or insoluble debris of INH-sensitive, -low and -high-resistant strains.

Test serum: A mixture of anti-α and -β rabbit sera after absorption
Indicator antisera: Anti-α and -β rabbit sera
Indicator antigens: α and β antigens (each 10 μg/ml)

★Erratum: The right and left photographs of the middle line should be exchanged.

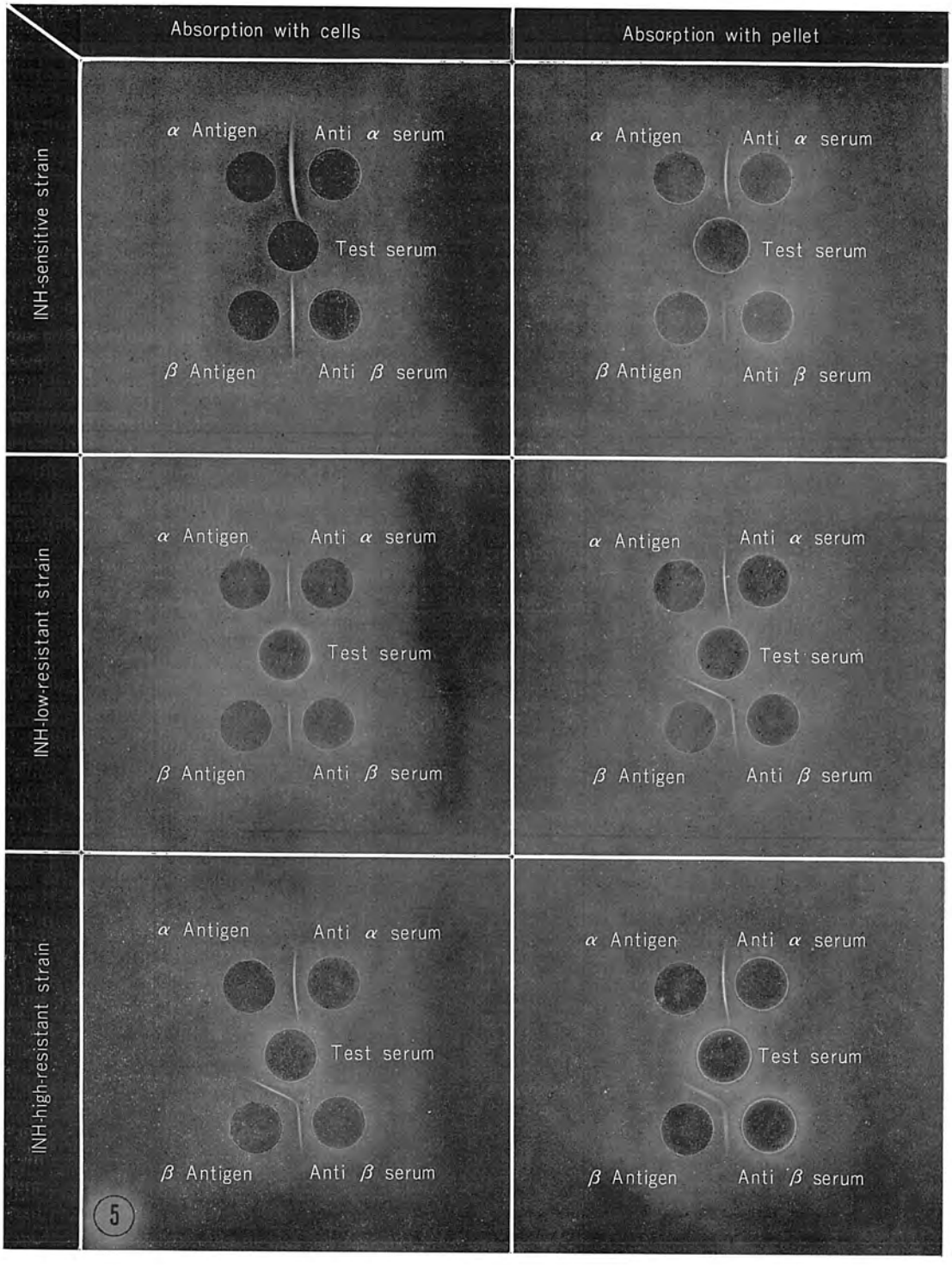
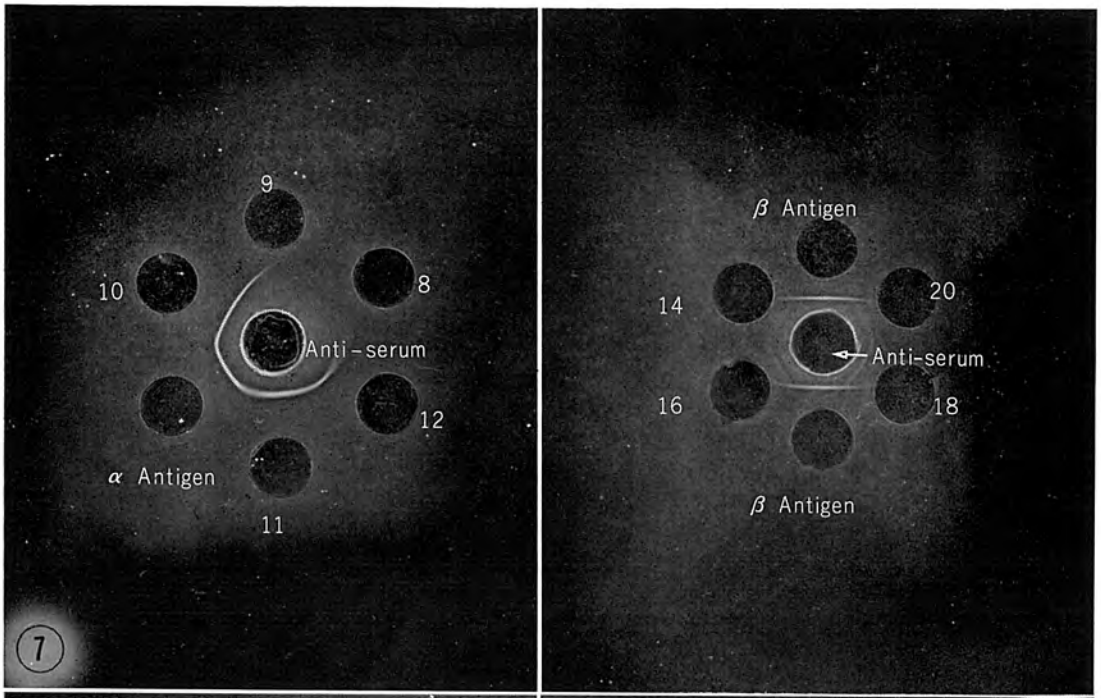
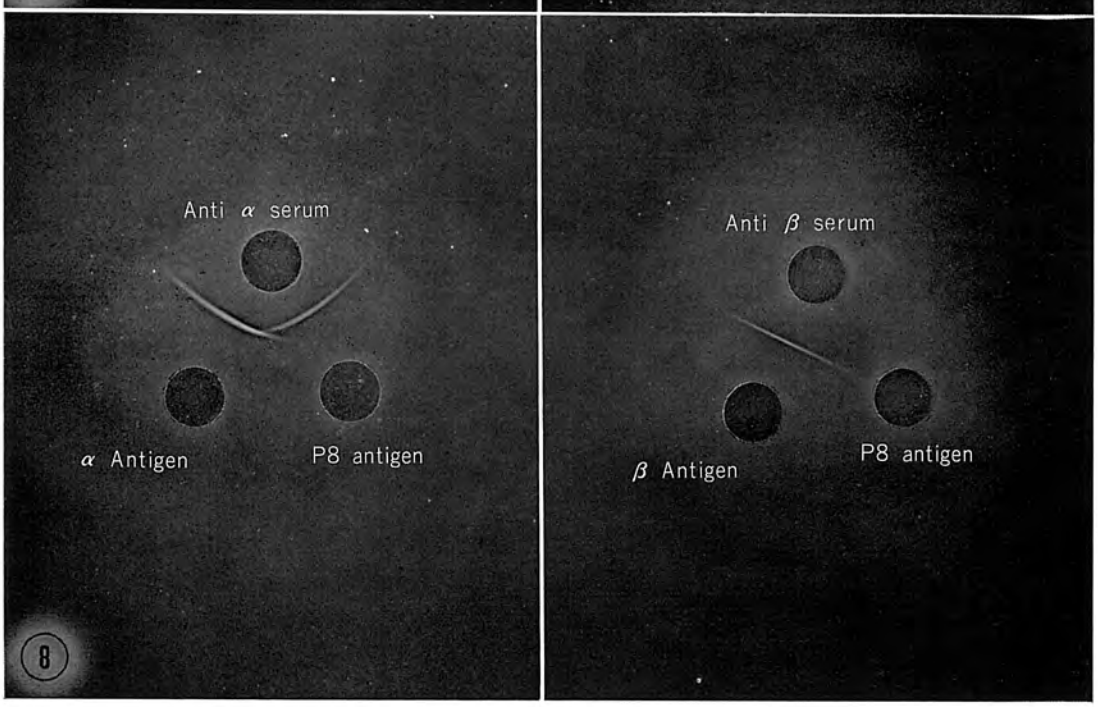

Absorption with cells
Absorption with pellet
INH-sensitive strain
INH-low-resistant strain
INH-high-resistant strain
α Antigen
Anti α serum
Test serum
β Antigen
Anti β serum
5

FIGURE 7 Immunodiffusion patterns demonstrating the absence of β antigen in an INH-resistant strain.
Test antigens: Eluates; the numbers indicate the segments
Indicator antigens: α and β antigens
Antiserum: Anti 30–50 Fr. rabbit serum

FIGURE 8 Immunodiffusion patterns demonstrating the presence of CRM with α antigen and absence of β antigen in the P8 strain.
Indicator antigens: α and β antigens (20 μg/ml)
Indicator antisera: Anti-α and -β rabbit sera
P8 antigen: Concentrated culture filtrate of P8 strain


9
10
8
Anti-serum
12
α Antigen
11
7
β Antigen
14
20
Anti-serum
16
18
β Antigen


Anti α serum
α Antigen
P8 antigen
8
Anti β serum
β Antigen
P8 antigen

mycobacteria" provided by Dr. RUNYON.

The presence of cross-reaction material with α antigen in these strains was demonstrated by both the agar diffusion precipitation and quantitative precipitation tests. Fig. 8 shows a typical precipitation pattern in Ouchterlony's plates with P8 (WOLINSKY *et al.*, 1957) antigen against anti-α or -β serum. The test antigen employed was concentrated, unheated culture filtrate of the P8 strain.

It may be seen that the single precipitation line between α antigen and anti-α serum fuses with the single precipitation line formed between P8 antigen and anti-α serum, with clear spur formation. This shows that the antigen in the crude protein fraction of this particular strain is cross-reaction material with some antigenic determinants in common with those of α antigen. On the other hand, no precipitation line is seen between P8 antigen and anti-β serum.

The presence of the cross-reaction material in strain P8 was also proved by quantitative precipitation tests. In this quantitative experiment the P8 antigen was a preparation of the protein fraction of strain P8, which was seperated by zone-electrophoresis from the unheated culture filtrate by the method used for α antigen and was found electrophoretically to correspond to the α component of the strain H37Rv.

To two series of 10 centrifuge tubes were added respectively increasing amounts of a preparation of α (from 8 to 50 μg N) and of P8 antigen (from 9 to 32 μg N) in a final volume of 1 ml. Then 1 ml of anti-α rabbit serum (Lot No. 2) was added to each tube with through mixing. The mixtures were placed in a water bath at 37°C for 1 hour and then in the cold for one week. They were then centrifuged and the resulting supernatants were used for assay of the antigen and antibody remaining unprecipitated. The precipitates were washed three times with 5 ml of cold physiological saline and then their nitrogen contents were determined.

Fig. 9 summarizes the results obtained. It may be seen that the determination of the nitrogen content in specific precipitates gave a typical quantitative precipitation curve in both cases, each representing the pattern of a single antigen and antibody system. However, as shown in the Figure, the amount of precipitate with P8 antigen was always much less than with α antigen. Thus, the amount of α antibody precipitated with P8 antigen was only about two thirds of that with homologous α antigen at their respective equivalence point. Moreover, it was found that appreciable antibody remained in the supernatant in the antigen excess region in this P8 antigen and α antibody system.

On the other hand, as expected, when anti-P8 antigen serum was used instead of anti-α serum, all the antibody was precipitated with α antigen as well as with P8 antigen at the equivalence point. This is shown in Fig. 10.

In this experiment, increasing amounts of antigen of from 4 to 30 μg N of both α and P8 antigen were placed in two series of 10 tubes.

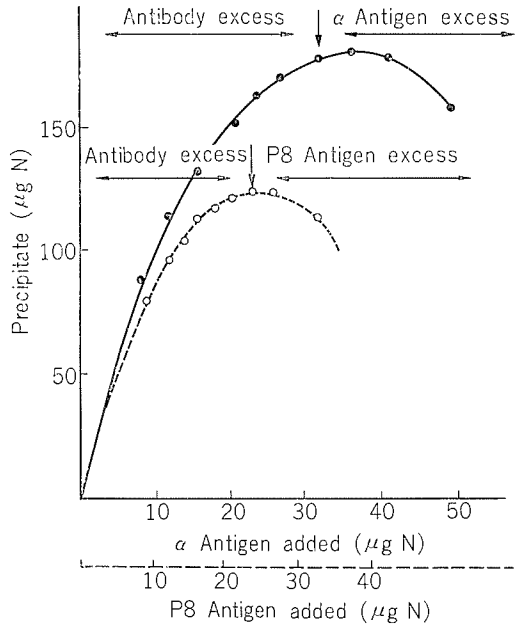


FIGURE 9 Quantitative precipitation reaction of α antigen and its CRM (P8 strain) with anti-α serum.

The antiserum (1 ml per tube) employed was prepared by immunizing rabbits with preparation of P8 protein fraction purified by zone-electrophoresis in incomplete Freund's adjuvant.

As can be seen in Fig. 10, the precipitation curve obtained either with α or with P8 antigen appears to represent a single antigen and antibody system and the two curves are nearly superimposable. It is thus evident from the results of the two experiments described above, that α antigen may have specific antigenic determinants as well as determinants in common with those of P8 antigen.

No antigen in common with β antigen was detectable in any of the strains of this group tested. In connection with this immunochemical observation, a finding of considerable interest was that no protein peak corresponding to β antigen (mtp-4) appeared on the zone-electropherogram of a crude protein fraction of strain P8. Fig. 11 shows a zone-electrophoretic diagram of the protein fraction of P8 strain precipitated by 30–50 per cent saturation of ammonium sulfate (30–50% Fr.).

If β antigen were present, the other main

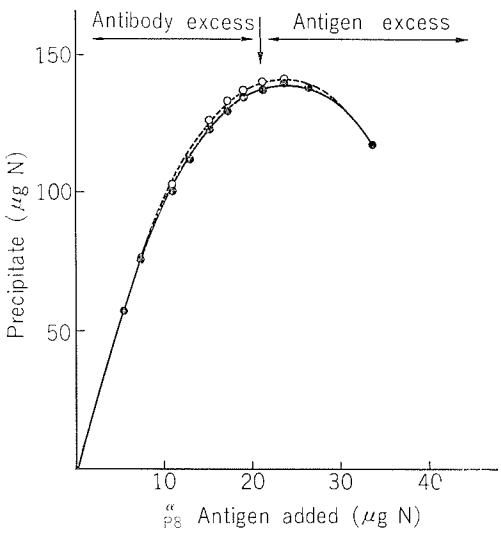


FIGURE 10 Quantitative precipitation reaction of α antigen and its CRM (P8 strain) with anti P8 serum.

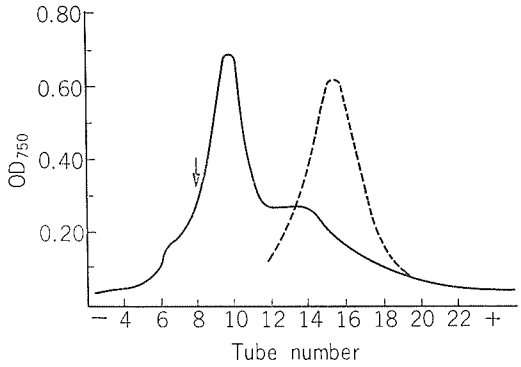


FIGURE 11 Zone-electrophoresis pattern demonstrating the absence of β antigen in strain P8.
Conditions: borate-phosphate buffer, pH 8.3, μ =0.1, 2 mA/cm^2, 16 hr run
Elution: 2 times with 10 ml of buffered saline
------ Position of β antigen fraction

peak indicated by the dotted line would appear. Actually, however, no such protein peak is seen in the diagram. It is thus apparent that this particular protein component is not present in strain P8.

Of particular interest with regard to the antigenic specificity of the cross-reaction material in these strains was the observation that the material which cross reacts with α in strains of *M. avium* is different from that found in strain P8. Thus, when both P8 and avian type antigens were allowed to react with anti-P8 serum in an Ouchterlony's plate, the single precipitation lines formed with the two antigens fused with each other with clear spur formation.

Fig. 12 illustrates a typical result of immunodiffusion analysis of the antigens of strains P8 and 4110 (*M. avium*) with anti-P8 serum, indicating that some of the antigenic determinants of the avian type antigen are in common with those of P8.

On the basis of this finding, an attempt was made to analyze the antigenic interrelationship of the strains in this group by Ouchterlony's technique using both the antigens of P8 and a typical strain (Kirchberg or 4110) of *M. avium* as tentative indicators. The results so

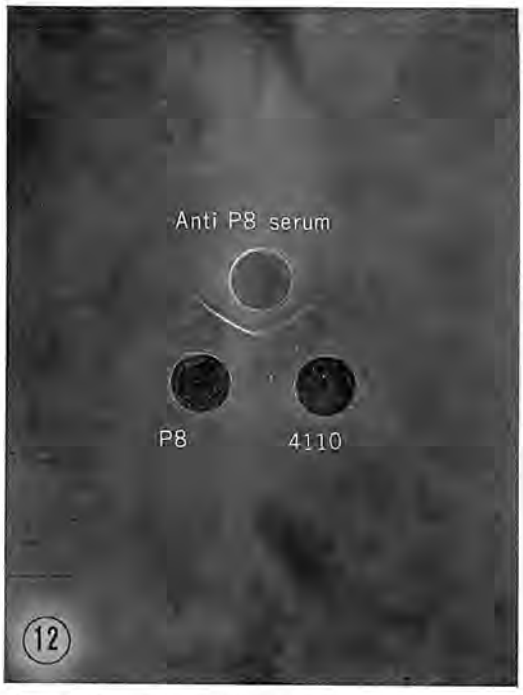


FIGURE 12 Immunodiffusion pattern demonstrating the presence of CRM with P8 antigen in strain 4110 (*M. avium*).
Indicator antigen: P8 α antigen
Test antigen: Concentrated culture filtrate of strain 4110
Antiserum: Anti P8 -α serum (20 μg/ml)

far obtained suggests that these strains may be divided into at least the following sub-groups:

A. (P8 group): P8, P18, P21, P22

B. (Avian group): all the strains of *M. avium* tested, *M. paratuberculosis*, *M. balnei*, *M. ulcerans*, P6, P15, P17, P29, P30, P36, P39, P44, P47, P48, P49, P50, P51, P55, 2057, 2059, 2060, 2062, 2064 and 2066.

The antigenic relationship between the cross-reaction material with α of the strains in each sub-group still remains to be clarified and must await further precise studies employing purified antigen and the specific antiserum of each strain.

The result of an immunodiffusion test with a strain (Hawaii) of *M. lepraemurium* is illustrated in Fig. 13.

As may be seen, the immunodiffusion patterns clearly demonstrate the presence of a cross-reaction material with α antigen and the absence of β antigen in the test antigen of the particular organism employed. A similar results was also obtained with another strain (Douglas) tested, but the question of whether or not these particular strains of *M. lepraemurium* belongs to the sub-groups described above remains unanswered. Extensive studies are now being carried on in collaboration with the Department of Leprology of this Institute on this point and on the problems of whether all the other strains of this species show a similar distribution pattern of α and β antigens to that observed with two strains tested.

4. *Strains in which neither α nor β antigen is detectable*

All saprophytic strains including *M. fortuitum*, *M. phlei*, *M. smegmatis*, Takeo, Dencho and Jucho strains, and 21 strains (P2, P3, P5, P16, P24, P26, P27, P31, P32, P34, P35, P37, P40, P41, P42, P45, P53, P54, 2058, 2061 and 2063) of "unclassified mycobacteria" tested can be included in this group. Immunodiffusion patterns demonstrating the absence of both α and β antigens in a strain of *M. phlei* are shown in Fig. 14.

Table 2 illustrates the tentative relationships between the biological and serological types of the various mycobacteria tested on the basis of the distribution patterns of α and β antigens so far found in this study.

DISCUSSION

In the present investigation, detailed antigenic analyses were made of 120 strains of mycobacteria employing α and β antigens as parameters. The aim of the study was primarily to see if these antigens play a significant role in serological typing for mycobacterial classification. Therefore, the results should be first evaluated from the point of whether the mycobacterial strains tested could be classified on the basis of α and β. Table 2 clearly shows

that this is indeed the case, and thus the 120 test strains were found to be divisible on the basis of the distribution pattern of α antigen, or its cross-reaction material (CRM) and β antigen without any serious discrepancies with the existing biological typing of the mycobacteria.

Merely from a combination of the presence or absence of α and β antigens, theoretically the strains should be divisible into the following four groups: 1) both α and β positive; 2) α positive but β negative; 3) β positive but α negative; 4) both α and β negative. It was found that they were actually divisible in this way but no strains of the 3rd group were found.

Examination of the immunodiffusion precipitation pattern in Ouchterlony's plates, however, revealed the existence in some strains of material cross-reacting (CRM) with α and consequently new group was formed for these mycobacteria (CRM positive but β negative). This CRM also enabled us to divide the new group into further sub-groups.

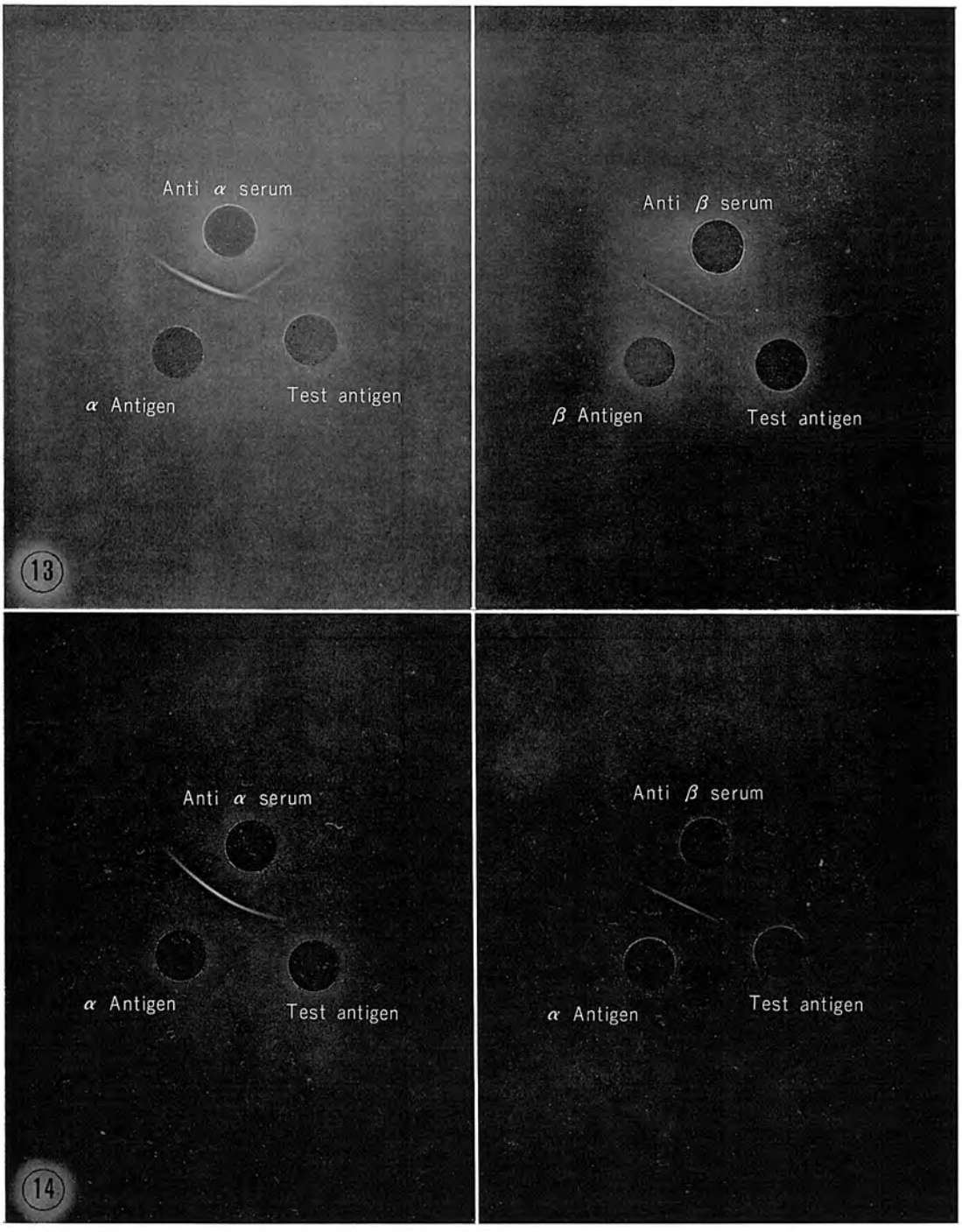
Incidentally, it should be noted that the es-

TABLE 2 *Distribution of α and β antigens in various mycobacteria*

Group	Sub-groups	α	β		Strains
			extra	intra	
I		+	+		*M. tuberculosis:* H37Rv, H37Ra, H_2, Toda, Takagaki, Kurono, Frankfult, Kamiike, Aoyama B, 9 strains of H37Rv resistant to drugs other than INH *M. bovis:* Ravenel, TC 50, BCG, Miwa *M. microti* (Vole bacillus)
II	A	+	−	−	*M. tuberculosis* and *bovis* (INH-high-resistant strains (>10-20 μg)): Kurono, Ravenel, BCG,
	B	+	−	+	*M. tuberculosis* and *bovis* (INH-low-resistant strains (0.1-0.2 μg)): H37Rv, Kurono, Ravenel, BCG, and other strains obtained from patients
III	P8	α^{P8}	−		p 8, p 18, p 21, p 22,
	Avium	α^{avium}	−		*M. avium:* Kirchberg, 4110, A71, Flamingo, A62, 4121 Demoiselle, E. 38686, Jueki *M. ulcerans, M. paratuberculosis,* *M. balnei,* p 6, p 15, p 17, p 29, p 30, p 36, p 39, p 44, p 47, p 48, p 49, p 50, p 51, p 55, 2057, 2059, 2060, 2062, 2064, 2066
	?	+spur	−		*M. lepraemurium* (Hawaii, Douglas)
IV		−	−		Saprophytic: *M. fortuitum, M. smegmatis, M. phlei,* Takeo, Dencho, Jucho, p 2, p 3, p 5, p 16, p 24, p 26, p 27, p 31, p 32, p 34, p 35, p 37, p 40, p 41, p 42, p 45, p 53, p 54, 2058, 2061, 2063

FIGURE 13 Immunodiffusion patterns demonstrating the presence of CRM with α antigen and absence of β antigen in *M. lepraemurium*.
Test antigen: Concentrated supernatant of cellular disintegrates of the Hawaii strain of *M. lepraemurium*
Indicator antigens: α and β antigens (30 μg/ml)
Indicator antisera: Anti-α and -β rabbit sera

FIGURE 14 Immunodiffusion patterns demonstrating the absence of both α and β antigens in *M. phlei*.
Test antigen: Concentrated culture filtrates of *M. phlei*
Indicator antigens: α and β antigens (30 μg/ml)
Indicator antisera: Anti-α and -β rabbit sera


Anti α serum
α Antigen
Test antigen
13
Anti β serum
β Antigen
Test antigen
Anti α serum
α Antigen
Test antigen
14
Anti β serum
α Antigen
Test antigen

tablishment of this new group is entirely attributable to immunochemical analyses of the nature of α or its CRM which revealed the difference in the antigenic determinants. It is thus apparent that, at least in serological typing of mycobacteria, precise analyses of the antigenic determinants of a common antigen are necessary together with the procedures for detecting differences in antigenic composition, which some investigators have already studied (PARLETT and YOUMANS, 1956; 1959; LIND, 1961; CASTELNUOVO *et al.*, 1958; 1959; 1960; 1965).

The exact relationship between the distribution of α or its CRM and β antigens in mycobacteria and their biological types must await further precise studies with more strains. However, from the fact that, except for most of the strains of " unclassified mycobacteria " tested, each group included strains of several mycobacterial species but, no difference was found in the distribution of α or its CRM and β in strains of a single species, it may be reasonably concluded that the distribution of these antigens is type specific rather than strain or species specific. Comments will be made on the unclassified mycobacteria later.

With the exception of isoniazid-resistant strains, which belong to the 2nd serological group, all strains of human and bovine types were found to posses both α and β antigens. It is of particular interest that the β antigen is only found in strains of *M. tuberculosis* and *bovis*, and not even its CRM has yet been found in other mycobacteria. Incidentally, the question of whether there is any difference between the antigenic determinants of α antigen in *M. tuberculosis* and *bovis* is still unknown. However, as was briefly noted in a previous section, preliminary immunodiffusion analysis employing a crude protein fraction of Revenel and its anti rabbit serum suggested that human type α antigen might be cross-reaction material with some antigenic determinants in common with those of Revenel α antigen. If this is so in all strains of *M. bovis*, it would be possible to differentiate bovine strains from those of human type on the basis of the immunochemical difference in their α antigens, as was done so well with the Niacin test (KONNO, 1956).

The absence of β antigen in strains of *M. tuberculosis* and *bovis* which are highly resistant to isoniazid is of great interest from both theoretical and practical points of view, but is not surprising if we consider the apparent association of catalase activity with β antigen, which was briefly reported in our previous communication (YONEDA and FUKUI, 1961 a). Since it is generally agreed that the most remarkable change in the properties of isoniazid-resistant strains of tubercle bacilli is the loss or great decrease in catalase activity (MIDDLEBROOK, 1954), one might naturally expect, assuming that β antigen is either catalase itself or its apo-protein (YONEDA, 1963), that a loss or great decrease in this particular antigen may also occur in the resistant-strains. Conversely, this concomitant loss or great decrease of β antigen with the acquisition of isoniazid-resistancy may be strong evidence in support of the above mentioned hypothesis. In this connection, it is obvious that the results observed with 9 human and bovine strains which were resistant to drugs other than isoniazid derivatives and had both α and β antigens, also provides circumstantial evidence for the above hypothesis. The fact, that, for instance, saprophytic mycobacteria show potent catalase activity, but have no β antigen appears contradictory to the above hypothesis. However, this may be easily accounted for by assuming that the antigenic specificity of the catalase (apo-protein) in the saprophytes is entirely different from that in strains of *M. tuberculosis* and *bovis*.

The discovery of a *M. tuberculosis* antigen (CRM with α) in *M. lepraemurium* is of particular interest since this is the first step in the elucidation of the immunochemical relationship betwen this species and tubercle bacilli. Based on this finding, an extensive study is now being made, in collaboration with the Department of Leprology in this Institute, on the immunochemical nature of CRM to see

if there are any unique antigenic determinants for this species. From the fact that β antigen was not detectable in the strains of *M. lepraemurium* tested, it seems reasonable to include these in the group of *CRM positive but β negative* strains. However, it must be remembered that the test antigen of the murine leprosy bacilli is derived from *in vivo* cultures, while that of all other strains is obtained from *in vitro* cultures. A possible important question to be studied is whether there is any distinction in the distribution of α and β antigens in *in vivo* and *in vitro* mycobacterial cultures.

Using α and β antigens as indicators, antigenic analyses showed that all strains of unclassified mycobacteria, could be either included in the *CRM positive but β negative* or in the *both α and β negative* group (Saprophytic group), as shown in Table 2, and that the strains belonging to the former group are further divisible into at least the following two subgroups on the basis of differences in their antigenic determinants of CRM: 1. strains having CRM identical with that of *M. avium* (Avian group); 2. strains having CRM identical with that of P8 (P8 group). According to the classification of Dr. Runyon (personal communication), of these 45 strains, 6 are included in Group I (=Photochromogens), 12 in Group II (=Orange-orange red scotochromogens) and the other 27 in Group III (=Non-photochromogens).

Table 3 shows comparison of our serotypes with Runyon's biotypes for the 45 strains. It can be seen that the serotype P8 group includes 4 Photochromogens; the Avian group,

TABLE 3 *A relation between the serotypes and Runyon's biotypes of 45 unclassified test strains*

Serological grouping		Test strains	Runyon's grouping	
Group	Sub-group		Group	Biotype
III	p 8 group	p 8 p 18 p 21 p 22	I	Photochromogens
	Avian group	p 6, p 15 p 29, p 30 p 36, 2066	II	Scotochromogens
		p 17, p 39, p 44 p 47, p 48, p 49 p 50, p 51, p 55 2057, 2059, 2060 2062, 2064	III	Non-photochromogens
IV		p 16, p 24, p 26	I	Photochromogens
		p 5, p 31, p 32, p 34, p 35, p 37	II	Scotochromogens
		p 2, p 3, p 27, p 40, p 41, p 42, p 45, p 53, p 54, 2058, 2061, 2063	III	Non-photochromogens

6 Scotochromogens and 14 Non-photochromogens, and the Saprophytic group (both α and β negative), 3 Photochromogens, 6 Scotochromogens and 12 Non-photochromogens. Thus there is no clear-cut correlation between the serotypes and Runyon's biotypes, though it is of interest that group P8 includes only strains of *M. kansasii*. However, it is uncertain whether the original biological properties of these test strains have been unchanged in their sub-cultures. Further studies are necessary before any final conclusion can be reached.

The present investigation is still in progress and further results will be reported elsewhere.

ACKNOWLEDGEMENTS

The authors wish to express their thanks to Drs. SHINJI NISHIMURA and TATSUO MORI of the Department of Leprology of this Institute for supplying the bacterial extracts of *M. lepraemurium*, to Dr. ERNEST H. RUNYON, Veterans Administration Hospital, Salt Lake City, USA, for providing 45 strains of unclassified mycobacteria, to Dr. LYDIA B. EDWARDS, Department of Health, Education, and Welfare, Washington, D.C., USA, for providing several strains of mycobacteria and to Dr. MITSUO HORI, chief of the Department of Tuberculosis Research II of this Institute, for his encouragement and helpful discussion.

REFERENCES

CASTELNUOVO, G., GAUDIANO, A., MORELLINI, M. and POLIZZI-SCIARRONE, M. (1958). Differenzizione antigenica dei micobatteri studiata con le techniche di diffusione in agar, immunoelecttroforesie deviazione del complemento. *Riv. Tuberc.* **6**, 303–308.

CASTELNUOVO, G., GAUDIANO, A., MORELLINI, M., PENSO, G. and POLIZZI-SCIARRONE, M. (1959). La constitizione antigenica di alcuni micobatteri. *Ann. Ist. Forlanini* 19 *fasc.* **1**, 1–18.

CASTELNUOVO, G. and MORELLINI, M. (1960). Sulla composizioni antigenica del mycobacterium avium studiata con la technica dell' immunoelecttroforese. *Ann. Ist. Forlanini* **20**, 190–195.

CASTELNUOVO, G., BELLEZZA, G., DUNCAN, M. E. and ASSELINEAU, J. (1964). Étude sur les Mycobactéries et les Nocardiae. I. Constitution antigenique. II. Relations sérologiques entre Mycobactéries et Nocardiae. III. Sensibilité auz phages. *Ann. Inst. Pasteur.* **107**, 828–844.

FUKUI, Y. and YONEDA, M. (1961). Extracellular proteins of tubercle bacilli. III. Column chromatographical purification of the proteins of a virulent strain (H37Rv) of *Mycobacterium tuberculosis. Biken's J.* **4**, 187–196.

FUKUI, Y., HIRAI, T., UCHIDA, T. and YONEDA, M. (1965). Extracellular proteins of tubercle bacilli. IV. α and β antigens as major extracellular protein products and as cellular components of a strain (H37Rv) of *Mycobacterium tuberculosis. Biken J.* **8**, 189–199.

KONNO, K. (1956). New chemical method to differentiate human-type tubercle bacilli from other mycobacteria. *Science* **124**, 985–986.

KUNKEL, H. G. and GLICK, D. (1954). *In Methods of Biochemical Analysis.* (*Interscience Publ. N.Y.*) Vol. **1**, pp. 141.

LIND, A. (1961). Serological studies of Mycobacteria by means of diffusion in gel techniques. *Goteborg* 1961. *Orstadius Boktryckeri AB.*

MIDDLEBROOK, G. (1954). Isoniazid-resistance and catalase activity of tubercle bacilli. *Am. Rev. Tuberc.* **69**, 471–472.

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

PARLETT, R.C. and YOUMANS, G.P. (1956). Antigenic relationships between mycobacteria as determined by agar diffusion precipitation technique. *Am. Rev. Tuberc.* **73**, 637–649.

PARLETT, R. C. and YOUMANS, G. P. (1959). Antigenic relationships between ninety eight strains of mycobacteria using gel-diffusion precipitation techniques. *Am. Rev. Tuberc.* **77**, 450–461.

RUNYON, E. H. (1955). Veterans Administration National Tuberculosis Association cooperative study of Mycobacteria (*correspondence*). *Am. Rev. Tuberc.* **72**, 866.

WOLINSKY, E., SMITH, M. M., MITCHELL, R. S. and STEENKEN, Jr. W. (1957). Atypical chromogenic mycobacteria associated with pulmonary disease. *Am. Rev. Tuberc.* **75**, 180–198.

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

YONEDA, M. and FUKUI, Y. (1961 b). Extracellular proteins of tubercle bacilli. II. Some serological properties of purified extracellular proteins of a virulent strain (H37Rv) of *Mycobacterium tuberculosis*. *Biken's J.* **4**, 121–130.

YONEDA, M. (1963). Immunochemical analyses of the proteins of *Mycobacterium tuberculosis*. *Kekkaku* **38**, 287–295. (in Japanese).