

# Characterization and Comparison of Mycobacterial Antigenes by Two-Dimensional Immunoelectrophoresis

DIANNA B. ROBERTS,<sup>1</sup> GEORGE L. WRIGHT, JR., LEWIS F. AFFRONTI,  
AND MELVIN REICH

*Department of Microbiology, The George Washington University School of Medicine, Washington, D.C. 20005*

Received for publication 27 April 1972

Two-dimensional immunoelectrophoresis (2D-IEP), in which a complex of antigens is subjected to electrophoresis first through an agarose matrix in one direction and secondly through an antiserum-agarose matrix at right angles to the first direction, was evaluated as a tool for analysis of mycobacterial antigens. Cell extracts from four species of mycobacteria, *Mycobacterium tuberculosis* (four strains), *M. bovis* strain BCG, *M. scrofulaceum*, and *M. phlei*, were assayed by 2D-IEP with four anti-mycobacterial antisera. Besides displaying the precipitin curves in a more easily interpreted format than did conventional immunoelectrophoresis (IEP), 2D-IEP offered greater sensitivity in terms of numbers of precipitin curves when like reactions were compared with IEP patterns. As many as 60 immunoprecipitates were observed on 2D-IEP slides compared to 18 on comparable IEP plates. Technical reproducibility of patterns from run to run was excellent. Other parameters, such as the influence of using different batches of antigen on the pattern, are discussed. Each of the cell extract antigens gave a unique pattern of precipitin peaks which could be easily differentiated from the patterns given by the other mycobacterial cell extracts when reacted with any of the antisera in 2D-IEP. Since both the species and strains of mycobacteria could be easily and reproducibly differentiated solely on the basis of two-dimensional immunoelectrophoretic patterns obtained with any of the antisera employed in this study, it may be possible, by using IEP, to differentiate and identify all species and strains of mycobacteria with one standard, highly sensitive antiserum, rather than a battery of antisera.

Immunoelectrophoresis (7) is a valuable technique for the analysis of mixtures of antigens. Its value, however, is limited in that, first, the precipitin patterns are not representative of quantitative variations among the antigens and, second, their interpretation requires considerable skill and experience. Ressler (18) showed that antigens could be forced by an electric current into an agar matrix containing antiserum, thus producing a number of narrow curves with a common base, the relative heights of which are proportional to antigen concentrations. Laurell (13) combined these methods so that the peaks no longer had a common base but were separated according to the electrophoretic mobility of the various antigens in agarose. This crossed electrophoresis technique is called two-dimensional

immunoelectrophoresis, quantitative immunoelectrophoresis (4, 11), or two-dimensional electroimmunodiffusion (A. J. Crowle, *personal communication*).

Two-dimensional immunoelectrophoresis was developed and has been used primarily for the analysis and quantitation of normal and abnormal serum antigens (5, 6) but should be applicable to other mixtures of antigens (2).

Although gel diffusion and immunoelectrophoresis have been used for the analysis of mycobacterial antigens (3, 14, 16, 17), they are not sensitive enough for studying all the antigenic moieties of a crude or multicomponent antigen mixture. For example, acrylamide pore gradient electrophoresis of a mycobacterial cell extract preparation results in the detection of between 40 and 60 bands which stain with protein-specific dyes (1); yet in immunoelectrophoresis only 11 to 20 bands appear after reaction with

<sup>1</sup> Part of this report was submitted in partial satisfaction of requirements for the M.S. degree, Department of Microbiology, The George Washington University, Washington, D.C. 20005.

homologous antiserum (L. F. Affronti and G. L. Wright, Jr., 1968, Amer. Rev. Resp. Dis. **98**:153). Furthermore, differences in the precipitin profiles of the various species are often difficult to detect.

Since two-dimensional immunoelectrophoresis appeared to be more sensitive and the two-dimensional immunoelectrophorograms were easier to interpret than was the case in conventional immunoelectrophoresis, it was thought worthwhile to assess the value of this procedure for the analysis of mycobacterial antigens. This report describes the adaptation of the technique to the analysis of mycobacterial antigens and its possible use in taxonomic classification of species and strains of mycobacteria.

### MATERIALS AND METHODS

**Preparation of mycobacterial antigens.** Selected strains and species of mycobacteria were obtained from the American Type Culture Collection, the Trudeau Culture Collection, and the culture collection of the Microbiology Department of The George Washington University School of Medicine. These included *Mycobacterium tuberculosis* strain H37Ra (H37Ra), *M. tuberculosis* strain H37Rv (H37Rv), *M. bovis* strain BCG (BCG), *M. scrofulaceum*, and *M. phlei*. The cells were grown as surface pellicles on Proskauer-Beck medium (Difco Laboratories, Detroit, Mich.) at 37 C for 6 to 8 weeks before they were separated from the culture fluid by filtration. The culture filtrates were concentrated by pervaporation to one-tenth their original volume. The concentrated filtrates were then dialyzed against 10 changes of 20 times their volume of cold distilled water. The protein concentration of the dialyzed culture filtrate (CF) was determined by using the method of Lowry et al. (15). The CF was used immediately or stored in 5- to 10-ml quantities at -80 C.

Bacterial cell extracts were prepared by diluting the washed cell mass with two volumes (w/v) of 0.88% saline containing 0.02% Merthiolate (PS). This mixture was placed in an ice bath and subjected to ultrasonic vibrations provided by a Biosonic sonifier for 10 min. The partially broken cells were allowed to cool for 5 min, and then sonically treated for an additional 10 min. The sonic extract was centrifuged at  $15,000 \times g$  for 45 min at 3 C. The supernatant fluid was drawn off and saved, and the pellet was resuspended in three volumes (w/v) of PS. The sonic treatment and centrifugation steps were repeated two more times. The pellet from the third  $15,000 \times g$  centrifugation was reserved for use in making antiserum as described below. The pooled supernatant fluids were centrifuged at  $100,000 \times g$  for 2.5 hr at 3 C. The pellet from this centrifugation was also reserved for use in making antiserum. The supernatant fluid was termed cell extract (CX) and was passed through a sterilizing membrane filter (0.45  $\mu$ m pore size, Millipore Corp., Bedford, Mass.). The protein concentration was ascertained as described

above for CF. The cell extract was divided into small quantities and stored at -80 C.

**Preparation of antisera.** With the exception of the antiserum directed against *M. tuberculosis* strain H37Rv, the antisera were prepared, with slight modifications, in accordance with the procedure described by Hirschfeld (9). At least three rabbits were used in making antiserum to each organism. That volume of CX which contained 5 mg of protein was mixed with that volume of CF which contained 5 mg of protein. One-half milliliter of a 10% aqueous solution of  $AlCl_3$  was added to the CX-CF mixture, the pH was adjusted to 7.2 with 10% aqueous NaOH, and the now turbid suspension was centrifuged at  $2,000 \times g$  for 20 min. The supernatant fluid was drawn off, reprecipitated, and centrifuged as above. These steps were repeated three more times. The pellets from the five centrifugations were suspended in three volumes of PS and centrifuged at  $2,000 \times g$  for 20 min. The supernatant fluid was discarded and the pellet was washed six more times.

With this pelleted precipitate were mixed 0.5 g of the  $15,000 \times g$  pellet, 0.5 g of the  $100,000 \times g$  pellet, and sufficient (approximately 2 ml) unprecipitated CX and CF (the protein contributions from each preparation being equal) to make a suspension which could pass through an 18-gauge hypodermic needle. The total volume of the suspension did not exceed 6 ml per rabbit.

The suspension, which contained 10 mg of alum-precipitated protein, about 6 mg of unprecipitated protein, and 1 g of centrifugation pellet material, was injected intramuscularly (im) into both hind quarters of the rabbit, half on each side. Forty days later, 2 mg of unprecipitated protein, half from CX and half from CF, was injected subcutaneously into the flanks of the rabbit. Seven to 10 days later, a small sample of blood was drawn from the ear and the serum was tested against CF and CX in the conventional agar gel double-diffusion method. The development of eight or more precipitin bands in 48 hr indicated the serum was ready to be harvested. If fewer than eight bands appeared, the rabbit received a second booster. The second booster was seldom necessary. Once the antiserum was deemed satisfactory, the rabbit was exsanguinated by cardiac puncture and the serum was drawn off. The antisera of the same specificity which gave about ( $\pm 1$ ) the same number of precipitin bands were pooled, divided into 10- to 20-ml quantities and stored at -80 C.

Three procedures and six rabbits were employed in making antiserum to *M. tuberculosis* strain H37Rv. Two rabbits were injected im with a mixture containing 10 mg of alum-precipitated protein (half from CF and half from CX), 30 mg of dried whole cells, and 3.5 mg of unprecipitated protein from CX. Two rabbits each received im an emulsion made from 3.3 ml of Freund's incomplete adjuvant, 10 mg of protein equally from unprecipitated CF and CX, and 30 mg of dried cells. A third pair of rabbits each received im half the dose of the alum suspension given each of the first pair of rabbits plus half the dose of the adjuvant emulsion given each of the second pair of rabbits. A booster injection of the

type described above was given to each of the six rabbits 40 days after the initial inoculation. Ten days later the rabbits were exsanguinated and their sera were pooled, divided into 10-ml quantities, and stored at  $-80^{\circ}\text{C}$ .

**Conventional immunoelectrophoresis.** A modification of the "macro" method described by Grabar and Williams (8) was used in performing immunoelectrophoresis (IEP). Fifteen milliliters of a hot ( $90^{\circ}\text{C}$ ) 1% agarose (Sea-Kem, Bausch & Lomb, Rochester, N.Y.) solution in IEP gel buffer (sodium barbital-barbital,  $I = 0.093$ ,  $\text{pH } 8.6$ ) was poured onto a glass lantern slide (8.2 by 10.1 cm). The antigen wells (0.7 cm in diameter) were cut with their centers 2.9 cm from the cathodic edge of the slide and 0.5 cm from the antiserum troughs, which were 0.2 by 9 cm and held 0.3 ml of antiserum. The antigen wells were charged with 70  $\mu\text{l}$  of CX containing  $200 \pm 35 \mu\text{g}$  of protein. Electrophoresis was conducted using IEP well buffer (sodium barbital-barbital,  $I = 0.06$ ,  $\text{pH } 8.6$ ) at 12 ma per slide at  $5^{\circ}\text{C}$  until the bromophenol blue tracking dye included in the antigen well was 0.5 cm from the anodic edge of the slide. The troughs were incubated at room temperature in a humid chamber for 72 hr.

**Two-dimensional immunoelectrophoresis.** Two-dimensional immunoelectrophoresis (2D-IEP) was employed, with slight modifications, as described by Laurell (13). The first electrophoresis was carried out as described above for conventional IEP. The center of the antigen well was 1.2 cm from the bottom edge and 2.9 cm from the cathodic side of the slide. After the first electrophoresis, the agarose above a line parallel to and 2.2 cm from the bottom edge of the slide was removed and replaced with 12 ml of a warm ( $50^{\circ}\text{C}$ ) mixture of one part 2% agarose in IEP gel buffer and one part antiserum, either undiluted (final dilution 1:2) or diluted 1 to 1 with IEP gel buffer (final dilution 1:4). After the agarose-antiserum mixture had solidified, the slide was returned to the electrophoretic chamber and electrophoresis was conducted at right angles to the first direction of separation at 10 ma per slide for 20 hr at  $5^{\circ}\text{C}$ . The steps required to perform 2D-IEP are illustrated in Fig. 1.

After the second electrophoresis, the slides were placed in a humid chamber and incubated for 24 hr at room temperature to increase the intensity of the precipitin curves. Once the curves had developed, the slides were washed with several changes of PS, dried under bibulous paper, stained with Ponceau S (1% in 3% trichloroacetic acid), destained in 5% acetic acid, again dried, and finally photographed.

The diagrammatic representations of the precipitin patterns were made by tracing the images of the tops of the peaks produced when the stained slide was projected through a photographic enlarger onto white paper.

## RESULTS

Typical two-dimensional immunoelectrophoretic patterns and the effect of dilution of antiserum on these patterns are presented in Fig. 2.

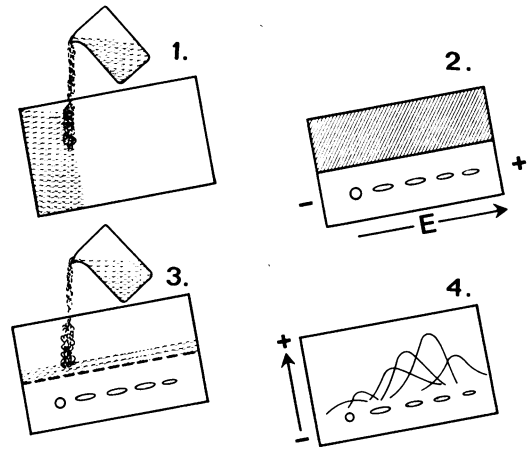


FIG. 1. Diagrammatic illustration of the procedure for performing two-dimensional immunoelectrophoresis. 1. A hot ( $90^{\circ}\text{C}$ ) solution of 1% agarose in barbital buffer is poured onto an 8 by 10 cm glass slide. 2. Electrophoresis in the first direction is carried out at 12 ma per slide. The agarose in the region of the diagonal lines is then cut away. 3. A warm ( $50^{\circ}\text{C}$ ) mixture of equal parts 2% agarose in barbital buffer and antiserum is poured onto the slide to cover the region above the dashed line. 4. The second electrophoresis is then carried out at right angles to the first at 10 ma per slide for 20 hr.

The higher the dilution of antiserum, the fainter and taller the peaks are for a given duration of electrophoresis in the second direction. At a dilution of 1:8 the peaks were too faint to be seen clearly. At a 1:2 dilution of the antiserum the peaks were quite opaque and easily seen, but tended to be grouped together near the line marking the beginning of the antigen-antiserum matrix above the line of the first direction of separation of the antigen. At the 1:4 dilution, the peaks were still easily seen, tall enough to be well separated from each other, and less antiserum was required. Because only half as much antiserum was required (3 ml per slide) at the 1:4 dilution and discrete patterns were obtainable, the 1:4 dilution was adopted for the comparison of mycobacterial cellular antigens from organisms homologous and heterologous to the specificity of the antiserum.

By comparing the 2D-IEP patterns obtained (Fig. 2) with either dilution (1:2 or 1:4) of antiserum with classical IEP patterns (Fig. 3) one may observe the ability of the 2D-IEP system to accentuate differences between antigen preparations from two mycobacterial species reacted against homologous and heterologous antisera. The classical IEP patterns (Fig. 3)

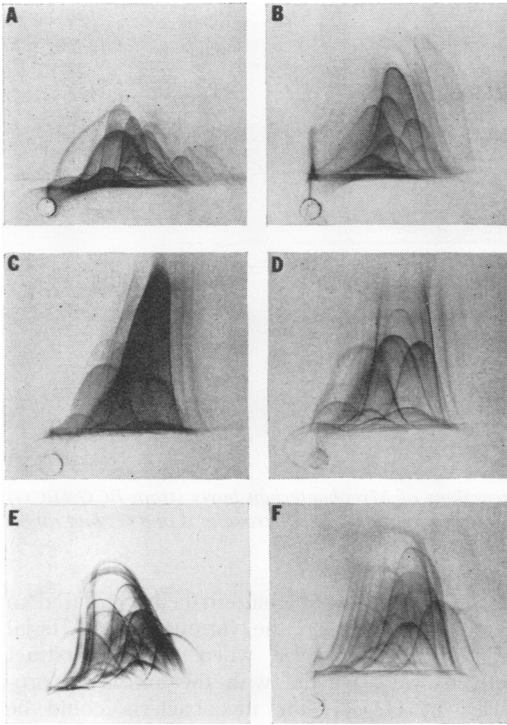


FIG. 2. Effect of dilution of antiserum on two-dimensional immunoelectrophoretic patterns. The antiserum dilution used in each of the patterns in the left column (A, C, E) was 1:2; a 1:4 dilution was used in each of the patterns in the right column (B, D, F). The antigen:antiserum reactions pictured are: A and B, *Mycobacterium tuberculosis* strain H37Ra cell extract versus rabbit anti-*M. tuberculosis* strain H37Ra; C and D, *M. bovis* strain BCG versus rabbit anti-*M. tuberculosis* strain H37Ra; E and F, *M. bovis* strain BCG versus rabbit anti-*M. bovis* strain BCG.

representing reactions of H37Ra cell extract (CX) and BCG CX with antisera directed against H37Ra and BCG CX are dissimilar. Yet the 2D-IEP patterns (Fig. 2) which represent some of these same reactions make the antigenic differences between the two species more apparent, and thus allow easier differentiation of the cell extracts from the two species. Besides displaying the precipitin curves in a more easily interpreted format, the 2D-IEP technique offers greater sensitivity than classical IEP. Ten to 18 immunoprecipitin arcs were detectable in each classical IEP pattern (Fig. 3), whereas 27 (Fig. 2B) to 48 (Fig. 2E) precipitin curves were detected in the 2D-IEP electrophorograms representing reactions of H37Ra CX and BCG CX, respectively, with their homologous antisera. Unfortunately, not all peaks which are visible on

the stained slides themselves are visible in the reproduced photographs.

The 2D-IEP electrophorograms presented in Fig. 4 were obtained with homologous mycobacterial antigen-antiserum systems. The patterns were distinct and unique for each species tested. Sixty immunoprecipitin curves were detected in the H37Rv CX-anti-H37Rv homologous system (Fig. 4B) which exemplifies the high degree of sensitivity obtainable with the 2D-IEP system.

By projecting the stained image of the two-dimensional pattern onto white paper and tracing just the peaks of the precipitin curves, a "fingerprint" profile was obtained. This permitted an even clearer interpretation and comparison of the two-dimensional patterns obtained with different species and strains of mycobacteria. Figure 5 is a representative example of some of the two-dimensional "fingerprints" obtained when the different mycobacterial CX antigen preparations were reacted against homologous and heterologous antisera. Regardless of the total number of precipitin curves detected, each "fingerprint" was unique for the species or strain CX antigens tested against any single antiserum. Somewhat similar patterns were obtained when the CX from a given strain was reacted with the homologous and the two heterologous antisera employed.

In an attempt to state quantitatively the observation that a pattern given by one strain or species could be differentiated from the pattern given by another strain or species of mycobacteria, the electrophorograms were divided arbitrarily into eight vertical zones as indicated in Fig. 5. The number of precipitin curves in each zone was counted and the results are illustrated in Fig. 6. Each mycobacterial species and strain used in this study could be differentiated by comparing the graphic profiles obtained with each antiserum.

When *M. phlei* CX was analyzed by the three antisera, precipitin peaks were not found in zones 1, 2, 6, 7, or 8. This pattern was easily differentiated from the 2D-IEP pattern of *M. scrofulaceum* in that the peaks in the latter were sometimes found in zone 1 or 2 and always in zone 6 or 7. The majority of peaks in the *M. bovis* pattern were centered around zone 4, whereas the majority of peaks in the *M. tuberculosis* strain H37Rv pattern were found in zones 4 through 6 and for the *M. tuberculosis* strain H37Ra pattern in zones 1 through 4.

No single zone was characteristic of the species or strain tested if the antigen preparations were reacted against either anti-BCG or anti-H37Ra

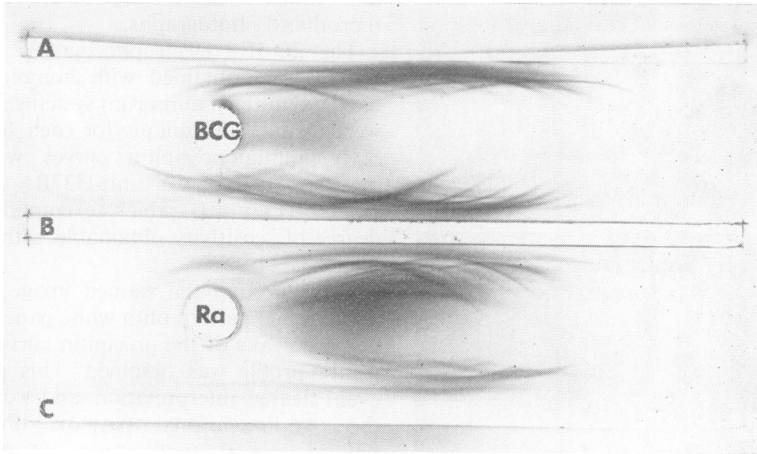


FIG. 3. Conventional immunoelectrophoretic patterns of reactions of *Mycobacterium bovis* strain BCG (BCG) cell extract (CX) and *M. tuberculosis* strain H37Ra (Ra) CX with rabbit anti-BCG (troughs A and C) and rabbit anti-Ra (trough B).

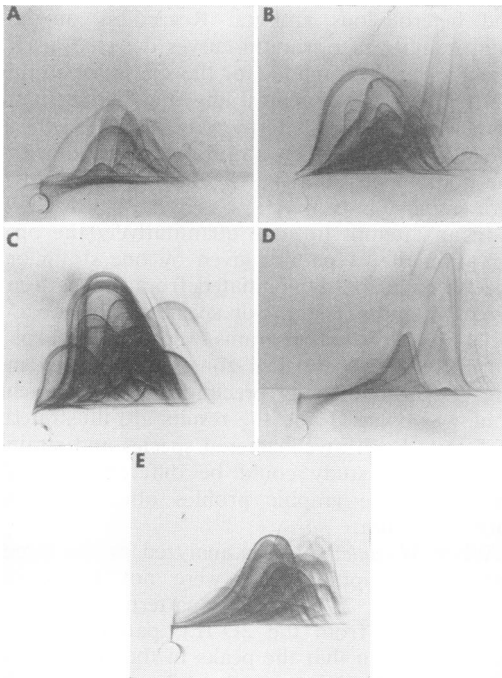


FIG. 4. Two-dimensional immunoelectrophoretic patterns obtained with homologous mycobacterial antigen-antiserum reactions at an antiserum dilution of 1:2. A, *Mycobacterium tuberculosis* strain H37Ra cell extract (CX) versus rabbit anti-*M. tuberculosis* strain H37Ra. B, *M. tuberculosis* strain H37Rv CX versus rabbit anti-*M. tuberculosis* strain H37Rv. C, *M. bovis* strain BCG CX versus rabbit anti-*M. bovis* strain BCG. D, *M. scrofulaceum* CX versus rabbit anti-*M. scrofulaceum*. E, *M. phlei* versus rabbit anti-*M. phlei*.

antiserum, the overall pattern being required to differentiate between the various mycobacterial cell extracts. However, when the cell extract antigens were reacted with the antiserum produced to H37Rv, the mycobacteria could be differentiated by the overall antigen profile as well as by the total number of precipitin curves and number of immunoprecipitins in zone 3.

It is interesting to note, as shown in Fig. 6, that at a dilution of 1:4, the three antisera tested gave more precipitin curves with at least one heterologous strain or species of mycobacteria than with the homologous strain or species. One possible explanation for this is that there may be certain antigens in the homologous CX which stimulate the synthesis of antibodies but, when analyzed with antiserum diluted 1:4, are in great antigen excess. Lesser amounts of these same antigens in heterologous CX might then be precipitable. If this accounts for the observation, a higher concentration of homologous antiserum would be expected to precipitate these antigens in the homologous cell extract, yielding a greater number of precipitin peaks. Sixty precipitin curves were detected in 2D-IEP electrophorograms of the homologous H37Rv-anti-H37Rv system when the antiserum was diluted 1:2 compared with 41 at a 1:4 dilution of antiserum. Forty-two were detected at 1:2 and 27 at 1:4 with the H37Ra homologous systems; and 48 at 1:2 and 34 at 1:4 with the BCG homologous system. Twofold concentration of the H37Ra CX resulted in a decreased number of precipitin curves at a 1:2 dilution of antiserum, and a fivefold concentration yielded only

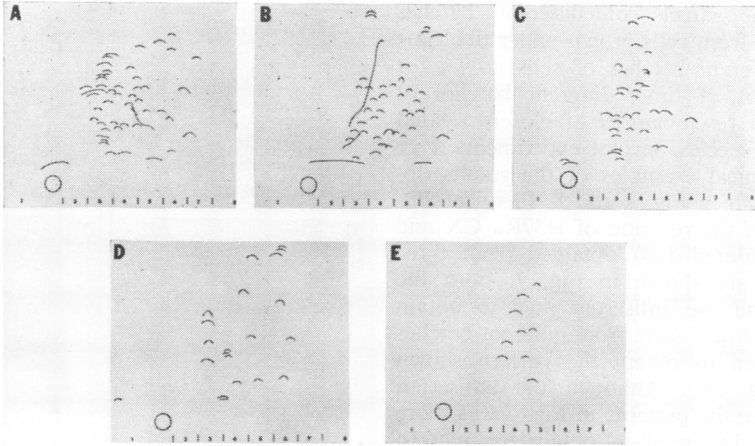


FIG. 5. Typical "fingerprint" profiles of two-dimensional immunoelectrophoretic patterns obtained with mycobacterial antigens tested with antiserum diluted 1:4. The numbers at the bottom of each profile are zone numbers; the vertical lines are the boundaries of the zones (see text). The antiserum employed was directed against *Mycobacterium tuberculosis* strain H37Rv. The antigens are: A, *M. tuberculosis* strain H37Rv cell extract (CX); B, *M. tuberculosis* strain H37Ra CX; C, *M. bovis* strain BCG CX; D, *M. scrofulaceum* CX; and E, *M. phlei* CX. (The continuous lines between two peaks indicate reactions of identity.)

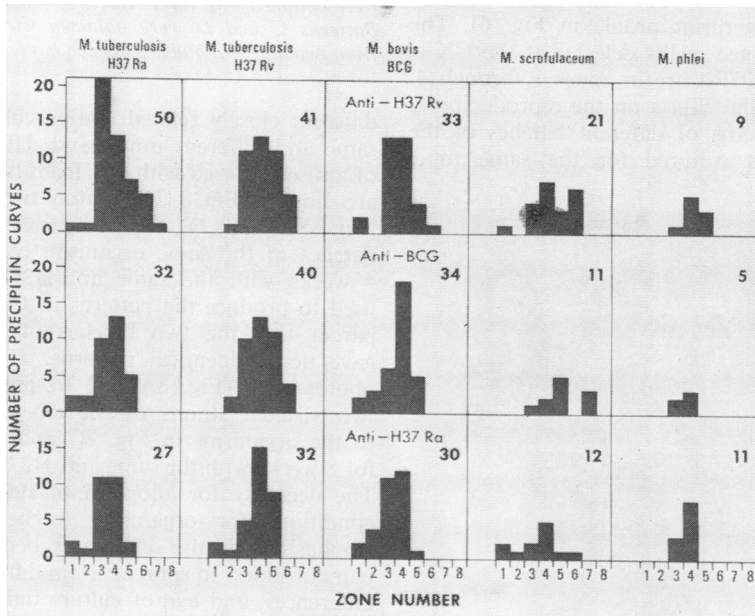


FIG. 6. Antigen profiles of mycobacterial cell extracts obtained by counting the number of precipitin peaks in each of the eight zones (illustrated by Fig. 5). The organism from which the antigen was made is written at the top of the column in which the profile appears. The antiserum used (all at 1:4 dilution) is written across the row in which the profile appears. The number in the upper right corner of each profile indicates the total number of precipitin curves obtained in that antigen-antiserum interaction. Note that the mycobacterial species and strains could easily be differentiated with any one of the three antisera.

10 curves at the 1:2 dilution of antiserum. In two heterologous systems, BCG-anti-H37Ra and BCG-anti-H37Rv, the same numbers of curves were detected with either a 1:2 or 1:4

dilution of antiserum. These results appear to support the hypothesis mentioned above.

To establish the reproducibility of 2D-IEP with respect to day-to-day or run-to-run re-

peatability, the effects of different batches of cell extracts from cells grown under the same conditions, different batches of cells grown for different lengths of time, different batches of antiserum, and resemblances of closely related strains of one species, several experiments were conducted. Typical examples of the results obtained are shown in Fig. 7 and 8. Identical 2D-IEP patterns of the reaction of H37Ra CX and anti-H37Ra (diluted 1:4) obtained from three different runs are shown in Fig. 7. Both the H37Ra CX and the antiserum used to obtain the patterns in Fig. 7 were from different batches than those used to obtain the pattern shown elsewhere (Fig. 2B). Although the antiserum used to produce the patterns in Fig. 7 was more sensitive than that used to obtain the pattern Fig. 2B (37 peaks compared to 27), the gross patterns are quite similar. Moreover, if the fingerprint profile of the patterns in Fig. 7 is determined (zone 1, 2 peaks; zone 2, 1 peak; zone 3, 10 peaks; zone 4, 20 peaks; zone 4, 3 peaks; zone 6, 1 peak; and zones 7 and 8, no peaks), it is found that the peaks are allocated to the zones in nearly the same proportions as in Fig. 2B (fingerprint profile in Fig. 6). The majority of antigen peaks detected if the CX is derived from H37Ra are in zones 1 through 4.

Examples of the effects on the reproducibility of 2D-IEP patterns of different batches of the same organisms cultured for the same time,

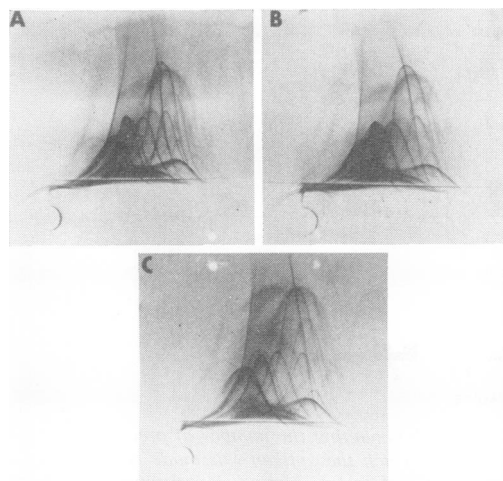


FIG. 7. Technical reproducibility of two-dimensional immunoelectrophoresis patterns. Plates were produced on different days. Reactions are of H37Ra cell extract and rabbit anti-H37Ra diluted 1:4. Both cell extract and antiserum are from different batches than those used to produce patterns described in other sections of the paper.

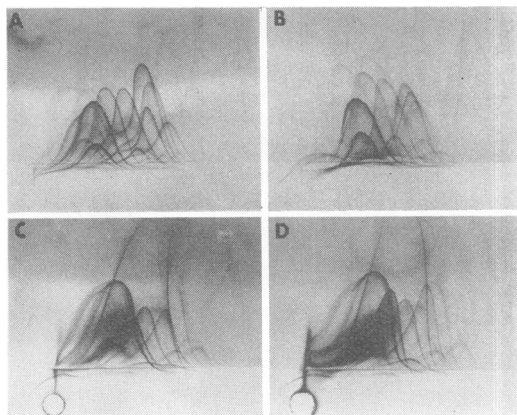


FIG. 8. Two-dimensional immunoelectrophoretic patterns of three strains of virulent *M. tuberculosis* reacting with the same anti-H37Ra antiserum used to obtain patterns in Fig. 7. Cell extracts are from strains: A, H37Rv, Trudeau Mycobacteria Collection (TMC) no. 103 (used elsewhere in these experiments and designated "H37Rv"); B, DT, TMC no. 119; C and D, H37Rv, TMC no. 102. Patterns A and B were obtained with cell extracts of 8-week-old cultures. Patterns C and D were obtained with cell extracts from two different batches of cells harvested at 4 weeks.

different closely related strains cultured for the same and different times, and different batches of antiserum used with CX from the same batch are shown in Fig. 8. The bottom two photographs (Fig. 8C and D) represent reactions of two batches of the same organism, each grown for 4 weeks with the same anti-H37Ra antiserum used to produce the patterns in Fig. 7. It is apparent that the two batches of the organism gave nearly identical patterns. The upper two photographs (Fig. 8A and B) are reactions of two virulent strains of *M. tuberculosis* related to the organisms in Fig. 8C and D but grown for 8 weeks with the same anti-H37Ra antiserum. The necessity for adoption of standard culture conditions for organisms to be analyzed is emphasized by the differences between the two pairs of slides. In spite of strain differences, batch differences, and age of culture differences, there seem to be overall resemblances among the four slides which reflect their close taxonomic relationship. In addition, their fingerprint profiles approximate those obtained when H37Rv CX reacted with a different batch of anti-H37Ra antiserum (Fig. 6). The majority of peaks fall into zones 4 through 6. It seems reasonable to conclude that, with standard culture conditions and sensitive antiserum, 2D-IEP patterns are reproducible from run to run and with different



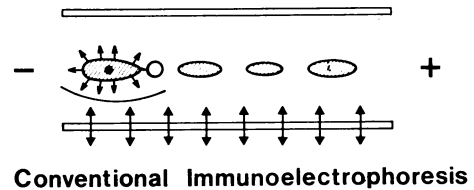
batches of antigen. In addition, although the power of 2D-IEP as a tool for identifying mycobacteria is exemplified by its ability to differentiate some strains within species of mycobacteria, strain resemblances within species are also apparent, making assignment of organisms to their proper species relatively easy without the use of batteries of monospecific antisera. Best results are obtained if the same, highly sensitive antisera are used throughout the analysis of all the cell extracts.

### DISCUSSION

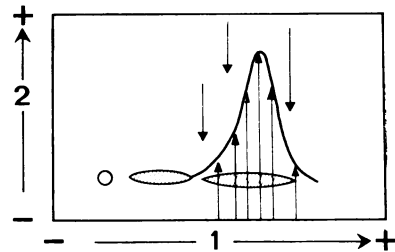
The two-dimensional immunoelectrophoretic technique as described in this report resulted in the detection of approximately four times the number of antigens detectable by a classical IEP procedure. The principal reason for the marked increase in sensitivity of the 2D-IEP system is that a large proportion if not all of the antigens in the CX are forced by the electric field into contact with the antibodies as illustrated in Fig. 7. Since in conventional IEP the antigens and antibodies make contact by means of simple diffusion which occurs in all directions, only a portion of the immunologic reagents meet and precipitate, others being lost in the surrounding agarose gel matrix (Fig. 9).

Because of this high degree of sensitivity the 2D-IEP system offers several possibilities for the qualitative and quantitative identification and characterization of the highly complex mycobacterial cell extract antigen mixtures. The great sensitivity and technical reproducibility (Fig. 7) of the technique plus the ease with which the two-dimensional immunoelectrophorograms could be interpreted made possible the differentiation of the mycobacterial species and strains employed in this study. This was especially true for the differentiation of the closely related species *M. tuberculosis* and *M. bovis* and differentiation between two strains of *M. tuberculosis*, H37Ra and H37Rv. It was difficult if not impossible to differentiate clearly the mycobacteria by classical IEP when only a single antiserum was used.

Although a greater number of antigens could be demonstrated by increasing the antiserum concentration, an antiserum dilution of 1:4 (3 ml) was adopted because reliable and reproducible two-dimensional patterns could be obtained which permitted easy identification of the mycobacterial species and strains without expending a large volume of antiserum. At this antiserum concentration, however, the number of antigens detected in the homologous system was always less than the number detected in at least



Conventional Immunoelectrophoresis



Two-Dimensional Immunoelectrophoresis

FIG. 9. Diagrammatic explanation for the greater sensitivity of two-dimensional immunoelectrophoresis (2D-IEP) compared to conventional immunoelectrophoresis (IEP). Because the antigens and antibodies diffuse in all directions (arrows) in IEP, there can be insufficient amounts of certain antigens and antibodies to allow the formation of a visible precipitate, or there can be sufficient loss of either an antigen or antibody species into the surrounding matrix so that an effective excess of the other reagent results in a prozone effect. But in 2D-IEP the antigens are forced electrically into the antibody-agarose matrix (arrows). Under these electrodynamic conditions, excess antigen or antibody effects are overcome, and small quantities of antigen are precipitated.

one closely related species or strain of mycobacteria. This was shown to be a result of considerable antigen excess in the homologous cell extract preparation, while lesser amounts of the same antigens in the heterologous cell extracts were in appropriate concentrations to produce a visible precipitin curve. As indicated above, this problem could be circumvented by increasing the concentration of antiserum (to 6 ml), but then the amount of antiserum consumed per test is no longer practical for screening or survey purposes. Even 3 ml (1:4 dilution) of antiserum is approximately 10 times greater than the amounts usually employed in classical IEP and may be considered by some investigators a quantity still too large for routine studies. It may be possible, however, to develop a "micro" 2D-IEP procedure for the characterization of mycobacterial antigens similar to that described by Krøll (11) and Crowle (*personal communication*) for the study of serum protein antigens in which the same amount of antiserum used in



classical IEP is employed. Experiments are being conducted in this laboratory to determine the applicability of such a procedure for analysis of mycobacterial antigens.

Because of the problem of antigen excess in homologous cell extracts when the 1:4 dilution of antiserum is used, a direct count of the total number of precipitin curves obtained with CX from an unknown species of mycobacterial antiserum or a battery of them may not serve to identify the unknown species. The useful parameter at this dilution of antiserum is the pattern of the precipitin curves. Both the species and strains of mycobacteria could be easily differentiated solely on the two-dimensional immunoelectrophoretic patterns (Fig. 4 and 5) obtained with any one of the antisera prepared against H37Ra, BCG, or H37Rv. This differentiation was also successful with different batches of antigen prepared from cells harvested at 4, 6, and 8 weeks cultured under the same conditions, although optimal ease of identification is obtained when the cells are grown for the same length of time and tested against the same batch of highly sensitive antiserum. The identification, therefore, of an unknown species or strain of mycobacteria on the basis of the 2D-IEP pattern would not require a battery of antisera. One standard, highly sensitive antiserum, such as the anti-H37Rv antiserum produced in the study, could possibly differentiate all the species and strains of mycobacteria. Of course, the analysis of cell extracts from a large number of mycobacterial species and strains would be necessary to determine if this were possible. The need for a suitable reference system to characterize mycobacterial antigens and a proposed reference system using classical IEP has recently been described (10; B. W. Janicki, S. D. Chaparas, T. M. Daniel, G. P. Kubica, G. L. Wright, Jr., and G. S. Yee., *Amer. Rev. Resp. Dis.*, *in press*).

The discussion thus far has centered on the suitability of the 2D-IEP procedure for the classification of mycobacteria. Although the various species and strains of mycobacteria could be simply identified by the 2D-IEP technique, it was impossible to determine which antigens were common and which were specific for the species or strain by the method as presented. However, Krøll (12) has recently described a modification of a 2D-IEP system in which two antigen mixtures are assayed against a single antiserum. In his technique, precipitin curves which fuse together between the two antigen mixtures are considered representative of identical antigen determinants, and those which intersect are considered nonidentical as is

usually described for reactions of identity and nonidentity in gel double-diffusion tests. It might, therefore, be possible to determine the antigenic relationships and differences between two species or strains of mycobacteria on a single 2D-IEP slide, once the patterns have been determined individually. Once individual mycobacterial antigens can be definitively identified, then it should be possible to quantitate the mycobacterial antigens in the same way as reported for serum protein antigens by using 2D-IEP (4-6, 11-13).

The 2D-IEP technique appears to have great potential for the qualitative and quantitative characterization of mycobacterial antigens as well as any complex mixture of antigens (2).

#### ACKNOWLEDGMENTS

We gratefully acknowledge the technical assistance of Karen Farrell, Linda Grow, and Mary Gaffney.

This investigation was supported in part by a grant from the John A. Hartford Foundation, Inc. and a grant from the United States-Japan Cooperative Medical Science Program administered by the National Institute of Allergy and Infectious Diseases.

#### LITERATURE CITED

1. Affronti, L. F., G. L. Wright, Jr., and M. Reich. 1972. Mycobacterial antigens: characterization and comparison by discontinuous pore gradient electrophoresis. *Infect. Immunity* 5:474-481.
2. Axelson, N. H. 1971. Antigen-antibody crossed-electrophoresis (Laurell) applied to the study of the antigenic structure of *Candida albicans*. *Infect. Immunity* 4:525-527.
3. Castelnuovo, G., M. E. Duncan, and G. Bellezza. 1964. Mycobacterial antigens: a study of antigens in bacterial extracts and in culture filtrates of different ages. *Tubercle* 45:246-254.
4. Clarke, H. G. M., and T. Freeman. 1966. A quantitative immunoelectrophoretic method (Laurell electrophoresis). *Proteides Biol. Fluids Proc. Colloq. Bruges* 14:503-509.
5. Clarke, H. G. M., and T. Freeman. 1968. Quantitative immunoelectrophoresis of human serum proteins. *Clin. Sci.* 35:403-413.
6. Clarke, H. G. M., T. Freeman, and W. Pryce-Phillips. 1970. Quantitative immunoelectrophoresis of serum from hospitalized chronic schizophrenic and epileptic patients. *J. Neurol. Neurosurg. Psychiat.* 33:694-697.
7. Grabar, P. 1959. Immunoelectrophoretic analysis. *Methods Biochem. Anal.* 7:1-10.
8. Grabar, P., and C. A. Williams. 1953. Méthode permettant l'étude conjuguée des propriétés électrophorétiques et immuno-chimiques d'un mélange de protéines. Application au sérum sanguin. *Biochim. Biophys. Acta* 10:193-194.
9. Hirschfeld, J. 1960. Immunoelectrophoresis procedure and application of the study of group specific variations in sera. *Sci. Tools* 7:18-25.
10. Janicki, B. W., S. D. Chaparas, T. M. Daniel, G. P. Kubicka, G. L. Wright, Jr., and G. S. Yee. 1971. A reference system for antigens of *Mycobacterium tuberculosis*. *Amer. Rev. Resp. Dis.* 104:602-604.
11. Krøll, J. 1968. On the immunoelectrophoretic identification and quantitation of serum proteins. *Scand. J. Clin. Lab. Invest.* 22:79-81.
12. Krøll, J. 1969. Immunochemical identification of specific protein lines in quantitative immunoelectrophoresis patterns. *Scand. J. Clin. Lab. Invest.* 24:55-60.

13. Laurell, C. B. 1965. Antigen-antibody crossed electrophoresis. *Anal. Biochem.* **10**:358-361.
14. Lind, A. 1961. Serological studies of mycobacteria by means of diffusion-in-gel techniques. *Arstadius Boktryckeri AB, Gothenburg.*
15. Lowry, O. H., N. J. Rosenbrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**:265-275.
16. Parlett, R. C., and G. P. Youmans. 1959. Antigenic relationships between ninety-eight strains of mycobacteria using gel-diffusion precipitation technique. *Amer. Rev. Tuberc.* **77**:450-461.
17. Pickett, M. J., M. M. Pedersen, D. I. Goldstein, and S. Froman. 1968. The antigens of *Mycobacterium tuberculosis*. Protein fractions from cytoplasmic and culture filtrates. *Amer. Rev. Resp. Dis.* **17**:415-422.
18. Ressler, N. 1960. Electrophoresis of serum protein antigens in an antibody-containing buffer. *Clin. Chim. Acta* **5**:359-365.