INFECTION AND IMMUNITY, Apr. 1972, p. 474-481
Copyright © 1972 American Society for Microbiology
Vol. 5, No. 4
Printed in U.S.A.

Characterization and Comparison of Mycobacterial Antigens by Discontinuous Pore Gradient Gel Electrophoresis

LEWIS F. AFFRONTI, GEORGE L. WRIGHT, JR., AND MELVIN REICH
The George Washington University Medical Center, Department of Microbiology, Washington, D.C. 20005

Received for publication 15 November 1971

Concentrated culture filtrates and cell extracts were prepared from selected mycobacteria and analyzed by a multistage polyacrylamide electrophoretic procedure. Various staining procedures were used to detect protein, carbohydrate, nucleic acid, and lipid constituents. Visual examination of the more prominently stained components indicated differences and similarities in each species of *Mycobacteria*. The majority of the individual components were of small molecular weight; however, there appeared in all culture filtrates and extracts examined a slowly moving protein component of relatively large molecular size. Evidence based on immunodiffusion suggests that it is a common mycobacterial antigen.

It has been demonstrated by a number of studies that the mycobacterial cell and its metabolic products are of an exceedingly complex nature. The biology of the tubercle bacillus and the relationship of its complex products within the host have been studied for many years to better understand the pathogenesis of tuberculosis. There have been many physicochemical techniques which have been applied to the separation and isolation of these complex cellular products. It is also important that their chemical composition be known so that the mechanism of tuberculous disease and, consequently, how to combat it may be better understood. Moreover, by employing more refined mycobacterial fractions of known composition, the similarities and differences that exist among the various acid-fast organisms could be more sharply defined and thus contribute to a more satisfactory understanding of mycobacterial relationships.

The present study is a direct outgrowth of our long-range investigations concerned with developing and standardizing methods for isolating and purifying mycobacterial antigens and elucidating their possible biological effects. Specifically, it has been one of the major aims of this study to identify and compare mycobacterial antigens by utilizing a modified pore gradient polyacrylamide electrophoresis method. The present report characterizes these biologically active components from various selected strains of mycobacteria utilizing this technique. Antigenic profiles from each of them are obtained and compared, and the possible interrelationships are examined.

MATERIALS AND METHODS

Source of organisms for culture filtrates and bacterial extracts. Selected mycobacterial species were obtained from the American Type Culture Collection and the culture collection of the Microbiology Department of The George Washington University School of Medicine. These included: *Mycobacterium tuberculosis* H37Ra, *M. tuberculosis* H37Rv, *M. bovis*, *M. bovis* BCG, *M. avium*, *M. kansasii*, *M. intracellulare*, *M. scrofulaceum*, *M. phlei*, *M. fortuitum*, and *M. smegmatis*. Cells were grown as surface pellicles on Proskauer-Beck medium at 37 C for 6 to 8 weeks before separation from the culture fluid by Seitz filtration. The culture filtrates were concentrated by pervaporation to one-tenth their original volume. The total volumes obtained were each divided into two equal portions. One portion was lyophilized and stored at −20 C, and the other was divided into 2-ml samples and also stored at −20 C. When ready for use, each of the lyophilized filtrates was reconstituted with sterile, distilled water.

The bacterial extracts were prepared by suspending the collected cells in isotonic saline and centrifuging them at 2,010 × *g*. Centrifugation was repeated twice, and one volume of the washed cells was suspended in 1.5 volumes of 0.15 M phosphate-buffered saline (PBS), *p*H 7.2. This suspension, which was maintained in an ice bath, was sonically treated for 20 min with a Branson model 575 sonifier. This sonic extract was centrifuged at 10,000 × *g* for 1 hr, and the sediment was resuspended in 1.5 volumes of PBS and subjected to sonic treatment for an additional 20 min. The sonic extract was centrifuged as above,

and the two supernatant fluids were pooled and centrifuged at 144,000 × *g* for 100 min. The final cell extract was then stored at −80 C in small volumes. Protein was analyzed by the Lowry method or by the Nessler reaction (6).

Discontinuous pore gradient polyacrylamide gel electrophoresis. Electrophoresis in polyacrylamide gel was performed as reported by Wright and Affronti (8) with tris(hydroxymethyl)aminomethane glycine buffer, *p*H 8.3, and 5 ma per tube. This method utilizes a multistage gel for the separation of mycobacterial cellular products and is a modification of the discontinuous pore gradient technique described by Wright et al. (9) for the separation of serum proteins. The complex ionic tuberculin mixture migrates through a four-stage separating gel in glass tubes (6 mm inner diameter by 12 cm). These gel columns consist of (total gel concentration followed by gel height): a 3.50% (1 cm) upper gel followed by a 4.75% (1 cm), a 7% (1.5 cm), and finally a 12% (5 cm) bottom gel. From 0.2 to 0.5 ml of sample containing 300 μg of protein and 20% sucrose was layered over the 3.75% gel. Electrophoresis was allowed to proceed at room temperature until the bromophenol blue tracking dye reached a point 1 cm from the bottom of the tube. The differential stains used for approximate identification of separated components were amido black and Coomassie Blue for protein, the periodic acid-Schiff reagent (PAS) for mucoproteins and polysaccharides, the Feulgen stain and acridine orange for nucleic acid, gallocyanin for ribonucleic acid (RNA), and the Oil Red O stain (ORO) for lipids. After the mycobacterial samples were subjected to electrophoresis and their patterns were well established, the R_F values for each of the components which had been developed by the Coomassie Blue stain were calculated.

Conventional disc polyacrylamide electrophoresis. The conventional disc electrophoresis procedure which was described by Ornstein and Davis (4) and which employs homogeneous gel columns of single concentrations was used.

Polyacrylamide gel densitometry. Polyacrylamide gels which had been stained for protein were scanned using a Gilford densitometer and multirange recorder (model 2000) used together with a Beckman DU spectrophotometer. This technique had the advantage of eliminating subjectivity involved in visual observation of the stained patterns.

Antisera. Twelve male albino guinea pigs weighing from 500 to 700 g were sensitized with a mixture of *M. tuberculosis* H37Ra culture filtrate and dried cells in oil (Bayol F). Each milliliter contained 10 mg of cells, and each guinea pig was given 0.4 ml subcutaneously in several sites on the back of the neck at weekly intervals for 2 weeks. Sensitization was evident since lesions of the Koch phenomenon were seen 1 week after the last injection. The injected animals were bled from the heart, and the sera were collected in the conventional manner.

Recovery of protein components from acrylamide gel. The protein components studied in precipitin tests and by immunodiffusion were obtained by pooling eluates from 50 gel columns after electrophoresis. The combined 3.50 and 4.75% gel portions were removed and pooled, and the components were eluted from the gel by grinding in a mortar together with an amount of distilled water equal to 10 times the volume of the gel. They were allowed to diffuse at 4 C for 48 hr, after which time the liquid was removed and concentrated by means of either ultrafiltration or pervaporation. The eluates from the pooled 7% gels and the eluates from the 12% gels were similarly prepared.

Precipitin tests. The precipitin tests used were as described in a previous report by Affronti (1). The concentrated eluates from the combined 3.50 and 4.75% gels and those from each of the pooled 7 and 12% gels were made to the following concentrations on the basis of their protein content: 50, 10, 5, and 1 μg per ml.

Double immunodiffusion. The concentrated eluates recovered from the gels were analyzed by double immunodiffusion in 1% agar and were buffered with

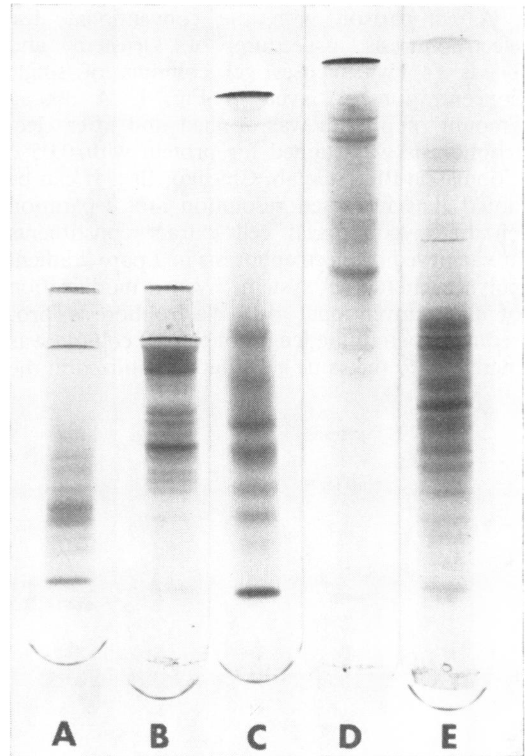

FIG. 1. *Comparison of protein-stained profiles of M. tuberculosis culture filtrate samples as obtained by the conventional disc and the discontinuous pore gradient electrophoretic methods. Samples were subjected to electrophoresis in gel columns as follows (total gel concentration followed by gel height): A, 4.75% (6.5 cm); B, 7% (6.5 cm); C, 4.75% (8.5 cm); D, 7% (8.5 cm); E, four-stage discontinuous pore gradient polyacrylamide gel.*

sodium diethylbarbiturate, *p*H 8.2. Plastic templates with wells 0.2 cm in diameter and 0.5 cm equidistant from each other were placed on glass plates. When the gel had hardened, the concentrated eluates and the antisera were placed into the wells. The units were then placed in a humidified chamber at room temperature until the precipitin pattern had developed. The plates were stained with amido black for proteins.

RESULTS AND DISCUSSION

With the introduction of a polyacrylamide electrophoretic multistage system for the separation and isolation of mycobacterial antigens, a simple method is available for the improved separation and resolution of mycobacterial products. Also, by utilizing various differential staining procedures, profiles of mycobacterial strains can be established for comparison, thus allowing a more refined characterization of their interspecific relationships.

A comparison with the conventional disc electrophoresis procedure of Ornstein and Davis (4) which uses gel columns of single concentrations is given in Fig. 1. A 300-μg amount of protein was applied and after electrophoresis was stained for protein with 0.05% Coomassie Blue (Colab, Chicago, Ill.). It can be noted that increased resolution and separation of the mycobacterial cell extract constituents are achieved by electrophoresis in a pore gradient polyacrylamide gel system. Even a modification of the conventional disc electrophoresis procedure, wherein the length of the gel column was increased to the same gel length as utilized in the pore gradient gel system, resulted in neither better separation nor better resolution of the mycobacterial samples. Because of these findings, the multistage method was selected as the procedure of choice for this study.

Electropherograms of culture filtrates and cell extracts from representative mycobacterial strains (*M. tuberculosis* H37Ra, *M. tuberculosis* H37Rv, *M. phlei*, and *M. fortuitum*) stained for protein by Coomassie Blue are given in Fig. 2. An increased number of protein-stained components was generally evident in cell extracts when they were compared with their respective culture filtrate samples containing an equivalent amount of protein (300 μg), as can be noted in Table 1. It could be demonstrated, however, that the protein-stained components of the originally electrophoresed culture filtrate could be made comparable in number and position with those of its respective cell extract samples. This was done by simply effecting a 100-fold greater concentration of the culture filtrate samples and adjusting the protein concentration optimally so that comparable profiles resulted. This indicated that there are several components in the originally electrophoresed culture filtrate samples which were below the sensitivity level of the protein stain; but when a greater concentration was effected, detectable levels were attainable. No increase in the number of stained bands could be detected when the cell extract samples were similarly concentrated and subjected to electrophoresis. In fact, some components coalesced to form broader bands, as a

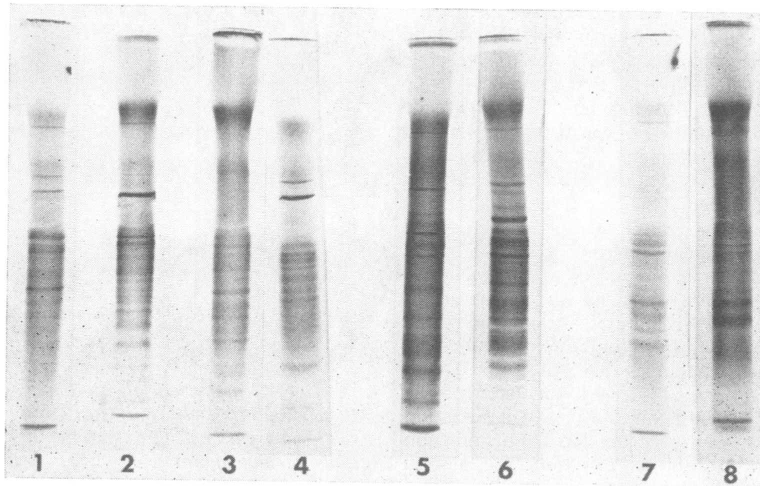

FIG. 2. *Representative electropherogram of nonlyophilized culture filtrate and cell extracts from various mycobacterial strains. (1 and 2) M. tuberculosis H37Ra culture filtrate and cell extract, respectively; (3 and 4) M. tuberculosis H37Rv culture filtrate and cell extract, respectively; (5 and 6) M. phlei culture filtrate and cell extract, respectively; (7 and 8) M. fortuitum culture filtrate and cell extract, respectively.*

result of column overloading, and thus yielded a protein profile with a decreased number of total bands.

It can also be seen from Fig. 2 that the majority of the culture filtrate and cell extract components are of low molecular weight since they migrate and separate in the small-pore gels (12%). The possibility that the fast-moving components, i.e., those found in the small-pore gel, might represent highly charged substances of large molecular weight can be discounted, particularly in view of the report of Tombs (7) which convincingly demonstrated that the mobility of a protein in a gel is related to the proportion of pores larger than the limiting size. Therefore, the position of the components within the gel can be accounted for mainly on the basis of a pore filtration mechanism instead of charge differences.

The number of protein, PAS, nucleic acid, and ORO-positive staining components demonstrated by the differential staining technique for each of the cell extracts studied is summarized in Table 2. These data reflect the finding that only a small number of protein-staining components migrated into the 3.50, 4.75, and 7% gel sections. This small number of components (from 1 to 13) probably represents molecules of large size since they are retarded by gels of large porosities.

TABLE 1. *Number of mycobacterial components separated by electrophoresis on polyacrylamide gel*

Component stained for	*Mycobacterium tuberculosis* H37Ra		*M. tuberculosis* H37Rv		*M. avium*		*M. bovis*		*M. kansasii*		*M. scrofulaceum*		*M. intracellulare*		*M. fortuitum*		*M. phlei*	
	CE[a]	CF	CE	CF	CE	CF	CE	CF	CE	CF	CE	CF	CE	CF	CE	CF	CE	CF
Protein	50	47	47	42	47	42	55	44	52	38	56	39	51	48	58	48	61	52
Carbohydrate	8	12	8	8	8	6	5	7	16	20	8	3	10	10	5	9	7	9
Lipid	8	10	10	11	6	2	9	12	15	6	15	7	9	8	6	7	12	10
Nucleic acid	2	2	3	1	1	1	3	1	4	2	3	2	2	2	2	1	2	1

[a] CE, cell extract; CF, culture filtrate.

TABLE 2. *Distribution of the number of components in mycobacterial cell extracts developed by various stains in polyacrylamide gel segments of different porosities*

Component	No. of components						
	Mycobacterium tuberculosis H37Ra	*M. tuberculosis* H37Rv	*M. bovis*	*M. avium*	*M. kansasii*	*M. intracellulare*	*M. phlei*
Protein							
3.5% Segment	3	1	1	2	2	2	3
4.75% Segment	10	10	12	4	7	10	13
7.00% Segment	11	11	13	11	13	13	10
12.00% Segment	26	25	29	30	30	31	35
Carbohydrate							
3.5% Segment	1	1	1	2	2	2	3
4.75% Segment	2	1	1	1	5	2	1
7.00% Segment	1	2	2	0	6	0	3
12.00% Segment	4	4	1	5	3	1	1
Lipid							
3.5% Segment	1	1	1	2	2	2	3
4.75% Segment	2	1	1	1	5	1	1
7.00% Segment	1	2	4	0	5	2	4
12.00% Segment	4	6	3	3	3	1	4
Nucleic acid							
3.5% Segment	0	0	0	0	0	0	0
4.75% Segment	0	0	0	0	0	0	0
7.00% Segment	0	0	0	0	0	0	0
12.00% Segment	2	3	3	1	4	2	2

PAS-positive components and those which stained for lipid were detected in each of the four polyacrylamide gel sections (3.50, 4.75, 7, and 12%). Most of the PAS-positive and ORO-positive components could be correlated with the protein-staining components, indicating that they were probably glycoprotein or mucoprotein lipid complexes. All mycobacterial culture filtrates and cell extracts analyzed contained PAS-positive and ORO-positive substances. The fact that the PAS-positive components were distributed in gels of four different pore sizes would suggest that these carbohydrates are extremely heterogeneous, ranging from complexes of large molecular weight to those of smaller shape and size. Moreover, as demonstrated by the specific gallocyanin RNA stain, RNA was only found in the 12% gel stage (Table 2). These data support the view, therefore, that both culture filtrates and cell extracts contain various chemical components of widely differing molecular sizes. Allowing molecules to be driven through gels of progressively decreasing pore size, such as occurs in the system described above, not only greatly enhances the resolution of these components but also suggests that this method might be a useful tool for molecular size determinations. In other words, it would be expected that large molecules would be retarded while smaller ones would migrate relatively unimpeded, depending upon the pore size of the gel. The relative distance that the components would migrate through the gels thus would serve as another criterion for further characterization of protein mixtures. In order to test this possibility, bovine serum albumin standards of known molecular weight varying from 67,000 to 268,000 (Table 3) were selected and subjected to electrophoresis in a single gel system containing 7, 9, and 12% polyacrylamide to compare their mobilities in these gel concentrations. For this tool to be valid, a linear function relationship between relative mobility and gel concentration is required. As shown in Fig 3, an approximate linear relationship exists in the region of the acrylamide concentration between 7 and 12%.

TABLE 3. *Standards used for polyacrylamide gel electrophoresis*

Protein and symbol		Molecular weight
Bovine serum albumin (BSA)	BSA-1	67,000
Dimer	BSA-2	134,000
Trimer	BSA-3	210,000
Tetramer	BSA-4	268,000

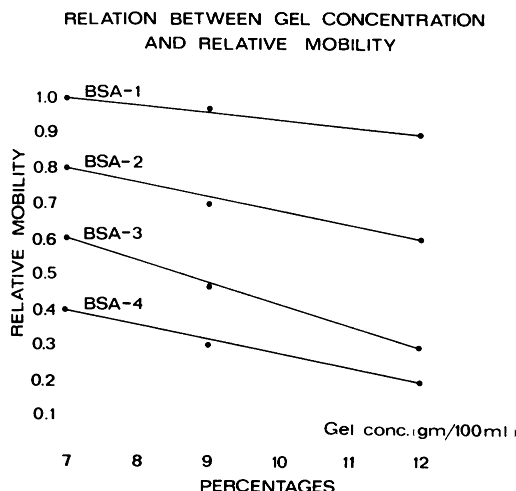

FIG. 3. *Relation between gel concentration and relative mobility.*

It can be seen that as the pore size decreases, the large-molecular-weight proteins exhibit a slower mobility than the smaller proteins. Relative mobility versus very low (<7%) or very high (> 12%) gel concentrations did not result in a straight line relationship, thus invalidating the use of gel concentrations in these extreme ranges. Therefore, within certain ranges, this demonstrates a relationship between molecular size and mobility in gels of different concentrations and permits the use of migration distance, or the conventional concept of R_F, as one possible criterion for characterizing protein components of a mixture separated by gel electrophoresis. Moreover, as was previously reported by Marchalonis (3), this method also provides a simple means of estimating molecular weights of protein in a mixture.

The applicability of using mobilities or R_F values in polyacrylamide gel electrophoresis as an aid in identifying similar or identical protein components which possibly would result in characteristic band patterns for each species and thus serve to complement the more conventional methods of bacterial classification appeared to be of limited value in this study. In an attempt to eliminate the subjectivity involved in visually reading these stained patterns, densitometric tracings were performed using a Gilford densitometer. Figure 4 gives representative tracings from three mycobacterial species including *M. tuberculosis* H37Ra, the reference strain. Fifty peaks corresponding to the total number of components separated by electrophoresis were easily noted. Although differences in strains were discernable with this technique, they were subtle

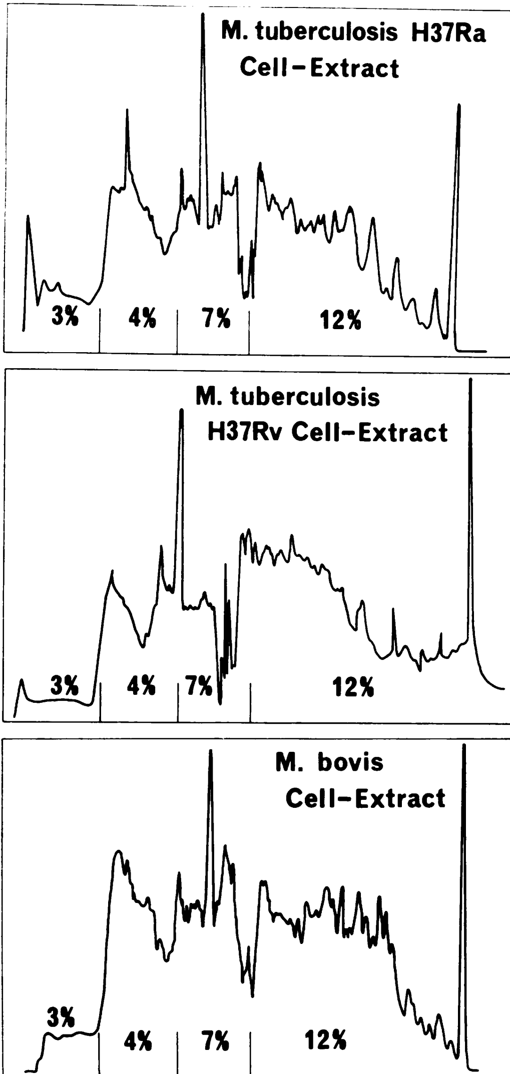

FIG. 4. *Representative densitometric tracing of M. tuberculosis H37Ra, H37Rv, and M. bovis Percentages indicate gel concentration.*

and their reproducibility was less than ideal. As will be described in the following paper, however, a method is now available which we believe has considerable merit in permitting one to establish definitive characteristic patterns for mycobacterial species and which could possibly be used as an aid in their classification.

Figure 5 is a schematic drawing which represents the electropherograms and R_F values of four strains of mycobacterial culture filtrates that were previously lyophilized and the protein components stained with Coomassie Blue. As can be noted, lyophilization apparently altered the electrophoretic protein patterns since the total number of stained components was reduced significantly, especially in the 3 and 4% gel regions, from that observed in the nonlyophilized culture filtrate samples (compare with Fig. 2). However, lyophilization did not reduce the major protein-stained components since these remained visually prominent in both nonlyophilized and lyophilized samples. Although differences in protein profiles are present among these mycobacterial species either due to a direct deleterious effect of the lyophilization process on susceptible protein components or due, in fact, to the existence of true protein compositional differences, it is clear that striking similarities are evident when the major components selected on the basis of visual prominence in corresponding gel segments are compared. This similarity in certain corresponding gel segments among culture filtrates from different mycobacterial species is also reflected by the presence in these filtrates of components with identical or nearly identical R_F values.

However, without a means of estimating the normal variation in R_F values from trial to trial, this approach would only have validity providing, as Rouatt et al. (5) have recently suggested, that homologous bands could be identified with certainty. Thus, if the migration of a protein in a gel derives essentially from a pore filtration mechanism, as Tombs proposes (7), then proteins with the same R_F values would be expected to be identical. This expectation was proven in the course of attempting to characterize the protein component in culture filtrates from four mycobacterial species which migrated into the lower portion of the 4.75% gel segment and had an average R_F value of 0.30. This band was considered the ideal candidate for selection for two reasons: first, it appeared as a single component devoid of any neighboring bands and thus, from a practical viewpoint, was relatively easily sectioned out; and secondly, it was present in all culture filtrates and extracts analyzed, that is, it can be considered as a very likely candidate for a possible "common antigen." Precipitin tests were performed on corresponding pooled gel segments to establish the presence or absence of antigenicity for the total components within each of the major gel portions, i.e., the 4.75, 7, and 12% gel segments. Precipitin tests were positive with the highest concentration (50 μg) of the eluates of electrophoresed culture filtrate samples from pooled 4.75% gel segments of *M. tuberculosis* H37Ra as test antigen against its homologous antiserum. Eluates from the pooled 7 and 12% gel segments also gave positive reactions, thus confirming the antigenicity of all of

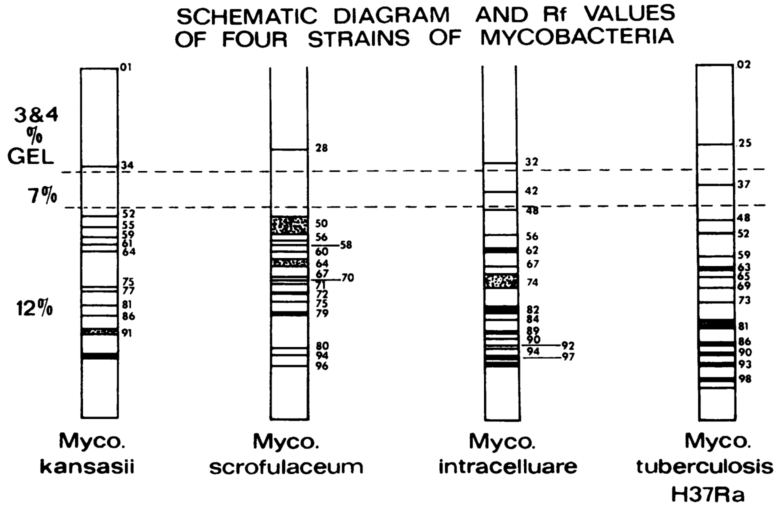

FIG. 5. *Schematic diagram and R_F values of four strains of mycobacteria.*

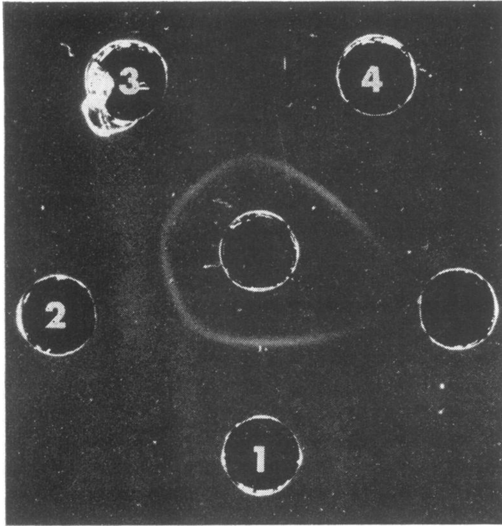

FIG. 6. *Gel diffusion results of culture filtrate eluates having nearly identical R_F values, from four strains of mycobacteria. The eluates gave reaction of identity indicating a "common antigen." Well no. 1, M. tuberculosis H37Ra; well no. 2, M. kansasii; well no. 3, M. scrofulaceum; well no. 4, M. intracellulare; center well, guinea pig anti-H37Ra serum.*

these fractions. After proof of their antigenicity, immunodiffusion studies were done with culture filtrate eluates from the pooled 4.75% gel segments from each of the four different mycobacterial strains, *M. tuberculosis* H37Ra, *M. kansasii*, *M. scrofulaceum*, and *M. intracellulare*. This was done in an attempt to establish possible interspecific relationships for this "common band" having almost identical R_F values. It can be seen from Fig. 6 that this component gave reactions of identity for each of these mycobacterial species, thus confirming immunobiologically the results obtained physicochemically. The component was found in the 4.75% gel segments of all the mycobacterial and culture filtrates and extracts analyzed, and therefore it can be considered a common antigen. It should be pointed out that re-electrophoresis of each of the common bands again yielded single components. The characterization of this antigen and its possible relationship with the common antigen which was present in all mycobacterial species which Castelnuovo (2) examined and reported will be the subject of a later communication.

ACKNOWLEDGMENTS

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

This research is supported by Public Health Service grants AI08529 and AI07099 from the National Institute of Allergy and Infectious Diseases, and by a grant from The John A. Hartford Foundation, Inc.

LITERATURE CITED

1. Affronti, L. F. 1959. Purified protein derivatives (PPD) and other antigens prepared from atypical acid fast bacilli and *Nocardia asteroides*. Amer. Rev. Resp. Dis. 79:284–294.
2. Castelnuovo, G., S. Yamanaka, M. Zoppis, and E. Dotti. 1969. Protein components of *Mycobacterium phlei*: fractionation procedures. Tubercle 50:194–202.
3. Marchalonis, J. J. 1969. Isolation and characterization of immunoglobulin-like proteins of the Australian lungfish. Aust. J. Exp. Biol. Med. Sci. 47:405–419.

4. Ornstein, L., and B. J. Davis. 1961. Disc electrophoresis. Distillation Products Industries, Rochester, N.Y.
5. Rouatt, J. W., G. W. Skyring, V. Pukayastha, and C. Quadling. 1970. Soil bacteria: numerical analysis of electrophoretic protein patterns developed in acrylamide gels. Can. J. Microbiol. **16**:202-205.
6. Seibert, F. B., and L. F. Affronti. 1963. Analytical chemical methods, p. 1-33. *In* R. Parlett (ed.), Methodology manual for investigation of mycobacterial and fungal antigens. American Thoracic Society, New York.
7. Tombs, M. P. 1965. The interpretation of gel electrophoresis. Anal. Biochem. **13**:121-132.
8. Wright, G. L., Jr., and L. F. Affronti. 1968. Gradient polyacrylamide electrophoresis of mycobacterial cell products. Amer. Rev. Resp. Dis. **98**:153.
9. Wright, G. L., Jr., K. B. Farrell, and D. B. Roberts. 1971. Gradient polyacrylamide gel electrophoresis of human serum proteins: improved discontinuous gel electrophoretic technique and identification of individual serum components. Clin. Chim. Acta **32**:285-296.