DISC ELECTROPHORETIC AND IMMUNODIFFUSION STUDIES OF MYCOBACTERIAL CULTURE FILTRATES

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ABSTRACT

DISC ELECTROPHORETIC AND IMMUNODIFFUSION STUDIES OF MYCOBACTERIAL CULTURE FILTRATES

by Thomas L. Roszman

Unheated, pervaporated culture filtrates were prepared from four mycobacteria, Mycobacterium bovis (strain 310, virulent, bovine origin), M. avium (strain 132, virulent, avian origin) and two strains of atypical mycobacteria. The atypical strains, strain 50 and strain 68, were of bovine origin and classified as Group III (slow growing, nonphotochromogens).

Disc electrophoresis was the best technique to separate the components in the mycobacterial culture filtrates. It was facile, economical and versatile and the results were highly reproducible. The culture filtrates contained between 18 and 24 protein components and from five to eight polysaccharide or glycoprotein components. Antigenic analyses of the culture filtrates with homologous and heterologous antisera (rabbit origin) were made with Ouchterlony immunodiffusion, immunoelectrophoretic and disc immunoelectrophoretic tests. With the Ouchterlony immunodiffusion technique, six to ten antigens were detected in the culture

filtrates. An antigen was present in each of the culture filtrates not detected in the other filtrates. The two Group III atypical mycobacteria were more closely related to M. avium than to M. bovis but were not identical to M. avium. No common antigen was detected.

The number of antigens detectable in the four culture filtrates was increased by immunoelectrophoresis and disc immunoelectrophoresis. Disc immunoelectrophoresis was better than immunoelectrophoresis. The greatest number of antigens occurred in the M. avium culture filtrates.

The components in culture filtrates were separated by preparative disc electrophoresis. Separation of the components in the culture filtrates by the preparative disc electrophoresis was as good or better than by analytical disc electrophoresis. The gels were sectioned and the components eluted. The elutes were dialyzed to remove tetramethylethylenediamine, a constituent used in preparing the gels, which reacts in the Lowry method for the determination of protein. The per cent of protein recovered from the eluates ranged from 50 to 60%. Re-electrophoresis of certain eluates in 10% analytical gels indicated that one amido black-staining component in 7% gel could be separated into as many as six components.

Examination of 20 different eluates by re-electrophoresis and with four reference antisera specific for the

culture filtrates indicated the reliability of the sectioning technique. One or more antigens were obtained from each filtrate which were not detected in the other filtrates. Examination of the antigens in the 20 eluates with 11 antisera specific for culture filtrate and beta-propriolactone-inactivated cells of M. avium, M. bovis, BCG and Group I, II and III atypical mycobacteria, indicated that group and species antigens were obtained by disc electrophoresis.

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INTRODUCTION

The mycobacteria are the oldest and most extensively studied bacteria, yet the basis of the pathogenicity of the tubercle bacilli is not understood. The tuberculin test aids in the detection of sensitivity induced during past or present infections. There is no serologic test which is conclusively, diagnostic for the disease. The final proof of active disease still depends on the isolation and identification of the causative agent.

Numerous factors have contributed to the impressive decrease in the mortality rate of man due to tuberculosis, but the decrease in the morbidity rate has been considerably less impressive. As a result, the problem of the atypical mycobacteria as possible infectious and sensitizing agents has been recognized and studied during the past fifteen years (89, 116).

An analogous situation exists in the field of bovine tuberculosis (39, 81). The number of cattle, which are tuberculin positive and have gross-lesions at slaughter, has been reduced to a very low number. The number of tuberculin positive cattle which have no-gross-lesions at the time of slaughter has remained relatively constant. Atypical

mycobacteria similar if not identical to those of human origin, have been isolated from both categories of cattle, lesions from swine, and inanimate sources (63).

Attempts have been made to isolate and characterize mycobacterial fractions which would be of value as specific sensitins or sero-diagnostic agents. Usually tuberculoproteins and tuberculopolysaccharides are obtained by chemical precipitation from culture filtrates. The antigenic complexities are readily detected by immunodiffusion tests. Newer physical techniques such as column chromatography and zone electrophoresis have been used. While the data obtained added considerably to the understanding of the chemical and physical properties of the mycobacteria, no specific antigen or sensitin has been isolated.

This is a report of the characterization and isolation of mycobacterial antigens from four concentrated, unheated culture filtrates. Ouchterlony immunodiffusion, immunoelectrophoresis and disc immunoelectrophoresis were used to study the antigenic composition and relatedness of the four culture filtrates. Analytical and preparative disc electrophoretic separation of the culture filtrate antigens afford a means of obtaining antigens from preparative disc gels for further study which are relatively pure and have promise of differential specificity.

LITERATURE REVIEW

At the present time, there is no conclusive test by which disease or sensitivity due to the classical pathogen can be differentiated from that due to atypical mycobacteria (89). There is no way by which pathogenic, atypical mycobacteria can be differentiated from saprophytic, atypical mycobacteria. A need exists for antigens with which the causative agent of disease or sensitivity can be identified while still in the host, or in pure culture.

The terms atypical, unclassified or anonymous, are used currently to indicate those mycobacteria other than the well defined species. Runyon (88) placed the atypical mycobacteria of human origin into four groups according to the number of days required for isolated colonies to grow on Lowenstein-Jensen medium at 37C and production of pigment.

Group I (photochromogen): slow growth, no pigment produced in the dark, dramatic production of yellow pigment in the dark after a short exposure to white light; one species, Mycobacterium kansasii.

Group II (scotochromogen): slow growth, yellow pigment produced in light or dark; many strains.

Group III (nonphotochromogen): slow growth, little or no pigment produced in light or dark; many strains.

Group IV (rapid growers): rapid growth; isolated colonies visible in five to six days; many strains.

Group I and some strains of Group III mycobacteria can produce pulmonary disease in man; some Group II and IV mycobacteria may cause disease in man (14, 88, 89, 110, 116). Some Group III mycobacteria of bovine origin can cause disease in cattle (62).

It is generally reported that little or no transmission of the atypical mycobacteria occurs among humans. It may be that transmission occurs frequently but that man has little susceptibility. Transmission has occurred from expermentally infected cows to their calves, and to swine housed with experimentally infected swine (J. A. Ray, Ph.D. Thesis, Michigan State Univ., East Lansing, 1966).

The chemistry of the mycobacteria and the biological preparations derived from them has been studied extensively since the use of concentrated culture filtrate, tuberculin, by Koch in 1891 (22). The pioneering studies by Seibert are classical (54, 92, 93, 94, 96). Chemical fractionation of culture filtrate indicated that the active component of tuberculin was protein (54, 91). Seibert (92, 93, 94, 97) developed the methods for preparation and standardization of the Purified Protein Derivative (PPD-S), an international standard. The PPD was fractionated into three protein components, A, B, and C and two polysaccharides, I and II (95).

Although further attempts were made to purify PPD (100), one of the paradoxes of protein chemistry is the change of definitions of purity. Purity is a dynamic state. Seibert recognized the dilemma and utilized new techniques as they were developed such as Tiseluis electrophoresis (95, 98), ultracentrifugation (98), gel immunodiffusion (99) and paper chromotography (100).

As the prevalence of tuberculosis declined, more dependence was placed on the tuberculin test as a means of detecting tuberculin sensitive individuals without apparent disease. Initially, the assumption was made that a positive test indicated tuberculosis due to M. tuberculosis, infrequently M. bovis, and less frequently M. avium. Veterinarians are credited with recognizing the high rate of false positive reactions, and the no-gross-lesion tuberculin positive animals, ten years before the physicians recognized the problem (22). The sensitivity was attributed to increased prevalence of M. avium and atypical mycobacteria (22, 26).

Numerous studies with PPD preparations including PPD's prepared from atypical mycobacteria proved that cross sensitivity occurred (1, 22, 24, 26, 40, 58, 59, 60). However, the homologous sensitins usually elicited a larger response than heterologous sensitins when injected into sensitized animals. This was presumptive evidence that specific antigen fractions isolated from PPD's or crude culture filtrate would be of value. In theory, the specificity

of the antigen fractions should be inversely proportional to the cross reactivity.

Unquestionably, recent advances in physical and biological techniques for the separation, isolation and characterization of proteins have been a stimulus for those interested in tuberculoproteins. Unfortunately, many researchers interested in the chemical and biologic properties of mycobacteria are traditionalists. The techniques and methods set forth by Seibert forty years ago which utilized chemical precipitation of crude culture filtrate are currently in use. These methods have served a purpose but no protein has been isolated which approaches the degree of purity or specificity sought. Researchers have been late in applying such techniques as immunodiffusion, immunoelectrophoresis, starch gel electrophoresis, disc electrophoresis, gel filtration and DEAE chromatography to mycobacteria.

Immunodiffusion was established as an analytical technique by Oudin (71). Until this time, multiple precipitate bands were regarded as rhythmic precipitates formed by one antigen-antibody system rather than reactions of different antigens with their specific antibodies.

The immunodiffusion technique which Oudin initially perfected (71) was the simple diffusion tube test. He established the basic tenets of immunodiffusion which were amplified by Elek (25) and Ouchterlony (69) by their independently developed double diffusion techniques. There is one

basic difference between the simple and double diffusion techniques. In the simple diffusion test, one reactant (usually the antiserum) is mixed with the gel and after solidification, the other reactant is added to diffuse into the gel mixture. In the double diffusion test, both the antigen and antibody diffuse simultaneously into a neutral agar area where precipitation occurs due to the formation of antigen-antibody complexes. While the theories developed initially by both Elek and Ouchterlony to explain their double diffusion systems were compatible, Ouchterlony's method is the technique of choice today. Elek's technique utilized paper strips impregnated with the reactants which were placed at right angles to each other on agar plates. In the Ouchterlony technique, wells were cut in the agar, and the well arrangements were varied so that many antigenic preparations and antisera could be compared. This allowed recognition of antigenic relationships that may exist between different preparations.

Reviews by both Ouchterlony (70) and Crowle (15, 16) describe immunodiffusion applications and techniques, and discuss the interpretation of the results. Crowle's monogram (16) is considered to be the handbook of immunodiffusion. He has considered both the applied and theoretical aspects of immunodiffusion.

Immunoelectrophoresis is a combination of electrophoresis and immunodouble diffusion in a gel. The technique, as devised by Grabar and Williams (33) was performed on large glass slides to separate and analyze human serum antigens. Scheidegger (90) adapted the technique to microscope slides which saves time and material.

Agar is the gel most widely used for immunoelectrophoresis. It has high gel strength, transparency, solubility in an aqueous medium and little or no reaction with proteins (16). Other supportive materials which have been employed are starch gel (77, 78), cellulose acetate (44), and more recently, polyacrylamide gels (42). Less electroendosmosis occurs in the polyacrylamide gels than in agar.

The acrylamide gels can be cast on glass slides for use as in agar immunoelectrophoresis (42) or as a sequential combination of disc electrophoresis and agar double diffusion (38, 101). After disc electrophoresis (68), the gels are placed on glass slides and covered with a melted agar solution. After solidifying, trenches are cut in the agar parallel to the full length of the gel columns. Antisera are placed in the trenches and the plates incubated at a predetermined temperature until precipitin lines form. Antigens can be cut from fresh gels for use in further studies.

Early zone electrophoretic techniques used only the difference in charges on molecules for separation. In 1955

Smithies (102) reported on starch gel electrophoresis which utilized not only the charge of the molecule but also the molecular weight and size. This added dimension of molecular-sieving increased immensely the number of separations heretofore obtained with other techniques. New techniques and media for zone electrophoresis continue to be reported. Bier (5) and Morris and Morris (66) have reviewed the applied and theoretical facets.

Polyacrylamide electrophoresis was reported independently by Raymond (83) and Ornstein and Davis (68). The investigators differed on the theoretical principles involved (82) but the underlying features are well accepted. The gel matrix is prepared by polymerization of two acrylamide compounds which form a cross-linked, transparent gel of defined pore sizes which can be controlled by the relative concentration of the two compounds. Materials electrophoresed in the gel are separated on the basis of molecular weight, structure and charge.

Ornstein and Davis have termed their technique disc electrophoresis. The name is derived from the discoid shape of the bands obtained and also from the dependence on discontinuities in the electrophoretic matrix (67). A controlled gel pore size and an electrophoretic step for concentrating the protein components into narrow bands prior to electrophoretic separation increases the resolution of the macromolecules (17). The average pore size of the 7-1/2% lower gel is approximately 50 Angstrom units (67).

Disc electrophoresis offers many advantages over most of the other types of zone electrophoresis. The gel is transparent, strong and flexible, qualities necessary for handling and making visual and spectrophotometric observations. Of even greater importance, is the molecular sieving properties that the gels possess by virtue of their pore size.

Changing the pore size of the separation gel can easily be accomplished by either decreasing or increasing the relative per cent of the two acrylamide monomers. This contributes to the versatility and value of disc electrophoresis. One very important attribute is the small quantity of biological materials required. As little as 250 μg of serum protein can be separated by this technique (17). Conversely, disc electrophoresis gels can be used for larger quantities of material. Preparative disc electrophoresis has been employed with as much as 40 mg of protein (41). The separated components were recovered by elution from the end of a polyacrylamide column into a fraction collector (21, 37, 41, 45, 61, 79, 106), or by cutting segments from the column of gel and eluting from the segments the proteins (45, 117).

Disc electrophoresis as originally described was designed for the separation of serum proteins. The buffer systems used were in an alkaline pH range above the isoelectric point of serum proteins. Williams and Reisfeld (108)

have reported on new cationic (pH 4.3) and anionic (pH 7.5) buffer systems. They have also described how the complex calculations of Ornstein's can be utilized to develop new buffer systems.

Mycobacteria have certain characteristics which dissuade many potential investigators. The pathogenic mycobacteria grow very slowly. To obtain sufficient amounts of protein, the organisms are usually grown for two to three months. Autolytic processes are relied upon to liberate the cellular proteins into the medium, during which time denaturization of some of the protein occurs. Frequently, the culture filtrates are heated which causes further denaturation. Heating the cellular suspensions before filtration originated as a precautionary measure and is continued as such. Heating destroys certain antigenic components (51). The high lipid content of mycobacteria causes auto agglutinations which renders them unsatisfactory for classical bacterial agglutination, a technique used frequently for antigenic analyses of other bacteria (6).

Among the first to employ zone electrophoresis for the separation of tuberculoproteins and polysaccharides were Rhodes, Sorkin and Boyden (86, 87, 103). Such media as glass, cellulose, and filter paper were used. By precipitation from a culture filtrate, a hemosensitin preparation was obtained which could be further purified by electrophoresis through glass beads (87).

Paper electrophoresis has been used to separate the components of PPD prepared from M. bovis (19). Four protein components were detectable. Starch-agar gel electrophoresis was less effective with the same PPD (20).

Yoneda and his associates have combined chemical and physical methods of separating tuberculoproteins from H37Rv, a virulent strain of M. tuberculosis (28, 111, 112, 113, 114). A culture filtrate of H37Rv was treated with ammonium sulfate and the three precipitates which occurred at 0-30%, 30-50%, and 50-80% were collected. These precipitates were subjected to starch block electrophoresis and the proteins eluted. Two components each were found in the 0-30% and 30-50% precipitates. The 50-80% precipitate contained many components. On further analyses of the four components, A, B, C, D from the 0-30% and 30-50% precipitates by the Oakley-Fulthrope immunodiffusion technique, one line of precipitation occurred with A and C, two with D and no lines with B. The serologic reactivity of all the antigens was sensitive to 70C for 10 minutes.

The physical and chemical properties of antigens A and C were similar, and identical by Ouchterlony analyses (112). The D antigen was not identical with the A or C antigen. Pure preparations of the three antigens were obtained by diethylaminoethyl (DEAE) cellulose chromatography. Chemical analyses indicated that A and C were pure protein (113). No carbohydrate or nucleic acid was detected.

Subsequently, the nomenclature of the three antigens was changed. Antigens A or C were referred to as alpha, and D as beta. The alpha and beta antigens comprised about 70% of the total protein released by the H37Rv strain into the medium (28). They indicated that these antigens might be on the surface of the cell.

Antisera against the alpha and beta antigens were used to study the distribution of these antigens in 120 strains of mycobacteria using the Ouchterlony technique (114). The 120 mycobacteria could be separated into four groups on the basis of the presence or absence of both alpha and beta, or the presence of one but not the other. All strains in which both alpha and beta were present constituted Group I. With the sole exception of the isoniazid-resistant strains, strains of M. tuberculosis, M. bovis and one strain of M. microti were in this group. Strains in which no extracellular beta but alpha was detectable constituted Group II. All isoniazid-resistant strains of M. tuberculosis and M. bovis were in this group. All the M. avium strains, M. paratuberculosis, M. balnei, M. ulcerans, M. lepraemurium and 24 out of 45 atypical strains were in Group III. This group was characterized by no beta detectable but cross-reacting material with antigenic determinants partially in common with alpha. Group IV contained all saprophytic strains including M. fortuituim, M. phlei,

M. smegmatis and 21 atypical strains. Yoneda's grouping of the atypical mycobacteria did not agree with Runyon's Groups.

Gel filtration has been used to separate the components of tuberculin (13). An unheated BCG culture filtrate was separated into a dialyzable and nondialyzable fraction (4). The nondialyzable fraction was precipitated with 1.0% acetic acid at pH 4. The supernatant fluid was designated fraction G and the precipitate fraction F. The G and F fractions were separated into three and two components, respectively, following separation on Sephadex G-50 columns. This resulted in a sharper separation of the protein and polysaccharide constituents. These five fractions elicited reactions when injected intradermally into sensitized guinea pigs. The complexity of these five fractions was readily detected by gel diffusion analyses. Each were composed of from five to seven antigens.

In the search for an immediate skin test antigen, Glenchur, Fossieck and Silverman (31, 32) fractionated bacillary extracts of H37Ra, an avirulent strain of M. tuberculosis, on columns of G-25 Sephadex and DEAE cellulose. Three fractions were obtained from the G-25 Sephadex chromatography. One of these fractions, I, was further separated into seven fractions by DEAE column chromatography. The results of skin testings with one of the DEAE fractions in pulmonary tuberculous and control patients suggested that

the procedure may have some merit from the standpoint of obtaining immediate type skin testing agents.

A continuation of this study indicated the PPD's could be fractionated in the same manner (32). Three fractions were obtained by G-25 Sephadex chromatography of PPD. The quantities of two of these fractions was considerably less than that found in H37Ra extracts. The seven H37Ra fractions obtained from DEAE separation were not pure but contained as many as five antigens in some of the fractions.

Kniker and LaBorde (43) used DEAE cellulose chromatography to separete the antigens of four strains of M. tuberculosis. Twelve fractions collected and pooled from each culture filtrate were concentrated, and analyzed by Ouchterlony immunodiffusion with their homologous antisera and with 14 heterologous antisera. Antisera were prepared for 12 atypical mycobacteria including Group I, II and III organisms. As many as twenty antigens were detected in the homologous immunodiffusion systems. Common antigens were found in various fractions as well as antigens specific only for the tubercle bacilli. No one antigen was isolated in pure form as evidenced by the immunodiffusion studies.

Complete separation of mycobacterial antigens by column chromatography methods has not been reported. Lind (51) used carboxymethyl cellulose to separate the antigens in an unheated culture filtrate. He suggested that good

separation of the antigens was not obtained because the proper variations in pH and molarities may not have been used.

A fraction obtained by Seibert's method (95) was used by Rhodes (85) for DEAE column chromatography. The fraction could be separated into three protein components by paper electrophoresis and into at least four components by DEAE chromatography. Re-electrophoresis of the fractions collected from the column using paper and starch indicated little separation of the antigens. All the fractions elicited about the same level of tuberculin type skin reactivity when tested in sensitized animals.

Immunodiffusion techniques have yielded excellent results when applied to studies on the antigenic relationships of mycobacteria (73, 74). Quantitative as well as qualitative data can be obtained. The Ouchterlony technique is the most favored because comparisons can be made between different antigenic preparations.

The first extensive study of the antigenic relationships between mycobacteria employing the modified Ouchterlony technique was by Parlett and Youmans (73). Concentrated, unheated culture filtrates were injected into rabbits to obtain the antisera, and used as antigen in immunodiffusion tests. The culture filtrates of forty-two mycobacteria, including strains of M. tuberculosis, M. bovis, M. avium

atypical and saprophytic mycobacteria, were analyzed for their antigenic relationships with 16 antisera. The greatest number of precipitate lines was four. On the basis of their findings they divided the 42 mycobacteria into four groups. Group I included one atypical mycobacterium, one attenuated bovine strain, one avian and all of the human strains. Group II consisted mainly of bovine strains and one avian strain. Group III consisted only of saprophytic mycobacteria. Group IV was one atypical strain which reacted only with its homologous antiserum. The cross reactivity reactions indicated the close relationship of the atypical strains to the human strains.

A more extensive study employed 98 mycobacteria and four fungi with 42 reference antisera (74). The methods and techniques were the same except the antigens in agar immunodiffusion tests were cell extracts. The greatest number of precipitate bands found was six and the number of groups expanded to eight. The major differences were that M. phlei was classed in a group separate from the other saprophytic mycobacteria, and the atypical strains were divided into five groups. The antigenic relationship reaffirmed their belief that the atypical strains were genetically related to the virulent human strains of mycobacteria. The atypical strains were more related antigenically to the avian strains. No common antigen was found for all of the 98 strains of

mycobacteria studies. No antigenic relationship was detected between the mycobacteria and fungi cultures. The results firmly established that immunodiffusion techniques were of value in classifying mycobacteria.

Further immunodiffusion studies have been reported, primarily by investigators in other countries. A common antigenic component was detected by immunodiffusion in all of 23 strains which included 11 atypical strains (34, 104). The antisera were produced in rabbits inoculated with viable cell suspensions. The antigens in the Ouchterlony immunodiffusion tests were unheated culture filtrates of organisms grown in Youman's medium (115) with 10% horse serum. No procedures were described to free the cell suspensions of the horse serum components before use for immunization. In addition to the common antigen, whether it was of mycobacterial origin or not, a close antigenic relationship existed between the mammalian tubercle bacilli and the atypical mycobacteria. More recently Tuboly (107) and Gempl (29), and Gempl and Weissfeiler (30) reported the presence of common antigens in the mycobacteria studies by immunodiffusion.

Other immunodiffusion studies have added materially to the information concerning mycobacterial antigenic relationships. Mycobacterium phlei was found to be antigenically distinct from all other mycobacteria including the saprophytic strains (29, 30, 52). Studies of M. avium, M. ulcerans,

M. balnei and M. marinum indicated that M. avium and M. ulcerans were closely related and that all four organisms possessed at least one common antigenic factor (53).

Lind (46, 47, 48, 49, 50, 51, 52) has made the most extensive and complete studies employing immunodiffusion techniques. His work has added immeasurably to the knowledge of the antigenic relationships among different mycobacteria. Equally important, he has investigated some of the variables and made a plea for the establishment of sound, uniform experimental techniques. He has studied extensively the methods of producing antisera, and the chemical and physical factors which affect the antigens of both culture filtrates and cell extracts. His plea is for standardization of both antisera and antigens among the various laboratories studying mycobacteria by immunologic methods.

The need for standardization of the procedures for the preparation of antigens and antisera is apparent. It is difficult to draw meaningful comparisons from reports of immunodiffusion studies of mycobacteria. The antigenic preparations differ and the antisera produced by different procedures. A difference of a few weeks in the age of the cultures and the growth medium used can change the antigenic content of the culture filtrate (12). The deleterious effect of heat and chemicals such as phenol on proteins is well known, yet phenol is used with mycobacterial antigenic preparations (107).

The Oakley-Fulthrope technique (99) and immunoelectrophoresis (8, 9, 12, 29, 51, 107) have been used with mycobacterial antigens. The Ouchterlony (29, 50) and tube double diffusion technique (3, 7, 84) are currently in use as sero-diagnostic tests; the latter is the more widely used (72, 75, 76). Reports vary on the reliability of the test. The number of positive human sera from tuberculous patients has ranged from 44% (50) to 60 to 80% (75, 105). Negative results have been reported by Long and Top (56) with sera from tuberculin positive cattle.

The antigenic preparations employed in the tube double diffusion sero-diagnostic studies were from different mycobacteria: H37Ra (18, 56), M. phlei and BCG, the attenuated Calmette-Guerin strain of M. bovis (56). As several investigators have suggested, the test will undoubtedly be more accurate when specific antigens are isolated and used in the test (18, 27). The inability to differentiate Group III's which are saprophytes and cause no disease, and those which can cause disease in man, cattle, swine and probably other animals indicates a need for antigens specific for each of the classical pathogens and representatives of Group III mycobacteria.

Edwards and associates (23) have delineated what an ideal skin test should be. "It would never indicate that an individual had not been infected if he had, or that he had been infected if he had not. Moreover, the ideal skin test

would never confuse infection by one organism with infection by another." They also state that the skin test should be able to differentiate between single and multiple infections. No skin test is available today which can meet these standards. This reaffirms the need for specific sensitins.

MATERIALS AND METHODS

Cultures. The four mycobacteria were isolated from animals: M. bovis, strain 310 from a gross-lesion cow; M. avium, strain 132 from a gross-lesion chicken; Group III, strain 68 from a tuberculin positive gross-lesion cow and Group III, strain 50 from a tuberculin positive no-gross-lesion cow. Strain 50 was categorized as of low or no virulence; strain 68 was of moderate virulence (64).

Culture filtrates. The organisms were grown on the surface of one liter of a modified Proskauer-Beck synthetic medium (115) in large diphtheria toxin bottles. After three months at 37C, the culture fluid was drawn off aseptically in 250 ml plastic bottles and centrifuged three hours at 2010 x g. The supernatant fluid was passed through a Berkefeld filter (N grade), the filtrates dialyzed against 0.015M phosphate buffer, pH 7.0, for three days at 4C and concentrated ten fold by pervaporation. The concentrate was filtered (Millipore, 0.45 μ pore size) merthiolate added (1:10,000) and stored at -80C in small amounts.

The amount of protein in the culture filtrates was determined by the method of Lowry (57). The amount of carbohydrate in the culture filtrates was assayed by the method of Morris (65).

For immunodiffusion and immunoelectrophoretic studies, the four culture filtrates were concentrated further as necessary with Ficoll (Pharmacia). Dialysis tubing (Visking Corp.) was filled with culture filtrate and covered with dry Ficoll.

Production of antisera. Twenty-nine Dutch Belted rabbits were used as the source of the four reference antisera, five rabbits each for culture filtrates 68, 50, and M. avium, and nine rabbits for M. bovis. A modified alum precipitation procedure as described by Carpenter (11) was used to prepare the inocula. To each 10 ml of culture filtrate, 0.46 ml of a 10% $AlCl_3$ was added. With constant stirring, the mixture was adjusted to pH 7 with 20% NaOH. Each rabbit was inoculated intramuscularly with 5 ml of the mixture containing 10 mg of protein. After 40 days, the rabbits were inoculated subcutaneously at three sites with a total of 1.5 ml of culture filtrate containing a total of 2 mg of protein. The rabbits were bled by cardiac puncture seven and nine days later. All antisera were tested with the homologous culture filtrate by the Ouchterlony immunodiffusion method and satisfactory antisera were pooled, merthiolate added (1:10,000), and stored in small amounts at -80C. These antisera are referred to hereafter as reference antisera.

Antisera were prepared against 11 mycobacterial culture filtrates (Table 1) containing homologous cells. The culture filtrates were prepared similarly to those described except that the organisms were grown two months in 50 ml of the medium in 250 ml flasks and the supernatant fluid filtered only with Millipore filters (0.45 μ pore size). Cells from each culture was inactivated with beta-propriolactone (BPL).

One mg of packed BPL inactivated cells was mixed with three ml of homologous culture filtrate containing 0.5 mg/ml protein. The suspension was mixed with an equal amount of Freund's incomplete adjuvant (Difco). Two Dutch Belted rabbits were inoculated for each of the 11 organisms (Table 1). Each rabbit received intravenously 0.25 ml of the preparation without Freund's incomplete adjuvant and at the same time a total of 0.5 ml at two sites subcutaneously of the preparation mixed with the adjuvant. Seven and 14 days later they were inoculated subcutaneously with 0.25 ml of the preparation with adjuvant. Two months following the initial injections, the rabbits received 0.5 ml subcutaneously of only culture filtrate on two successive days. Thirty-six days later they were inoculated with 0.75 ml of only culture filtrate subcutaneously and bled six days later by cardiac puncture. The sera were pooled and stored at -80C until used. These antisera are referred to hereafter as cell-culture filtrate antisera.

Table 1. Eleven mycobacteria from which cells and culture filtrates were prepared for inocula to obtain antisera

Designation	Classification
P4	Photochromogen (*M. kansasii*) Group I (human origin)
P15	Scotochromogen, Group II (human origin)
P39	Nonphotochromogen (Battey Type) Group III (human origin)
50	Nonphotochromogen, Group III (bovine origin)
51	Nonphotochromogen, Group III (bovine origin)
62	Nonphotochromogen, Group III (bovine origin)
68	Nonphotochromogen, Group III (bovine origin)
172	Nonphotochromogen, Group III (swine origin)
131	*M. avium* virulent (avian origin)
310	*M. bovis* virulent (bovine origin)
BCG	*M. bovis* attenuated (bovine origin)

Ouchterlony immunodiffusion. Glass slides 3-1/4 by 4 inch were washed with detergent, rinsed in distilled water and dried. The slides were covered with 10 ml of 1% melted agar (Difco) in a 0.15 M phosphate-saline buffer, pH 7.2 containing merthiolate (1:10,000). After the agar had solidified, the slides were placed in a humidified chamber for at least three hr before wells were cut in the agar according to a drafted pattern placed beneath the slide. The diameter of the wells was 6 mm and the diffusion distance between wells 6 mm. After the reactants were added to the wells, the plates were incubated at 28C for two to five days in a humidified chamber and observed daily. Following incubation, the plates were washed in phosphate-saline buffer solution, pH7.0, for 24 hr followed by a second rinse in distilled water for 24 hr to remove unreacted protein. The slides were air dried and then stained in triple stain (16) for 15 min and destained for 30 min in 2% acetic acid.

The four culture filtrates were placed in peripheral wells arranged around a center antiserum well in varying concentrations to determine the optimal conditions for precipitin line formation. Each culture filtrate was tested at the following concentrations: undiluted, two, five, ten and 20 fold. In addition, culture filtrates 50 and 68 were tested at 30, 50, and 100 fold and M. bovis and M. avium at 40 fold. In tests thereafter, 30 fold concentrations of 50 and 68, 20 fold concentrations of M. bovis and ten fold

concentrations of M. avium were used. The amount of protein in the ten fold concentrated culture filtrate is given in Table 2. By varying the arrangement of the culture filtrates, each culture filtrate was placed in a well adjacent to every other culture filtrate to detect bands of identity and nonidentity.

Immunoelectrophoresis. Immunoelectrophoresis was performed according to the Hirschfeld procedure (35) with certain modifications. The barbital buffer, pH 8.6, consisted of the following:

Constituents	Buffer in electrode vessel	Buffer in agar
Diethylbarbituric acid	1.38 gm	1.66 gm
Sodium barbital	8.76 gm	10.51 gm
Distilled water to	1 liter	1 liter

A 2% purified Bacto agar (Difco) solution was prepared as described by Hirschfeld (35). After the final washing, the agar cubes were stored at 4C in distilled water until needed.

The agar buffer solution was prepared by mixing two parts of the barbital buffer with one part of distilled water, heating to 60C, and adding three parts of melted 2% purified agar. Microscope slides which had previously been cleaned, rinsed and dried were covered with 2.5 ml of the mixture of melted agar and buffer and allowed to stand at least three hr in a humidified chamber before use.

In the center of the long axis the slide, an antigen well 2.5 mm in diameter was cut 25 mm from the cathode end of the slide and the agar plug removed. At a distance of 5 mm on both sides of the antigen well, the agar was cut so that after electrophoresis, two 2 x 66 mm antiserum troughs were made by removing the agar.

A Shandon migration chamber (Colab) was fitted with a 6 x 8-1/2 inch plexiglass plate to hold four agar slides on each side. Filter paper impregnated with the well buffer served to establish electrical contact between slides. The antigen was added to the antigen well. Strains 68 and 50 were used in 100 fold concentrations, M. bovis at 40 fold and M. avium at ten fold.

The chamber was placed at 4C. A current of 1.25 ma per slide was applied for 110 min using a constant current DC power supply (Volkam). After the agar plugs were removed to form the antiserum troughs, the antisera were added. Incubation was at 28C in a humidified chamber for 24 to 48 hr.

The slides were washed for 24 hr in a 0.15 M phosphate-saline buffer, pH 7.0 followed by a second washing in distilled water for 24 hr. The slides were covered with a moistened bibulous paper and air dried. Triple stain (16) was used followed by destaining in 2% acetic acid.

Analytical disc electrophoresis. The apparatus and technique was the same as described by Ornstein and Davis (68). The preparation of the stock solutions used in preparing the gels was followed as outlined by Davis (17). A spacer gel one cm in thickness containing 40% sucrose was layered over the lower gel, and no sample gel used. From 0.05 to 0.2 ml of culture filtrate containing approximately 280 μg of protein was then added on top of the spacer gel by displacement. Electrophoresis was carried out at room temperature at 5 ma per tube using a 50 ma DC power supply (Volkam) until the bromphenol blue marker dye had migrated exactly 3.6 cm into the lower gel (approximately 90 min). The gels were stained for protein or polysaccharide. A 0.5% solution of amido black in 5% acetic acid was used to stain the proteins in the gel for 30 min at room temperature. Excess stain was removed electrophoretically in 5% acetic acid. Polysaccharides and glycoproteins were stained by the periodic acid-Schiff (PAS) reaction method according to Canalco (10).

Preparative disc electrophoresis. Glass columns, 1.1 x 10 cm, were filled to 7 cm with the 7% lower gel and a 1 cm spacer gel added following polymerization of the lower gel. After polymerization of the spacer gel, the columns were placed in the electrophoretic chamber and buffer was added to the buffer chambers. From 0.4 ml to 1.5 ml of culture filtrate containing about 2.0 mg of protein was layered

onto the spacer gel. Electrophoresis was at 11 ma per tube until the bromphenol blue marker dye had migrated 6.5 cm into the lower gel (approximately 4-1/2 hr). An amido black stained reference gel of each culture filtrate was prepared. The staining and destaining procedure was the same as that for the analytical disc gels. Line drawings of each of the reference gels were drawn to scale to serve as templates for cutting unstained gels.

Eight columns were used for each of four culture filtrates. Immediately following electrophoresis the columns were immersed in an ice bath for 1 to 5 min. Each gel was placed on a glass lantern slide with a template beneath it and the gel cut into segments with a razor blade following the predetermined pattern. For each component to be recovered, the eight similar segments from each gel were placed, four segments to a tube, in 19 x 100 mm plastic tubes. Each tube contained 2 ml of 0.05 M phosphate-saline buffer solution, pH 7.0. The segments were cut into fine pieces with a microspatula and the mixture allowed to stand at room temperature for 48 hr. The contents of each of the two tubes containing the eight similar segments were poured into a Filterfuge tube (International Equipment Company). The Filterfuge tubes were assembled without filters and the gel separated from the eluate by centrifugation at 2010 x g for 20 min. The eluate from each segment pool was removed

and dialyzed for 48 hr against 0.05 M phosphate-saline buffer, pH 7.0. The protein concentration was determined with correction for residual tetramethylethylenediamine (TEMED).

Selected eluates from the segments cut from the gels of the four culture filtrates were examined by analytical disc electrophoresis. The sample volume was from 0.1 to 0.2 ml with enough 40% sucrose added to give the sample sufficient density. One eluate from each of the four culture filtrates was also examined by analytical disc electrophoresis using a 10% lower gel, prepared with 40 gm acrylamide, 0.4 gm N,N'-methylenebisacrylamide, and distilled water to 100 ml.

Selected eluates obtained from the preparative disc gels were analyzed with the four reference antisera and 11 cell-culture filtrate antisera. In the case of the four reference antisera, the homologous eluates were placed in the peripheral wells in such a way that each eluate was compared against every other homologous eluate. The homologous reference antiserum was placed in the center well. This was repeated for the heterologous reference antisera.

To test the eluates with the 11 cell-culture filtrate antisera, the antisera were placed in the peripheral wells and the eluates in the center well.

Disc immunoelectrophoresis. This is a combination of disc electrophoresis and immunodiffusion in agar. After electrophoresis, one of each of the four culture filtrates was

placed on 3-1/4 by 4 inch slides. A buffered agar solution was prepared by mixing one part melted 2% agar prepared according to the method of Hirschfeld (35) with one part tris-glycine buffer, pH 8.3. The tris-glycine buffer was the same as that used for the reservoir buffer in the disc electrophoresis procedure but was diluted, one part distilled water to two parts stock buffer. Ten ml of the hot agar solution at 60C containing merthiolate (1:10,000) was pipetted onto the clean 3-1/4 by 4 inch slides with the four gels. The disc gels were immediately centered and spaced 12 mm apart. The plates were incubated at 28C for 24 hr. Four antisera trenches, 2 x 66 mm, were cut the full length of the gels and spaced 5 mm from the outer edges of the gels. These trenches were filled with the four reference antisera. The plates were incubated for five days and observed daily. The plates were washed, dried and stained in the same manner as the Ouchterlony plates.

RESULTS

Analytical disc electrophoresis. The amounts of protein and carbohydrate in the four culture filtrates are listed in Table 2. From 0.25 to 0.30 mg of protein in not more than 0.2 ml was the optimal concentration for disc electrophoresis. The same concentration of protein was used in the duplicate gels which were stained with PAS. Representative patterns of the protein components of the culture filtrates are presented in Fig. 1. Schematic drawings of the protein and PAS positive components are presented in Fig. 2 and 3.

The number of protein components in the culture filtrates are as follows: 24 in M. bovis and 22 in M. avium (Fig. 3); 18 in both strains 68 and 50 (Fig. 2). The protein bands were more distinct in the M. avium and M. bovis culture filtrates. All the culture filtrates had a protein band at the surface of the lower gel. The band nearer the anode was in approximately the same position for all filtrates. The bands in culture filtrates 68 and 50 contained approximately twice as much of this most anodic component as culture filtrates M. bovis and M. avium. No protein components were detected in the spacer gel.

Table 2. Amounts of protein and carbohydrate in the unheated, ten fold concentrated mycobacterial culture filtrates

	mg/ml of	
Culture Filtrate	Protein	Carbohydrate
50 (Group III)	1.4	0.47
68 (Group III)	1.4	0.55
310 (*M. bovis*)	2.3	0.47
132 (*M. avium*)	6.0	0.30

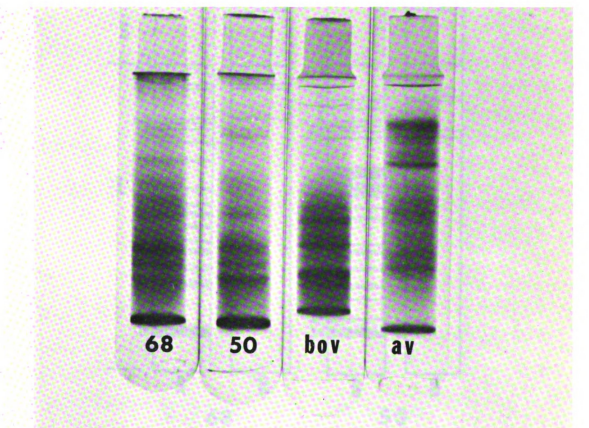

Fig. 1. Analytical disc electrophoretic patterns of culture filtrates 68, 50, Mycobacterium bovis (bov), and M. avium (av) stained with amido black.

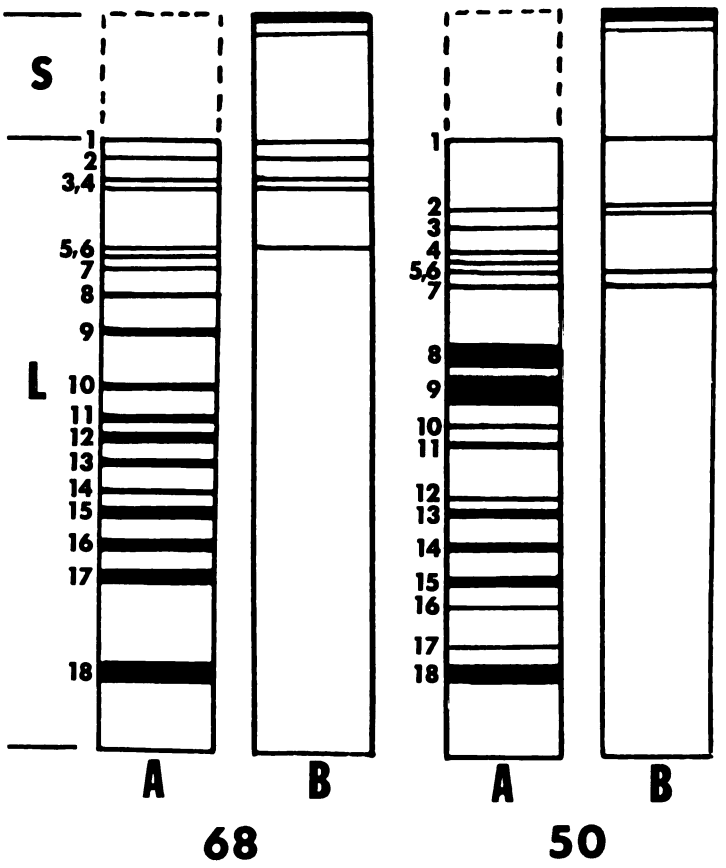

Fig. 2. Schematic representation of the protein (A) and PAS positive (B) components in culture filtrates 68 and 50. The letters S and L refer to the spacer and lower gel areas, respectively.

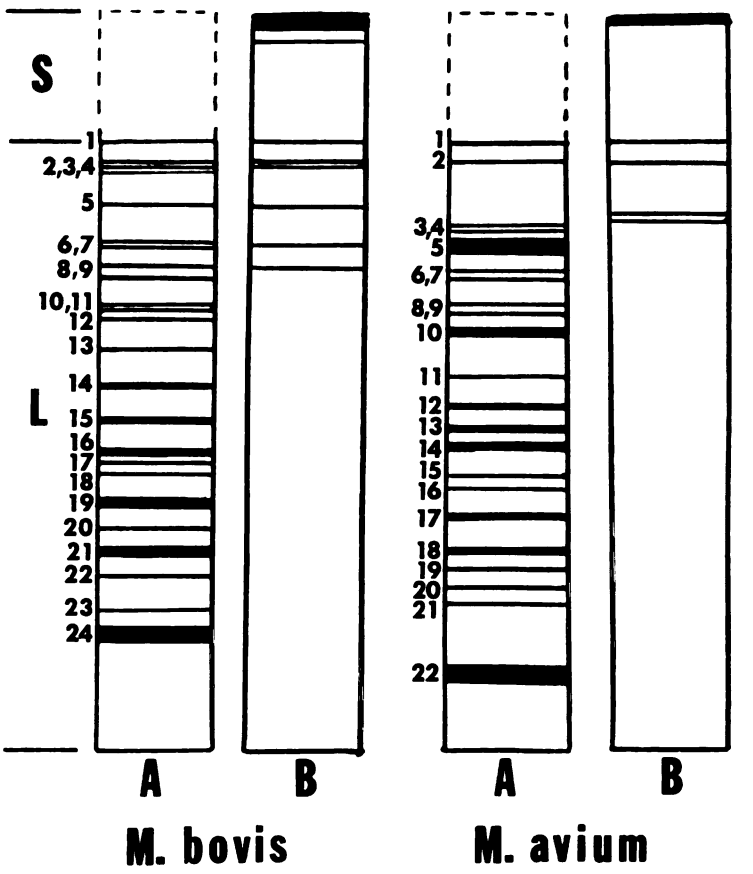

Fig. 3. Schematic representation of the protein (A) and PAS positive (B) components in culture filtrates Mycobacterium bovis and M. avium. The letters S and L refer to the spacer and lower gel areas, respectively.

All the culture filtrates contained PAS positive material (Figs. 2 and 3). The number of PAS positive components in the culture filtrates were as follows: eight in M. bovis and five in M. avium (Fig. 3); seven in both 68 and 50 (Fig. 2). All the culture filtrates had a PAS positive component at the surface of the spacer gel and at the interface of the spacer gel and lower gel. Three of the culture filtrates, 68, 50, and M. bovis had an additional PAS positive band in the spacer gel. The other PAS positive bands in the four culture filtrates were located in the upper third of the lower gel. Most of these PAS positive components could be correlated with amido black-staining components. The PAS positive material in the culture filtrates did not react strongly with Schiff's reagent. The strongest reactions occurred with the material located in the spacer gel.

Immunodiffusion analyses of the four culture filtrates. The results obtained with the four reference antisera systems are summarized in Table 3. When reference antiserum 50 was reacted in various combinations with the four culture filtrates from two to eight immunoprecipitates were observed (Figs. 4 and 5). The homologous system had eight immunoprecipitates. Seven were obtained with culture filtrate 68 and two each with M. bovis and M. avium. All the immunoprecipitates of culture filtrate 68 were in common identity with those of culture filtrate 50. Only one of the M. avium immunoprecipitates was in common identity with those

Table 3. Number of immunoprecipitates in Ouchterlony gel diffusion tests of mycobacterial culture filtrates with the reference antisera

	Antisera			
Culture Filtrate	50	68	132	310
50 (Group III)	8	7	1	0
68 (Group III)	7	10	3	0
132 (*M. avium*)	2	4	7	0
310 (*M. bovis*)	2	2	0	6

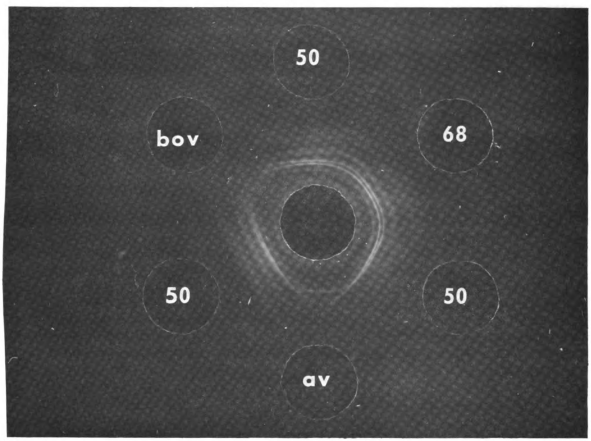

Fig. 4. Ouchterlony immunodiffusion of culture filtrates 50, 68, Mycobacterium avium (av), and M. bovis (bov) with reference antiserum 50 (center well).

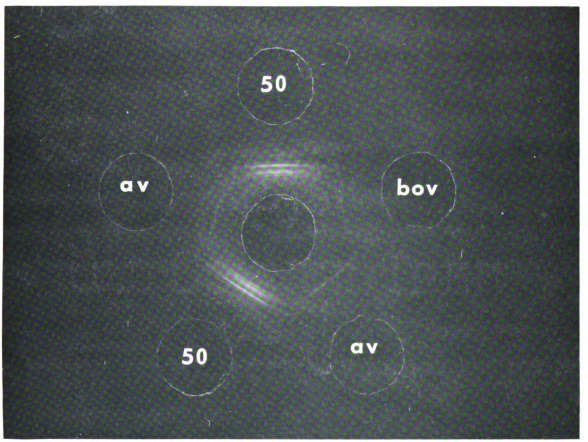

Fig. 5. Ouchterlony immunodiffusion of culture filtrates 50, Mycobacterium avium (av) and M. bovis (bov) with reference antiserum 50 (cetner well).

of 50 and 68. The other M. avium line was in common identity with one line produced by M. bovis. None of the M. bovis immunoprecipitates were in common identity with those of culture filtrate 50.

From two to ten immunoprecipitates were formed with culture filtrate 68 reference antiserum (Figs. 6 and 7). The greatest number of immunoprecipitates was obtained with the homologous system. Seven, four and two separate immunoprecipitates occurred with culture filtrates 50, M. avium and M. bovis, respectively. All culture filtrates with the exception of M. bovis had lines of common identity with the homologous system. One line of common identity occurred among culture filtrates M. avium, 68 and 50. The M. avium culture filtrate possessed one other antigen in common identity with culture filtrate 68, not in common identity with 50. None of the immunoprecipitates of M. bovis were in common identity with any others.

With the M. avium reference antiserum, eight immunoprecipitates occurred with the homologous culture filtrate (Fig. 8). No reaction was detected with the M. bovis culture filtrate. Of the three immunoprecipitates obtained with culture filtrate 68, two were in common identity with those of M. avium. One immunoprecipitate formed with culture filtrate 50. The one immunoprecipitate with culture filtrate 50 and the third immunoprecipitate with culture filtrate 68 were not in common identity with each other or with M. avium.

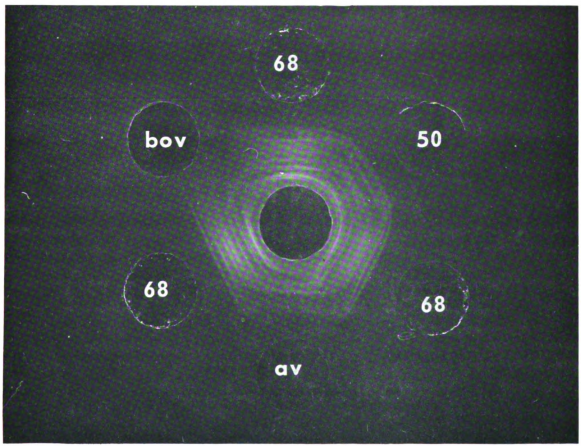

Fig. 6. Ouchterlony immunodiffusion of culture filtrates 50, 68, Mycobacterium avium (av) and M. bovis (bov) with reference antiserum 68 (center well).

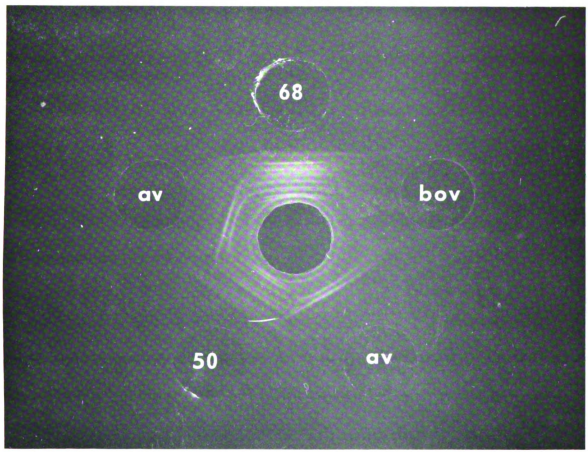

Fig. 7. Ouchterlony immunodiffusion of culture filtrates 50, 68, Mycobacterium avium (av) and M. bovis (bov) with reference antiserum 68 (center well).

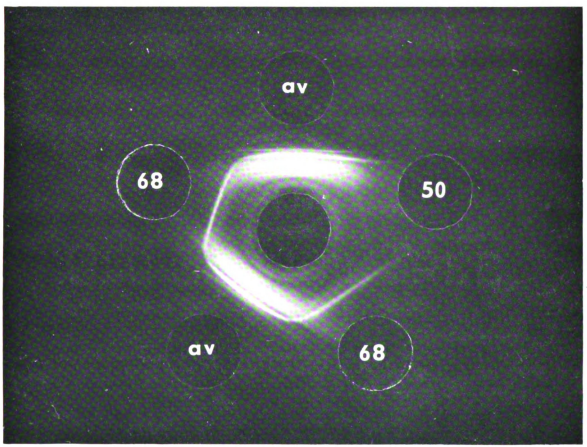

Fig. 8. Ouchterlony immunodiffusion of culture filtrates 50, 68 and Mycobacterium avium (av) with the M. avium reference antiserum (center well).

The M. bovis reference antiserum reacted only with the homologous culture filtrate (Fig. 9). Six immunoprecipitates were observed.

Immunoelectrophoresis. Nine antigens were detected in the culture filtrates 50 and 68 by immunoelectrophoresis (Table 4). The immunoprecipitate patterns of the two culture filtrates were very similar. No immunoprecipitates were found on the cathode side of the origin. Two of the immunoprecipitates extended almost to the sample origin. At least two immunoprecipitates were close to the sample origin with slight lateral displacement. Some of the immunoprecipitates had similar positions and curvatures, and were stained very lightly.

Eight immunoprecipitates were present after electrophoresis of the M. bovis culture filtrate (Table 4). None of the immunoprecipitates was on the cathode side of the sample origin. There was only slight lateral displacement from the center of diffusion with three of the immunoprecipitates. Those antigens which migrated the least, stained lightly.

Fifteen antigens were detected in the M. avium culture filtrate by immunoelectrophoresis (Table 4). Only one antigen was found on the cathode side of the sample origin (Figure 10). One very darkly stained immunoprecipitate began at the cathode side of the sample origin and extended far toward the anode side. The lateral displacement of one

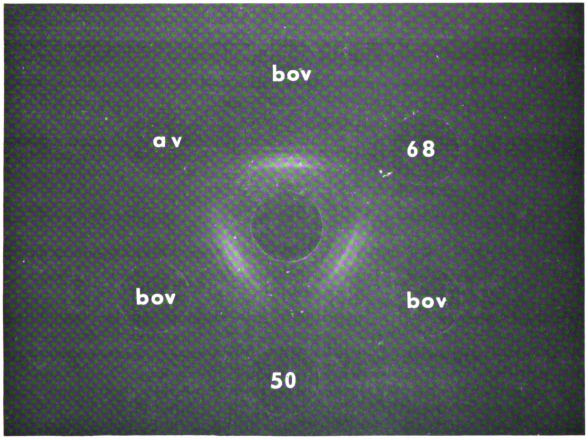

Fig. 9. Ouchterlony immunodiffusion of culture filtrates 50, 68, Mycobacterium avium (av) and M. bovis (bov) with M. bovis reference antiserum (center well).

Table 4. Number of immunoprecipitates in immunoelectrophoresis (IE) and disc immunoelectrophoresis (DIE) tests of the four mycobacterial culture filtrates with the reference antisera

Culture Filtrate	DIE Antisera				IE Homologous Antisera
	50	68	132	310	
50 (Group III)	13	11	1	0	9
68 (Group III)	12	12	7	0	9
132 (*M. avium*)	4	6	16	0	15
310 (*M. bovis*)	2	4	0	9	8

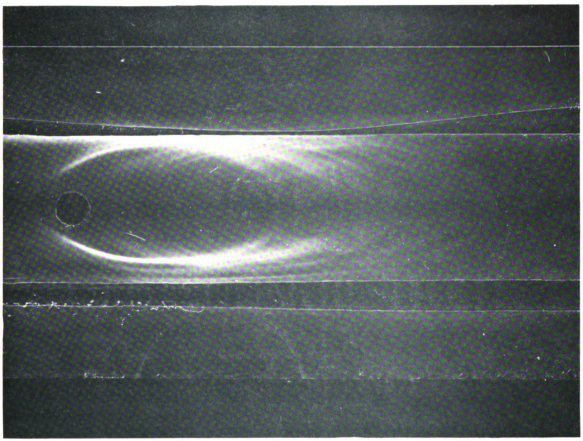

Fig. 10. Immunoelectrophoresis of Mycobacterium avium culture filtrate with homologous reference antiserum.

immunoprecipitate was slight in comparison to the others. Most of the immunoprecipitates stained sharply.

Disc immunoelectrophoresis. When the reference serum 50 was tested against gels in which the four culture filtrates had been electrophoresed, culture filtrates 50, 68, M. avium, and M. bovis had 13, 11, four and two antigens, respectively (Table 4). The lateral displacement of the immunoprecipitates away from the gels generally was progressively greater for antigens farther from the top of the lower gel and was consistent for all of the culture filtrates reacted with the four reference antisera.

The immunoprecipitate patterns of culture filtrates 50 and 68 were very similar. There were at least three antigens located in approximately the same position in the most distal part of the gel. One antigen was located at the surface of the lower gel, and the immunoprecipitate was extremely faint and formed close to the gel. The four antigens of M. avium and two antigens of M. bovis which reacted with the reference serum 50 were located in the lower half of the gels. There was no evidence of immunoprecipitates with the components found in the spacer gels with any of the four reference antisera.

Culture filtrates 68, 50, M. avium and M. bovis formed 12, 11, six and four immunoprecipitates, respectively, with the reference serum 68 (Table 4). The position and lateral displacements of the immunoprecipitates were similar

to those with the reference serum 50. With the exception of one, all the immunoprecipitates formed by M. avium and M. bovis antigens were in the lower half of the gel.

When the M. avium culture filtrate was electrophoresed 16 immunoprecipitates formed with the homologous reference antiserum (Fig. 11; Table 4). Seven and one antigens were detected in the culture filtrates 68 and 50, respectively, when reacted with the M. avium reference serum. There was no reaction with the M. bovis culture filtrate. Most of the antigens of the M. avium culture filtrate and culture filtrate 68 were located in upper two-thirds protion of the gel. Several of the M. avium immunoprecipitates extended almost the full length of the gel.

Consistent with the other immunodiffusion tests, the M. bovis reference antiserum reacted only with the electrophoresed M. bovis culture filtrate (Table 4). Nine immunoprecipitates were observed. Three of these immunoprecipitates were in the upper third of the gel with slight lateral displacement and were lightly stained. The remaining six immunoprecipitates were in the lower half of the gel, more laterally displaced and stained sharply.

Preparative disc electrophoresis. The optimal concentration of protein for preparative disc electrophoresis was between 2.1 and 2.6 mg, in no more than 1.5 ml, approximately ten fold more than for analytical disc electrophoresis. Exceeding 2.6 mg caused broadening and overlapping

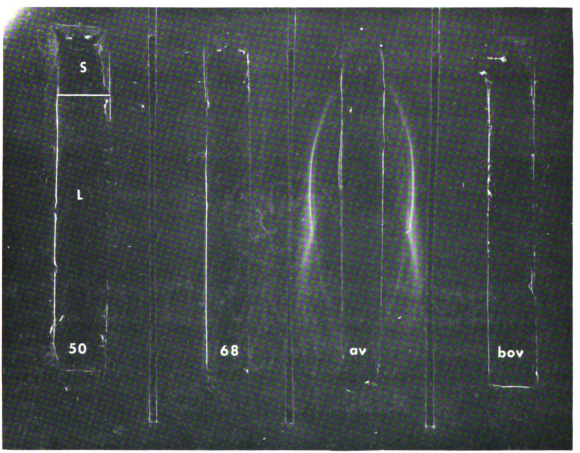

Fig. 11. Disc immunoelectrophoresis of disc gels of culture filtrates 50, 68, Mycobacterium avium (av) and M. bovis (bov) with M. avium reference antiserum (long, narrow trough). The letters S and L refer to the spacer and lower gel areas, respectively.

adjacent components. The resolution of the components was equal to that in the analytical gels and by increasing the gel length many of the components were separated further from adjacent ones.

The components in the eluates from the preparative gels were numbered as in the analytical disc electrophoretic schematic drawings (Figs. 2 and 3). The bands were numbered from the top of the gels to the bottom. The eluates were coded so that the first number indicated the culture filtrate and the second number the number of the disc band from which the antigen was eluted. Re-electrophoresis of the eluates obtained from gel segments affirmed the accuracy of the sectioning process. A representative disc electrophoretic pattern of the eluates obtained from five of the segments cut from a culture filtrate 50 disc preparative gel is presented in Figure 12. All of the culture filtrate 50 eluates appeared as single bands when re-electrophoresed in analytical disc gels.

The number of amido black-staining components in each of the 20 eluates is presented in Table 5. Two of the culture filtrate 68 eluates had two amido black-staining components, while the remaining three had only one component. Of the five M. avium eluates, one had three components and another two. Three of the M. bovis eluates contained only one amido black-staining component. Two of the eluates contained two components.

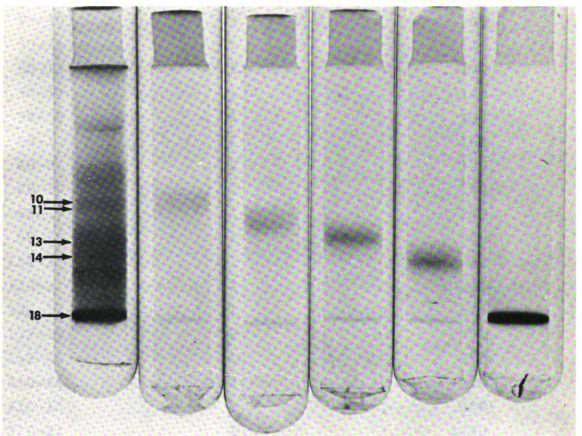

Fig. 12. Analytical disc electrophoretic patterns of culture filtrate 50 (extreme left) and five components re-electrophoresed after sectioning from preparative gels stained with amido black.

Table 5. Number of protein-staining components detected by re-electrophoresis and protein concentrations of the 20 eluates obtained from preparative disc electrophoresis

Eluate #	Number of Amido Black-Staining Components	Protein Concentration µg/ml
50-10[1]	1	80
50-11	1	110
50-13	1	170
50-14	1	140
50-18	1	340
68-1	1	160
68-8	2	120
68-10	2	140
68-17	1	200
68-18	1	300
132-2	1	80
132-3	3	130
132-12	1	270
132-19	2	290
132-22	1	370
310-14	2	120
310-15	1	260
310-21	2	1120
310-23	1	410
310-24	1	270

[1]The first number indicates the strain. 50 and 68 are Group III mycobacteria, 132 is M. avium, 310 is M. bovis. The second number is the number of disc band from which the antigen was eluted.

The protein concentrations ranged from 80 µg to 1120 µg per ml (Table 5). In general, the concentration was greater for those components found progressively more anodic in the gel. Based on theoretical calculations, 20 to 60% of the protein was recovered. The values obtained for the protein concentration of the eluates were corrected for residual TEMED, a chemical component of the gels which reacted in the Lowry procedure for the determination of protein.

The four most anodic components which were found in eluates 50-18, 68-18, 132-22 and 310-24, were re-electrophoresed in 10% disc analytical gels. In 7% analytical gels, these eluates appeared as single amido black-staining components. A representative disc electrophoretic pattern of the eluate 50-18 electrophoresed in a 7 and 10% gel is presented in Fig. 13. In the 10% gel, the single component was separated into six. The single components in 7% gels, in the eluates 68-18, 132-22, and 310-24 were separated into five, three and components respectively in the 10% gels. Those components separated in 10% gels which did not correspond to the original band that was cut were stained very faintly with the amido black dye.

Immunodiffusion analyses of the eluates. The number of immunoprecipitates obtained when the 20 eluates were reacted with the four reference antisera are presented in Table 6. Eluate 50-13 had two antigenic components one of which was in identity with the single antigen in eluate

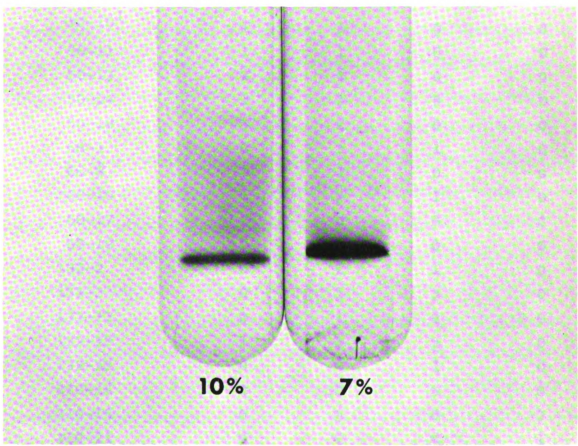

Fig. 13. Analytical disc electrophoretic patterns of eluate 50-18 obtained from culture filtrate 50 preparative gels on re-electrophoresis in 7 and 10% gels. Stained with amido black.

Table 6. Number of immunoprecipitates in Ouchterlony gel diffusion tests of 20 eluates obtained by preparative disc electrophoresis of mycobacterial culture filtrates with four reference antisera

Eluate	Antisera 50	68	132	310
50-10[1]	1	1	0	0
50-11	1	1	0	0
50-13	2	2	0	0
50-14	1	1	0	0
50-18	3	2	0	0
68-1	0	1	1	0
68-8	1	1	0	0
68-10	1	2	1	0
68-17	2	2	0	0
68-18	2	3	0	0
132-2	0	1	1	0
132-3	0	1	3	0
132-12	0	1	3	0
132-19	1	1	3	0
132-22	0	0	1	0
310-14	0	0	0	0
310-15	0	0	0	2
310-21	0	0	0	1
310-23	0	0	0	1
310-24	0	0	0	1

[1]The first number indicates the strain. 50 and 68 are Group III mycobacteria, 132 is M. avium, 310 is M. bovis. The second number is the number of disc band from which the antigen was eluted.

50-14. One of the eluates, 50-18, electrophoresed as a single band but formed three immunoprecipitates with the homologous reference antisera. All of the culture filtrate 50 eluates reacted with the culture filtrate 68 reference serum, but none reacted with the culture filtrate M. avium or M. bovis reference antisera. Eluate 50-18 had one antigen more when reacted with the homologous reference antiserum than with the culture filtrate 68 reference serum.

Nine separate immunoprecipitates occurred in the five eluates of culture filtrate 68 with the homologous antiserum. In one eluate, two amido black components were present in the disc gel but only one was antigenic. Eluate 68-18 contained three antigens but electrophoresed as a single component. Using the culture filtrate 50 and M. avium reference antisera, six and two immunoprecipitates were observed, respectively, with the five culture filtrate 68 eluates. No immunoprecipitates formed with the M. bovis reference antiserum. Two of the culture filtrate 68 eluates had at least one antigen not in common with the other culture filtrates.

The five M. avium eluates formed 11 separate immunoprecipitates with the homologous reference antiserum. Three of the eluates contained three antigens each. The reference antisera 50 and 68 reacted with the antigens found in the M. avium eluates yielding one and four immunoprecipitates respectively. No reaction was observed with the M. bovis

reference antiserum. Four of the eluates contained specific antigens but they were mixed with antigens which cross reacted with reference antisera 50 and 68. Only eluate 132-22 had an antigen which did not cross react with the other three reference antisera.

The M. bovis eluates reacted only with their homologous reference antiserum. Five separate immunoprecipitates occurred. Eluate 310-14 had two amido black-staining components but neither of them reacted with the reference antiserum. Two antigens were detected in the eluate 310-15 but only one component was observed in the disc gel after re-electrophoresis. The opposite was true of eluate 310-23; there were two amido black-staining components but only one was antigenic.

The reactions of the 20 eluates with 11 cell-culture filtrate antisera are recorded in Table 7. All eluates of culture filtrate 50 reacted with the three Group III antisera 50, 68, and P39. Four reacted with the Group I antiserum P4. Each of the eluates formed two immunoprecipitates with the Group III P39 antiserum. Antigens common only to Group III mycobacteria were found in eluate 50-18.

Eluates 68-1 and 68-8 reacted with four of the Group III antisera and to M. bovis and to attenuated M. bovis (BCG) antisera. An immunoprecipitate formed with the 68-10 eluate and BCG antiserum. Eluate 68-18 contained antigens specific for the Group III mycobacteria.

Table 7. Number of immunoprecipitates in Ouchterlony gel diffusion tests of 20 eluates obtained by preparative disc electrophoresis of mycobacterial culture filtrates with 11 cell-culture filtrate antisera

	Antisera										
Eluate #	P39[2]	50	68	51	62	172	132	310	BCG	P4	P15
50-10[1]	2	1	1	0	0	0	0	0	0	1	0
50-11	2	2	2	0	0	0	0	0	1	1	0
50-13	2	1	2	0	0	0	0	0	0	1	0
50-14	2	2	1	0	0	0	0	0	0	1	0
50-18	2	1	1	0	0	0	0	0	0	0	0
68-1	1	1	1	1	1	0	0	1	1	0	0
68-8	2	2	2	1	1	0	0	1	1	0	0
68-10	1	2	2	0	0	0	1	1	1	1	0
68-17	2	2	2	0	0	0	1	1	0	1	0
68-18	1	2	2	1	0	0	0	0	0	0	0
132-2	0	0	0	1	1	0	2	2	1	0	1
132-3	0	0	0	0	0	0	1	1	1	0	0
132-12	1	1	0	0	1	0	1	1	0	0	0
132-19	2	2	2	1	1	1	2	0	0	1	0
132-22	1	1	1	1	0	0	0	0	0	0	0
310-14	1	0	1	1	1	0	1	1	1	0	0
310-15	1	1	1	1	1	0	0	1	2	1	0
310-21	1	1	1	0	0	0	0	1	2	1	0
310-23	1	0	1	0	0	0	0	1	1	0	0
310-24	0	0	0	0	0	0	0	1	1	0	0

[1]The first number indicates the strain. 50 and 68 are Group III mycobacteria, 132 is M. avium, 310 is M. bovis. The second number is the number of disc band from which the antigen was eluted.

[2]Mycobacterial stains: P39 (human origin), 50, 68, 51, 62 (bovine origin) are Group III; 132 is M. avium; 172 (swine origin) is Group III; 310 is M. bovis; BCG is attenuated M. bovis; P4 (human origin) is Group I; P15 (human origin) is Group II.

Some antigens in the M. avium eluates reacted with all of the 11 cell-culture filtrate antisera. No one antigen was found to be common by the antisera. Of the 20 eluates, only 132-2 contained an antigen which reacted with the Group II P15 antiserum. Eluate 132-3 contained an antigen which formed an immunoprecipitate with the 132, M. bovis and BCG antisera only. The antigens in eluate 132-19 reacted with the greatest number of antisera. Immunoprecipitates formed with all antisera except M. bovis, BCG and P15. The antigen in the eluate 132-14 reacted with four of the Group III antisera. It did not react with the remaining seven antisera.

The antigen in the eluate 310-24 reacted only with the M. bovis and BCG antisera. Eluate 310-23 formed an immunoprecipitate with M. bovis and BCG antisera and one with the P39 and 68 antiserum. Eluates 310-15 and 310-21 formed two immunoprecipitates with the BCG antisera and one immunoprecipitate with the remaining antisera. Eluate 310-14 reacted with only four of the antisera.

DISCUSSION

The mycobacterial components in the unheated culture filtrates were readily separated by disc electrophoresis. Agar, starch gel, and cellulose acetate electrophoresis or polyacrylamide electrophoresis as described by Raymond (82), were considerably less effective. The unique properties of disc electrophoresis make it a very suitable technique for the separation of mycobacterial components. Moreover, the technique is facile, effective, economical and the results highly reproducible.

Another advantage of disc electrophoresis is that culture filtrate does not need to have a high protein concentration to be electrophoresed. Protein concentrations in the range of 1 mg per ml were satisfactory, which eliminates the need for precipitating the protein from culture filtrate by various methods.

The complexity of the four culture filtrates is readily apparent in the disc electrophorograms. The average number of amido black-staining components was 20. This is in close agreement with the number reported by others using an H37Ra culture filtrate after ammonium sulfate precipitation (2). Disc electrophoresis is currently the most satisfactory technique to separate mycobacterial culture filtrate antigens.

The PAS reaction stains not only polysaccharides but also glycoproteins. Having the same relative position in the gels when stained with either amido black or PAS, indicates the presence of glycoproteins or the lack of similar amido black-staining components indicates a polysaccharide. All polysaccharides in the culture filtrate were located in the spacer gel. Inability of the polysaccharides to migrate was probably due to their high molecular weight and/or neutral charge. The glycoproteins were always located in the upper one-third of the lower gel indicating that their ability to migrate was influenced by the same two factors. Molecular weight would more probably be the more important factor because the protein moiety of the complex would generally have a relatively negative charge. Furthermore, other studies by this author have demonstrated that the glycoproteins in the culture filtrates are of large molecular weight.

Densitometric recordings of the gels obtained with a microdensitometer (Canalco) were not as satisfactory as visual observation. If the technique can be improved, it would be highly desirable in determining quantitatively the differences in the concentrations of the components in the culture filtrates from different species, strains and preparations of the same strains.

When culture filtrate reacted with homologous reference antisera in the modified Ouchterlony technique the number of immunoprecipitates ranged from six to ten. The two Group III atypical strains were shown to be very closely related antigenically. They were more closely related to M. avium than to M. bovis but were not identical to those of M. avium. The antigenic relationship to M. avium was greater for the Group III, strain 68 than for strain 50. Antigens in the M. bovis culture filtrate which reacted with the reference antisera 50 and 68 were not in common identity with the antigens detected in the culture filtrate 68 or 50. The M. bovis antiserum reacted only with homologous filtrate indicating that the culture filtrate 50 and 68 antigens were below a detectable level in the homologous systems.

Results of Ouchterlony immunodiffusion studies indicate that antigens are present which should be investigated for strain and species specificity. Although the strains 50 and 68 were antigenically closely related, at least one antigen in each of the culture filtrates was detected in only that strain. One antigen was in common identity for the two Group III strains, 68 and 50, and M. avium when reacted with the reference antisera 50 and 68.

There was no common antigen for all four organisms. The absence of any antigenic relatedness between M. bovis and M. avium was particularly puzzling since other immunodiffusion studies have indicated that these organisms do

have antigenic relatedness (73, 74). The difference in the results of the present study is probably due in part to the methods used to produce the four reference antisera. Hyperimmunization is known to decrease progressively the specificity of the antibodies which results in greater cross-reactivity (80). In this study, the method of obtaining the reference antisera was chosen deliberately to avoid hyperimmunization and to obtain antibodies of high specificity.

Three of the culture filtrates formed immunoprecipitates in heterologous systems which were either not in common identity or only in partial identity with those immunoprecipitates of the homologous system. Ouchterlony (70), Crowle (16), and Hirschfeld (36) have interpreted similar results using model systems. Usually immunoprecipitates of non-identity indicate the presence of unrelated antigens, although not always true, especially if the concentration of the two antigen solutions are highly disproportionate. To avoid this, insofar as possible, preliminary tests were made and the culture filtrates were standardized to obtain the greatest number of immunoprecipitates. As a consequence and because there are multiple antigen-antibody systems, all systems could not be at the optimal ratios.

Immunoprecipitates of partial identity between different antigenic preparations generally indicate that the antibody to one of them is cross-reacting (16). This is called a pattern of partial intersection or sometimes

"spurring" (109). Patterns of this type occur when the antigens carry determinants of two or more separate specificities or the antibodies are either bi- or multi-specific (70). The amount of "spurring" may vary considerably and is difficult to detect and analyze (16). "Spurring" occurred with all the culture filtrates and indicated that certain antigens probably had more than one determinant site.

The number of immunoprecipitates observed in the properly performed Ouchterlony tests represents the minimum number of antigen-antibody systems. The antigenic analyses of the culture filtrate antigens by immunoelectrophoresis and disc immunoelectrophoresis substantiated that the number of lines were not the maximum number of antigen-antibody systems. A greater number of antigens in three of the culture filtrates, 50, M. bovis, and M. avium was observed in immunoelectrophorograms than in the Ouchterlony slides. In the Ouchterlony technique, immunoprecipitates can be masked by other immunoprecipitates, particularly by antigens having similar diffusion coefficients. By electrophoresis, the antigens are first separated by their differences in electrical charge, thus when allowed to react with antisera they are less apt to be masked.

An example of the masking of immunoprecipitates by another was the reaction of M. avium culture filtrate with the homologous antiserum. In the Ouchterlony technique, only seven immunoprecipitates were observed. The lines were

close together and the one centrally located was broad and diffuse. Electrophoresis of the M. avium antigens effectively separated them and the number of immunoprecipitate lines was increased to 15. The same was true for antigens of M. bovis. Masking was not as apparent for culture filtrates 68 and 50. The culture filtrates 68 and 50 had approximately the same number of antigens when assayed by either Ouchterlony immunodiffusion or immunoelectrophoresis.

The disc immunoelectrophoretic technique proved to be the best for enumerating the culture filtrate antigens. Culture filtrates 68 and 50 had the greatest increase in the number of antigens detected. The number of antigens in the M. bovis and M. avium culture filtrates was only one more than that detected by immunoelectrophoresis. The increase in the number of antigens detected, especially in the culture filtrates 68 and 50, may have been due to the greatest effective protein concentration. In the disc gels the antigens migrate as narrow bands rather than broad diffuse ones as in immunoelectrophoresis and therefore are more concentrated.

In disc immunoelectrophorograms, the lateral displacement of the immunoprecipitates away from the edge of the disc gels into the agar indicated that antigens of lower molecular weight were found progressively more anodic. The approximate location of the antigen can be determined.

Because the lower molecular weight antigens diffuse out of the gels more rapidly than the larger molecular weight ones, a longer incubation period was required to develop the immunoprecipitates formed by the large molecular weight antigens. During this incubation, the low molecular weight antigens formed immunoprecipitates which subsequently became diffuse and faint.

There are few guide lines in the analyses of disc immunophorograms because it is a relatively new technique. The few reports in the literature do not discuss the interpretation of the results from either a practical or theoretical standpoint (38, 101).

The antigenic complexity of the culture filtrates is greater than that detected by the Ouchterlony technique as evidenced by the results obtained with the two techniques of electrophoretic separation. More importantly, disc electrophoresis affords better separation than the other methods which have been reported. The reports on the separation of mycobacterial antigens by gel filtration (13) and DEAE cellulose chromatography (43, 51, 85) have not been encouraging. The separation of the antigens was not complete and no one antigen was effectively isolated. Present attempts to separate the components of the culture filtrates with DEAE cellulose and the various grades of Sephadex were also unsatisfactory. Because of this and the successful delineation by analytical disc electrophoresis of the components

found in culture filtrates, the disc electrophoretic technique was modified to accommodate larger volumes, which is referred to as the preparative disc electrophoresis.

Separation of culture filtrate components by preparative disc electrophoresis resolves the components as well or better than analytical disc electrophoresis. It is consistent and reproducible which is important for the successful sectioning and removing of the unstained bands. To locate the position of the unstained bands in the gel, a schematic drawing of the amido black stained gels was made to scale for each culture filtrate. The accuracy of the cutting procedure depended upon the proper performance of the electrophoresis and preparing the schematic drawing appropriately. If too much current is applied to electrophorese the sample, excessive heat occurs. Due to the large diameter of the tubes used in the preparative technique the heat cannot be rapidly dissipated. As a consequence of this heating the lower gel has a tendency to swell. When the gel is aligned with the schematic drawing to be used as a sectioning guide the position of the components do not correspond with the drawing and the sectioning will be inaccurate.

There is some swelling of the stained gels when they are stored and preserved in acetic acid. A correction in schematic drawing must be made when these are to serve as sectioning templates.

The preparative disc electrophoretic technique is preparative in the sense that ten fold more protein can be electrophoresed than in the analytical technique. Application of more than 2.1 mg of protein contained in 0.4 to 1.5 ml of sample to the gels causes overloading of the gel, heating, distortion and lack of resolution. If performed properly, preparative disc electrophoresis provides an excellent method for separating components, sectioning gels and eluting the components. The major drawback is that large quantities of proteins cannot be fractionated on a single column. However, in order to employ larger quantities of protein, eight gel columns were used at one time for each culture filtrate.

Antigens were eluted in a minimal amount of buffer to avoid the need of subsequent concentration. The protein concentration of the eluates could not be measured accurately unless they were dialyzed to remove the TEMED used in preparing the gels which reacted in the Lowry procedure. The protein recoveries ranged from 50 to 60%. A progressively greater per cent of protein was recovered for antigens more anodic in the gels. This was undoubtedly due to the fact that the more anodic an antigen, the lower the molecular weight and the more readily eluted.

Re-electrophoresis of the eluates by analytical disc electrophoresis was a rapid and accurate means of determining the purity of the antigens in the eluates as well as the accuracy of the sectioning process (Fig. 12). As little as

16 micrograms of protein could be detected indicating the great sensitivity of the technique. Fourteen of the eluates contained only one amido black-staining component. Six eluates had at least two components.

Electrophoresing in different concentrations of gel can indicate the relative purity of the antigens as well as provide an additional method of separation. Comparison of the Rf value of a component in the eluates with the Rf value of the same component in the original reference gel can also confirm the accuracy of the sectioning technique.

The antigenicity of the components in the 20 eluates was determined by reacting the eluates with the four reference antisera. Nine of the eluates contained only one antigen. The remaining eluates contained one or more antigens. The results were consistent with those obtained from the Ouchterlony immunodiffusion analyses of the antigenic character of the culture filtrates. Antigens were isolated which occurred only within a group or within a species.

A discrete band in a disc gel does not establish that this band is composed of only one antigen. For example, with eluates 50-18 and 68-18 there were only single bands in the gels. Each contained three antigens. The same three antigens were found in the culture filtrates 68 and 50 by reactions with homologous antiserum. Therefore, these antigens are not the result of fragmentation during electrophoresis. The fact that disc electrophoresis probably does not

cause fragmentation is a particularly important aspect of the technique since the other separatory techniques have been questioned as giving rise to many smaller molecules as a result of the technique itself. These antigens probably have a large negative electrical charge and a small molecular weight which does not restrict their movement in the gels to any great extent. They migrated rapidly in the gel and the molecular sieving in the gel was less effective than for larger molecules. Increasing the total per cent of polyacrylamide decreases the mean pore size of the lower gel and therefore, the probability of separating low molecular weight components can be increased by increasing the concentration of the gel. When the concentration of the gel was increased to 10%, the single amido black-staining band from a 7% gel was separated into 6 bands (Fig. 13). Obviously, the separation obtained with polyacrylamide gels must be evaluated for each band. It can be expected that eluates from some of the amido black-staining components may contain more than one antigen. Preparative electrophoresis in increasingly higher percentage of acrylamide would afford more complete separation.

The eluates were analyzed for their specificity with the 11 cell-culture filtrate antisera for atypical and classical mycobacteria. These antisera were produced in rabbits which received culture filtrate and homologous, beta-propriolactone inactivated cells. The inoculation schedule was

such that the antibodies elicited were probably not as specific as those of the four reference antisera.

The results obtained indicated that several antigens had marked degrees of specificity. The antigen in eluate 310-24 reacted only with the M. bovis and BCG cell-culture filtrate antisera. Antigens in eluates 68-18 and 50-18 were group specific. The antigens reacted with at least three of the different Group III cell-culture filtrate antisera but none of the other antisera. No specific antigen was detected in the M. avium eluates. Preparation of the antisera to the specific antigens would provide a means for screening large numbers of cultures. The cultures could be classified on the presence or absence of the antigen. Data such as these must be tempered with the knowledge that the quality and quantity of mycobacterial proteins can vary markedly in different batches of culture filtrate (53). This will, of course, affect the specificity of the antibodies elicited. It is important that standardized procedures be developed and employed in all laboratories studying mycobacteria. The resulting data and products would be comparable.

The results obtained are encouraging for the isolation of specific antigens by disc electrophoresis. Antigen eluted from 7% preparative gels can be further purified by using varying percentages of polyacrylamide gels. It may be possible to use the preparative disc technique to obtain

large quantities of antigen by increasing the gel dimensions or by continued electrophoresis and eluting off the end of the gel into a fraction collector. These pure antigens could be used for either sero-diagnostic tests or specific sensitins. The specificity of the antigens can be tested by preparing reference antisera to each. Mycobacterial culture filtrates or cell extracts from classical, atypical and saprophytic organisms could be tested with the reference antisera to determine the presence or absence of the antigens. Data from this type of study may afford some insight into a means of serologic classification of mycobacteria.

Such antigens can be used for identification of the mycobacteria isolated from man and animal for which the need is great. They may be used as antigen in sero-diagnostic tests which also are needed. Perhaps most importantly, an antigen or a hapten of the same specificity can be used for skin tests. The urgency of such a preparation is apparent from the following.

> The purpose of giving a skin test is to determine whether an individual--man or animal--has or has not been infected by a specific microorganism. An ideal skin test, then, would be one that would make no mistakes; it would never indicate that an individual had not been infected if he had, or that he had been infected if he had not. Moreover, the ideal test would never confuse infection by one organism with infection by another (23).

> None of the skin tests now in use meets these requirements, and from our own work on mycobacterial infections we know that an important cause is that almost all human populations are mixtures--that is, they contain some persons that have been infected with one type of mycobacterium, some of who have been infected with another type, some who have been infected with more than one type, and some who have never been infected at all. Moreover, the identity of many of the infecting strains is unknown (23).

The following quotation is from the same report by Edwards, Hopwood and Palmer (23) who are unchallenged in their records of tuberculin testing of human populations and the statistical analyses of the results:

> Sooner or later it seems likely that a way will be found to make more specific mycobacteria antigen than are now available. While preliminary estimates of the specificity of these products may be obtained for testing experimentally infected animals in the laboratory, their ultimate usefulness will have to be assessed in the human populations in which they are to be used. The situations illustrated in this report, taken from studies in the laboratory, where the infecting organisms are known, will have to be faced in the evaluation of new products in human population, where the infecting organisms are not known. At that time, it will be essential to know which of possibly many mycobacterial infections are important in sensitizing human beings and where we would expect to find them, and also to have some preliminary notions of their prevalences. We venture to suggest that work along the lines indicated in this paper--in other words, a combined laboratory and field research program--should furnish just that kind of information.

The quotation emphasizes the need for antigens in the laboratory and in field testing programs. This is as true in the field of animal tuberculosis as it is in the

field of human tuberculosis, which are ultimately inseparable. Such an antigen with the methods of preparation are presented in this report. It is hoped that this research will lead to the eradication of tuberculosis of man and animal.

SUMMARY

Four unheated, concentrated, mycobacterial culture filtrates M. bovis, M. avium and two Group III atypical strains of bovine origin, 68 and 50, were studied. Disc electrophoresis was the best method for separating the culture filtrate components. Between 18 and 24 amido black-staining components were in the culture filtrates and five to eight PAS staining components. The technique is facile, economical and highly reproducible. Only 250 to 280 ug of protein in 0.2 ml or less of sample is required for optimal results.

Reference antisera were produced against the four culture filtrates. The antigenic character and relatedness of the four culture filtrates were determined with gel diffusion techniques. Ouchterlony immunodiffusion analyses revealed the presence of from six to ten antigens in the culture filtrates when reacted with homologous reference antisera. Comparative Ouchterlony immunodiffusion analyses established that the two Group III atypical strains, 68 and 50, were closely related but at least one antigen was not in common identity with each other or M. bovis or M. avium. There also existed specific antigens for M. avium and M. bovis. The Group III atypical strains were more closely

related antigenically to M. avium than to M. bovis. There was no common antigen in the four culture filtrates nor was there any antigenic relatedness between M. avium and M. bovis.

A greater number of antigens was detectable in the culture filtrates by immunoelectrophoresis and disc immunoelectrophoresis than by the Ouchterlony immunodiffusion technique. Disc immunoelectrophoresis separated more antigens than immunoelectrophoresis. With disc immunoelectrophoresis 16, 13, 12 and 9 antigens were found in M. avium, 50, 68 and M. bovis culture filtrates, respectively, when reacted with their homologous reference antisera.

A preparative disc electrophoretic technique was developed by increasing the length and diameter of the gel column. From 2.1 to 2.6 mg of protein could be electrophoresed on each gel approximately ten fold more than on the smaller gels used in analytical disc electrophoresis. The resolution of the components was as good or better than in analytical disc electrophoresis. Components were sectioned from the gels following a schematic drawing made from stained gels. The gel segments were placed in buffer and the component(s) eluted. The eluates were dialyzed to remove TEMED, a chemical in the gels, which reacts in the Lowry method for determining protein. Re-electrophoresis in analytical disc electrophoresis and comparative Ouchterlony immunodiffusion

analyses confirmed the accuracy of the technique in separating the bands. Some of the eluates contained only one antigen, others contained more than one antigen. Re-electrophoresis in 10% analytical disc gels established that one amido black-staining band in 7% gel could be separated into as many as six components.

The eluates were examined with 11 cell-culture filtrate antisera collected from rabbits inoculated with homologous beta-propriolactone inactivated cells and culture filtrates of M. avium, M. bovis, BCG, Group I, II, and III atypical mycobacteria. The results indicated that specific antigens had been isolated from the preparative disc gels. The preparative disc electrophoretic technique can be used for the successful isolation of mycobacterial antigens and sensitins. By further purification and characterization, the differential specificity of these mycobacterial products can be established. There is a need for these products in sero-diagnostic tests and skin tests.

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