ABSTRACT

THE PRECIPITINOGENIC RELATIONSHIPS AMONG MYCOBACTERIA OF BOVINE, PORCINE, HUMAN, AND SOIL ORIGINS

by Calvin Armstrong Davenport

The precipitinogenic relationships of 49 representative mycobacteria of human, bovine, porcine, and soil origins were compared by use of an agar slide immunodiffusion technique. The organisms studied included the classical pathogens and saprophytes, and "atypicals" of human, animal, and soil origins.

Antigenic differences exist among species and groups, and in addition, among strains within a group. No one precipitinogen was found to be common to all mycobacterial strains.

From two to six precipitinogens were detected in homologous antigen-antibody systems, and in all tests, the homologous systems reacted to give equal or greater numbers of precipitate bands than the heterologous systems.

The Group III strains of bovine and porcine origins were closely related but not identical to M. avium.

A new grouping of "atypicals," based on antigenic relationships to known mycobacterial species, is presented.

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INTRODUCTION

Many unsuccessful approaches have been made to the problem of differentiating mycobacterial species. Primarily, the mycobacteria have been classified according to their morphologic characteristics and virulence for experimental animals; such classification systems have numerous shortcomings. The many strains and variants of acid-fast bacilli constantly being isolated from various sources and the consequent difficulties in attempting to group them emphasize the need for new criteria for their identification.

Recent advanaces in the interpretation of the immunodiffusion tests offer an excellent means of studying the precipitinogenic relationships of acid-fast bacilli. Sufficient information on these relationships may provide the foundation for a reliable system of identification and classification of these organisms. Moreover, some adaptation of gel diffusion tests may eventually serve as an aid for detecting mycobacteriosis in infected individuals.

In this thesis are presented observed precipitinogenic similarities and dissimilarities of acid-fast bacilli isolated from man, cattle, swine, and soil, as interpreted from the results of slide immunodiffusion tests.

LITERATURE REVIEW

The Precipitin Reaction

Kraus (1897) observed that a precipitate formed when the sera of animals immunized with typhoid or cholera bacilli were mixed with filtrates of broth cultures of the homologous organism. This marked the discovery of the precipitin reaction and led to the further observation that most proteins and many polysaccharides may elicit antibody responses when injected into suitable animals.

Under proper physico-chemical conditions, antigen and antibody react to form a precipitate in two distinct stages (Crowle, 1961). The first stage is a rapid and invisible combination of antigen and antibody molecules to form a specific but readily reversible complex. The second or aggregation stage develops slowly and marks the initial formation of the visible precipitate. The antigen-antibody complexes, or lattices of these complexes, become too large to remain either invisible or soluble (Crowle, 1961).

The first stage of antigen-antibody complexing results from an attraction of the two reactants due to one or several of five types of forces (Crowle, 1961): (1) attraction by van der Waals forces, (2) dipole-dipole attraction,

(3) dipole-ion attraction, (4) positive-negative chemical group attraction, and (5) hydrogen bonding. Visible precipitates result from combinations of certain groups on a given reacting molecule. If the molecule is disrupted into relatively complex fractions, each possessing two or more reactive sites, visible precipitation may still occur. If the molecule is disrupted into simple fragments possessing no more than one reactive site, combination may be possible but visible aggregation will not occur. Fragments will not be able to form the lattices necessary for developing visible precipitates.

Animals may produce "incomplete" or "blocking" antibodies, which, like the simple fractions, combine with antigens but fail to cause aggregation (Crowle, 1961). Combinations of reactant sites without aggregation, termed "incomplete precipitation" (Crowle, 1961), may interfere with specific precipitation by deviating one of the reactants in a mutual reaction area (Oudin, 1948).

Recent studies (Ouchterlony, 1958) have suggested that in most precipitation tests both antibody and antigen are impure. Untreated antigenic material such as a culture filtrate is usually a mixture of molecules carrying various groups. After injection into an animal, each determinant

group may induce the formation of corresponding antibodies.

If a culture filtrate is fractionated, the fractions obtained may be chemically pure but not neccessarily immunologically pure. If the individual particles of a specific fraction carried only one kind of reactive site, the fraction would be immunologically pure. But since each particle is a molecule of considerable size and complicated structure, other assumptions must be made: certain particles may carry several serologically related groups, some particles may carry several serologically non-related groups, and other particles may carry both related and non-related determinant groups. Furthermore, these groups may be in a hidden, partially hidden, or an exposed position on the particle. During immunization, antibodies may be produced that correspond to each type of originally exposed determinant, or to one which became exposed during the immunization. The degree of antibody response will depend on the predominance, exposure, and antigenicity of the determinant groups (Ouchterlony, 1958).

Studies (Ouchterlony, 1958; Oudin, 1952) of precipitation systems have established this classification: (1) simple, in which a single antigen reacts with an antibody; (2) complex, in which several antigens cross-react with a

given antibody; and (3) multiple, in which several antigens and several antibodies react simultaneously.

The Influence of Physico-chemical Factors on Precipitation

Antigen-antibody combinations can occur in the absence of electrolytes, but the rate and intensity of precipitation is enhanced by their presence (Aladjem and Lieberman, 1952). The optimal range of electrolyte concentrations for precipitation tests varies and should be determined for each antigen-antibody system studied. Studies have shown that in liquid media, horse antitoxin and diptheria toxin (Boyd, 1956), precipitate best at a NaCl concentration of between 0.05 and 0.25 M. Chicken precipitins require 1.5 M NaCl for optimal precipitation in liquid media (Goodman et al., 1957). Yet, the same chicken sera precipitate the same antigens much better at 0.85 M NaCl in gel media whether these are conducted in agar, gelatin, or cellulose acetate (Crowle, 1961). Minute quantities of the salts of cadmium and lanthanum have been found to enhance or inhibit specific precipitation (Crowle, 1958c).

The optimal temperature for antigen-antibody combination varies from system to system. Boyd (1956) reported that incubation temperatures of 15 C to 40 C permitted optimal

precipitation for most antisera obtained from warm-blooded animals, and that 10 C was best for antibodies produced in the frog. Rheins et al. (1956) observed that certain specific precipitates of antigens and antibodies of rabbit origin that formed in a gel medium at 4 C disappeared on warming to room temperature. Precipitation tests in semi-solid media must be conducted at a constant incubation temperature to prevent formation of artifacts resembling precipitate bands (Oudin, 1946, 1948; Crowle, 1961).

Studies by Grabar (1959) suggest that the ideal pH for a particular precipitation system is within the range of 6.5 to 8.2. At a pH lower than 6.5 nonspecific precipitation of serum proteins often occurred, and at a pH greater than 8.2 specific precipitation was hindered and certain antigen-antibody complexes tended to dissociate.

Other factors affecting specific precipitation are the presence of lipids in the reactants, the presence of proteins foreign to the desired reaction, and the relative proportions of antigens and antibodies (Crowle, 1961). Maximum precipitation in minimum time results when both reactants are used in optimal proportions.

It was reported (Oudin, 1948) and confirmed (Becker and Munoz, 1949; Munoz and Becker, 1950) that there is a

straight-line relationship between the movement of a precipitate band and the square root of the time. This movement is also influenced by the size, shape, and molecular weight of the particles (Crowle, 1961); the initial concentrations of reactants (Munoz and Becker, 1950; Oudin, 1948; Ouchterlony, 1958); and the gravitational field (Crowle, 1961).

Immunodiffusion

Immunodiffusion, a term which replaced the phrase "agar diffusion precipitin test," refers to serologic tests conducted in or on semi-solid media (Crowle, 1961). The first use of an immunodiffusion technique was made by Bechhold (1905). Anti-goat serum of rabbit origin was mixed with 1% gelatin in test tubes. After the mixture was gelled, goat serum was poured onto the gel. Precipitates were observed that differed from the types that appear from inorganic substances. Two heavy, distinct precipitates were described but the possibility that these could have been caused by separate antigen-antibody systems was not mentioned.

Oudin (1946) made the interpretations that established immunodiffusion in its present form as an analytic technique for mixed antigen-antibody systems. Employing a method of

simple diffusion, he placed an antigen solution over a solid immune serum-agar mixture that was contained in a thin-bore tube. An antigen-antibody precipitate band formed and appeared to migrate down the tube as more antigen diffused into the antibody-agar mixture. At equivalent concentrations of reactants, one band formed when a single antigen-antibody system was present, and multiple bands formed when more than one antigen-antibody system was present.

Oudin's studies (1948, 1952) led to these observations: (1) positive correlation exists between precipitate intensity and antibody concentration; (2) sudden changes in incubation temperature may cause the appearance of artifacts which usually have characteristics that differentiate them from primary precipitates; and (3) cross-reacting antigen can influence the formation of precipitate by the homologous antigen.

The Oudin tube technique has been applied to the study of many antigen-antibody systems. Becker and Munoz (1949) used this method to study the multiplicity of antigens in ragweed pollen extracts, and Telfer (1953) used it to study the antigenic differences existing between the larval, pupal, and adult forms of the Cecropia moth.

Shortly after the application of simple or single-diffusion to antigenic studies, techniques of double-diffusion in two dimensions were developed independently by Ouchterlony (1948) and Elek (1948). Ouchterlony, studying the toxin-producing ability of various strains of Corynebacterium diphtheriae poured 1% agar prepared with saline into petri dishes and allowed it to harden. Wells were cut in the agar and filled with antigen and antibody. Both antigen and antibody diffused into a common reactant area to form precipitate bands. The gel technique of Elek (1948) has been called the "Double-diffusion Gradient Test." It differs from Ouchterlony's method in that no wells were cut in the agar. Two filter paper strips impregnated with the reactants were placed on the agar at right angles to each other to form an L. Chen and Meyer (1955) utilized this technique for studying antigens of Pasteurella pestis and Pasteurella paratuberculosis. No antigen specific for P. pestis was detected, but two antigen-antibody bands were common to P. pestis and P. paratuberculosis, indicating that two identical or closely related antigens were present in both species. Modifications have increased the usefulness of immunodiffusion techniques. The inhibition plate technique (Bjorklund, 1952) made use of the principle that pretreating the diffusion medium with

a sufficient amount of a component from a complex immunologic system completely inhibited the subsequent appearance in the medium of precipitates corresponding to this particular component. Furthermore, the pretreatment of the agar did not interfere with the diffusion and precipitation of other components in the system.

In experiments designed to compensate for the large volumes of reactants required by Ouchterlony's double-diffusion plate technique, Preer (1956) developed micro-gel diffusion methods. The use of modified micro-immunodiffusion methods by Mansi (1958) and Crowle (1958a, 1958b, 1960) have proved that micro-techniques are of greater sensitivity than the agar plate method.

Immunoelectrophoresis, a combination of agar-diffusion with electrophoresis, has increased resolution of precipitate bands formed by complex antigen-antibody systems in agar media (Crowle, 1961; Grabar, 1959; Grabar and Williams, 1955). Techniques for drying and staining improved the definition of bands in photographs made for permanent records (Crowle, 1961).

Immunodiffusion tests are useful in resolving multiple precipitating systems into their individual components, and in comparing two antisera against the same antigen or two

antigens against the same antiserum. The resolution of multiple precipitating systems into individual components is based on the assumption that specific precipitates in semisolid media permit the diffusion of unrelated antigens and antibodies. The number of bands developing in a multiple precipitating system represents the minimum number of antigen-antibody systems present, not the maximum number. A band formed by one system may mask other bands (Becker, 1953; Ouchterlony, 1958; Oudin, 1948). When two identical antigens are compared by immunodiffusion tests on a single plane, the precipitation lines developing from individual components fuse to form a continuous band called the "Reaction of Identity." Diffusion of two serologically related antigens against an antiserum containing specific antibodies results in a precipitation pattern consisting of a continuous arc with a spur extending above the precipitation line and is termed "Reaction of Partial Identity." The comparison of two serologically unrelated antigens with an antiserum containing homologous antibodies results in the formation of precipitation lines that cross each other. This pattern is termed "Reaction of Non-Identity" (Ouchterlony, 1958).

Studies of Mycobacteria

The Need for Identification and Classification. Fourteen species of the genus Mycobacterium are listed in the Seventh Edition of Bergey's Manual for Determinative Bacteriology (Breed et al., 1957): M. phlei, M. smegmatis, M. fortuitum, M. marinum, M. thamnopheos, M. platypoecilus, M. ulcerans, M. tuberculosis, M. bovis, M. microti, M. avium, M. paratuberculosis, M. leprae, and M. lepraemurium. These species are differentiated primarily on their virulence for laboratory animals and their ability to grow on non-living media at various temperatures.

The existence of other strains and variants of the genus Mycobacterium has been established (Mallmann, Mallmann, and Robinson, 1961; Parlett and Youmans, 1956, 1958; Runyon, 1960; Xalabarder, 1961), yet the significance of these atypical organisms remains controversial. Some "atypicals" are pathogens; others may be mere saprophytes.

Studies by Runyon (1960) of "atypicals" of human origin led to a classification system based primarily on the rate of growth and the presence or absence of pigmentation when the organisms were grown in the light or dark. Runyon's group designations and characterizations are: Photochromogens

(Group I) develop a yellow pigment after a short exposure to light; Scotochromogens (Group II) develop a yellow or orange pigment when grown in the dark which deepens when grown in the light; Non-photochromogens (Group III) and Rapid-growers (Group IV) do not produce pigment under any growth conditions, but are differentiated by variations in their growth rates. Organisms in Groups I, II, and III require one to two weeks for visible growth and organisms in Group IV need only two to four days.

Runyon (1960) reported that the tendency of the "atypicals" to form strands or cords was proportional to the roughness of the cultures. All Group I strains and Group IV rough strains formed cords, but strains of Groups II and III rarely exhibited cording.

Runyon's virulence studies (1960) indicated that "atypicals" of human origin do not produce progressive disease in guinea pigs, rabbits, or birds. In mice, strains of Group I and some strains of Groups III and IV produced lesions, and sometimes death.

On the basis of the frequency with which an organism was isolated from a patient, from resected lung tissue and from sputum, and the absence of other disease agents, Runyon (1960) concluded: Group I strains, with few exceptions, are

agents of disease in man; Group II strains are generally non-pathogenic in man; and strains of Groups III and IV are sometimes pathogenic for man.

Similar problems prevail in the field of animal tuberculosis (Johnson, Balsden, and Frank, 1961; Mallmann, Mallmann, and Robinson, 1961; Ranney, 1961). Mallmann et al. (1961) reported the isolation of large numbers of acid-fast bacilli from bovine and porcine tissues that differ from the classical pathogens. Studies of these "atypicals" from the standpoint of their growth and cytochemical characteristics, and their ability to sensitize and infect animals, substantiated earlier observations that one set of characteristics is insufficient for the identification of any one mycobacterium. Furthermore, compilations of the characteristics did not always establish conclusive identification. The use of the Runyon classification system was not adequate for differentiating "atypicals" from animals. Mallmann et al. (1961) isolated a group of organisms that was intermediate between Group I and Group II in its pigment response to light exposure. Other tests indicated that this group, designated as pseudochromes, was most closely related to Runyon Group III, and may be more accurately designated as pigmented Group III. "Atypicals" of bovines and porcines were heterogeneous,

highly variable, and adaptable. Increased virulence of Group III organisms by animal passage suggested that some possess a markedly higher virulence potential than has been reported for the "atypicals" from human beings. Tuberculosis, or tuberculosis-like disease, in domestic animals may be caused by a variety of acid-fast organisms.

The problems of grouping mycobacteria on the basis of morphologic characteristics and virulence for laboratory animals are well known (Negré 1947; Chapman, 1960; Guy and Bernard 1960; Runyon, 1960; Xalabarder, 1961). Strains may increase in virulence by animal passage or lose virulence by continued growth on artificial media. Cultures of the same strain may vary somewhat in their growth and pigmentation characteristics, depending on such an insignificant factor as the size of the inoculum (Mallmann et al., 1961).

Recognizing the shortcomings of classification by morphologic characteristics and virulence tests only, investigators have employed many serologic methods for the study of mycobacteria.

A variety of crude antigens, such as cell extracts, whole cells (heat-killed or viable), and Old Tuberculin have been employed to stimulate antibody production in suitable animals. The antisera obtained have been allowed to react with crude

antigens properly prepared for precipitation (Meynell, 1954; Schaefer, 1940, 1947), agglutination (Bando, 1962; Furth, 1926), complement-fixation (Vardeman and Larsen, 1961; Toda, 1956), hemagglutination (Boyden, 1951; Middlebrook and Dubos, 1948) and hemolytic tests (Buehler and Rheins, 1959). None afforded a method for the systematic classification of the mycobacteria.

Other workers (Coghill, 1931; Creighton and Anderson, 1944; Creighton, Chang, and Anderson, 1944; Heidelberger and Menzel 1934, 1937; Pepys, Augustin, and Paterson, 1959; Seibert, 1949, 1958; Seibert, Crumb, and Seibert, 1950; Seibert and Soto-Figueroa, 1957) approached the problem by attempting to chemically isolate specific antigens from mycobacteria. Two polysaccharide antigens and three protein antigens have been isolated from the mycobacterial cell, but no one has been successful in isolating a series of antigens from which a serological classification could be established. This may be due, in part, to the difficulties encountered in the isolation and purification of antigenic stubstances, to the low antibody response in animals inoculated with mycobacterial antigens (Parlett and Youmans, 1956), and to the adaptability and mutability of the tubercle bacillus (Darzins, 1958).

Applications of Immunodiffusion to the Study of Mycobacteria. Successful analyses of complex antigenic mixtures by immunodiffusion led to its application to the studies of acid-fast bacilli. Parlett and Youmans (1956) studied the antigenic relationships among 42 strains of mycobacteria by means of the Oudin tube test and a modified Ouchterlony plate method. Employing unheated culture filtrate antigens, and antibodies elicted following the subcutaneous inoculation of rabbits, they found that the modified Ouchterlony method was superior to the Oudin test for determining antigenic patterns. The presence or absence of cross-reacting antigens and antibodies permitted a division of the various strains of mycobacteria into four antigenic groups.

Group I consisted of known virulent and avirulent M. tuberculosis, atypical acid-fast organisms from human beings, an attenuated strain of M. bovis, and a strain of M. avium. Four different precipitating antigens were common to all strains placed in this group.

Group II was composed of several strains of M. bovis and one strain of M. avium. The filtrates of the group contained two precipitating antigens, both of which were identical to two of the four antigens found in organisms of Group I.

Group III consisted mainly of saprophytes. Filtrates of these strains contained four antigens which were identical for the strains within the group, but unrelated to the antigens of Groups I, II, and IV.

Group IV consisted of a single strain of an atypical, chromogenic acid-fast organism originally isolated from man. The filtrate of this strain contained two precipitating antigens unrelated to those produced by organisms in Groups I, II, and III.

A common antigen among all strains of mycobacteria was not detected. The classical pathogens, M. tuberculosis, M. avium, were closely related, and none of the antigens of the saprophytic strains cross-reacted with the antibodies specific for the human, bovine, or avian species.

In a study of mycobacterial antigens, Rheins, Burrell, and Birkeland (1956) employed two procedures for eliciting antibodies in rabbits: (1) a single intravenous injection of whole cells of mycobacteria was made with M. bovis (Ravenel), M. tuberculosis (H37Rv), M. phlei, and the Bacillus of Calumette and Guerin (BCG), and the blood collected at intervals; (2) three injections of 2.0 mg of BCG were made at three-day intervals, and the blood collected one week following the last injection. Agar plate diffusion

tests were conducted with the antisera, with Old Tuberculin as the source of antigens. The antisera collected following either procedure for eliciting antibodies contained a low titer of a single antibody.

Studies of Parlett and Youmans (1956) suggest that the intravenous injection of whole mycobacterial cells elicited antibodies in rabbits which was markedly less effective for detecting antigen by gel-diffusion methods than the antiserum obtained when cell products or culture filtrates were incorporated in a water-in-oil emulsion, and several subcutaneous inoculations in rabbits made. Furthermore, Old Tuberculin and P P D (Purified Protein Derivatives) were poor indicator antigens in gel-diffusion tests (Parlett and Youmans, 1958).

Seibert and Soto-Figueroa (1957) utilized a tube double-diffusion technique to study the reactions of tuberculoproteins and polysaccharides with antibodies (rabbit origin) elicited by BCG. Proteins were extracted from unheated culture filtrates, and polysaccharides from heated culture filtrates. Different protein extracts formed two or more bands with the anti-BCG serum, which indicated that the extracts consisted of mixtures of antigens. The polysaccharide extract produced two distinct bands with the anti-BCG serum.

Immunoelectrophoretic studies (Burtin, 1959; Burtin and Kourilsky, 1959) revealed that four different protein antigens and two polysaccharide antigens are present in M. tuberculosis.

Parlett and Youmans (1958) studied the antigenic relationships among 98 strains of mycobacteria and 4 fungi by the agar-plate diffusion method. The organisms studied, predominantly from human beings were: 20 strains of M. tuberculosis; 54 atypical mycobacteria isolated from man; 4 M. bovis; 2 M. avium; 10 saprophytic mycobacteria; 7 miscellaneous mycobacteria, including M. ulcerans, M. balnei, M. marinum and M. fortuitum; and 4 species of fungi: Nocardia pelliteriae, Nocardia braziliensis, Penicillium notatum, and Candida albicans.

Antisera (rabbit origin) were prepared from 43 strains of mycobacteria and the 4 species of fungi. Subcutaneous injections of water-in-oil emulsions of concentrated culture filtrates were made weekly for six weeks and after a 10-14 day waiting period, blood was obtained by heart puncture. Viable mycobacterial cell suspensions served as a source of antigens in the immunodiffusion tests. The number of precipitins detectable for any one strain varied from 2 to 6, but no single antigen was common to all of the mycobacteria.

There were no cross-reactions between the 4 fungi and the mycobacteria. On the basis of the precipitation bands that were formed, the 98 strains of mycobacteria could be divided into 8 antigenic groups.

Parlett and Youmans (1959) conducted studies of the specificity and sensitivity of a gel-diffusion tube test to determine its usefulness in detecting antibodies in human sera specific for mycobacterial antigens. Sera were obtained from tuberculous and non-tuberculous hospitalized patients. Gel double-diffusion tube precipitation tests were performed with concentrated culture filtrates as sources of antigens. From 465 patients hospitalized for various diseases other than tuberculosis, ten specimens were positive. These ten were assumed not to be true biologic false positives, but were accounted for by technical factors, or undiagnosed or healed tuberculosis. Of the serum from patients with extrapulmonary tuberculosis, 43.9% were positive; of sera from persons classified as having inactive tuberculosis or a history of "cured" tuberculosis, 56.4% were positive; of sera from patients from whom only atypical acid-fast bacilli had been isolated 74.3% were positive; of sera from cases of moderately advanced active pulmonary tuberculosis 73.5% were positive; of sera from cases of far advanced active

pulmonary tuberculosis 84.2% were postive; and in nine cases of primary tuberculosis in infants, 10 to 120 months of age, all of the sera were positive.

The specificity of the gel-diffusion tube technique was demonstrated and also the need for a more sensitive diagnostic method. Sushida et al. (1961) utilizing the Ouchterlony plate method to determine antibodies in the sera of tuberculous persons, found that the plate technique also lacked the sensitivity required for a diagnostic test.

Parlett (1961) recognized the influence of serum antigen on precipitation tests conducted with serum from patients with chronic disease. He reported that when an antigen resides in the tissues, as happens in mycobacterial infections, varying amounts of antigen may enter the circulation. He assumed that there is an inverse relationship of serum antigen to serum antibody concentration; therefore, in chronic disease, serum would contain free antibodies only in the cured individual. At other times, it is probable that some antigen will be present in the serum, either free or as part of an antigen-antibody complex. In vitro tests for antibodies are performed by adding antigens to the serum. With serum which contains significant antigen, this addition would shift the antigen-antibody ratio further away from the

equivalence ratio and into an area in which antigen-inhibition may occur (Parlett, 1961). Parlett (1961) tested the sera of 373 tuberculous patients for the presence of serum antigens, and antibodies. Results showed that 40.8% were positive for both antigen and antibody; 3.7% were negative for both; 47.6% were positive for antibody only; and 7.7% were positive for antigen only. It may be necessary that diagnostic tests for the presence of serum antibodies elicited by mycobacteria will require various antigen concentrations.

The gel double-diffusion test is more promising from the standpoint of specificity and sensitivity than hemagglutination and comlement-fixation tests. Testing each serum with four concentrations of antigens, Parlett examined serum specimens from 1,379 non-tuberculous persons and from 1,452 persons in all stages of tubercular infection and receiving various therapeutic treatments. Of sera from the non-tuberculous persons 6.5% were positive; and from tuberculous patients 82.3% of the sera positive.

Kniker and LaBorde (1962) and Kniker and Heiner (1960) made antigenic analyses of 20 strains of mycobacteria representative of the major mycobacterial groups. A culture filtrate of each strain was placed on an ion-exchange column of DEAE - cellulose and followed with gradient elution.

From each filtrate 11 to 16 fractions were collected and the antigenic composition of each fraction was determined by a micro-immunodiffusion technique. In each culture filtrate 20 to 30 antigens were present. Antigens common to the 20 organisms were detected and moreover, 2 fractions were found that contained 1 to 5 antigens specific for each organism or its group.

The separation of different fractions of the culture filtrates by elution might have resulted in the differentiation of antigens (in immunodiffusion tests) that could have been undetectable without previous fractionation. There is also the possibility that fractionation resulted in the dissociation of large antigenic molecules into fragments. Such fragments may have possessed two or more functional groups, and would therefore be capable of combining with antibodies to form visible precipitates.

MATERIALS AND METHODS

Cultures

Fifty representative strains of mycobacteria were studied. These strains, with their hypersensitivity and infectivity characterizations,* are listed in Tables 1 and 2. The strains from which antisera were prepared are listed in Table 3.

The 5 atypical strains of human origin and the 8 known laboratory strains were obtained from the Tuberculosis Unit, Communicable Disease Center, Atlanta, Georgia. The remaining 37 strains were "atypicals" isolated from soil, cattle, and swine in 1960-1962 by members of the Bovine Tuberculosis Project, Michigan State University, East Lansing, Michigan.

*Studies were made by Mallmann and Robinson (1961, 1962). Infectivity studies were made by intradermally inoculating 0.1 ml of suspensions containing 0.1 mg wet wt of the organisms into guinea pigs. The development of a lesion of any size was considered evidence of virulence.

Tuberculin sensitivity tests were performed by two procedures: (1) 30 days post-inoculation of guinea pigs intramuscularly, quantities of 0.1 ml of avian O.T. diluted 1:20, 0.1 ml of mammalian O.T. diluted 1:10, and 0.1 ml of Edward's PPD-B were injected intradermally. Reactions were recorded after 48 hours. (2) Approximately 50 days post-inoculation, guinea pigs inoculated intradermally in the infectivity studies were tuberculin tested as in (1) above.

Table 1. Atypical strains from which culture filtrate antigens were prepared prepared

Group	Source	Strain	Hypersensitivity in guinea pigs	Infectivity for guinea pigs
I	man	R-P4	mammalian	virulent
		R-P8	mammalian	virulent
II	man	R-P15	nondefinitive	avirulent
	soil	X27-1	no sensitivity	avirulent
		X28-1	no sensitivity	avirulent
		X37-1	no sensitivity	avirulent
	cattle	368E-1	no sensitivity	avirulent
III	man	R-P39	avian	virulent
	cattle	50B-0	avian/battey	virulent
		62D-0	avian/battey	virulent
		B102E-0	avian	virulent
		107E-0	nondefinitive	virulent
		83F-0	nondefinitive	virulent
		94F-0	nondefinitive	virulent
		98F-0	avian	virulent
		B124F-0	nondefinitive	virulent
		B66F-1	no sensitivity	avirulent
	swine	$152A_1$-1	avian	virulent
		93C-0	avian	virulent
		151C-1	avian	virulent
		$172C_1$-1	mammalian	virulent
		186C-1	avian	virulent
		$193C_2$-1	mammalian	virulent
		198C-1	avian	virulent
		228C-1	mammalian	virulent
		242I-1	avian	virulent
		244I-1	avian	virulent
		350-1	avian	virulent
		352-1	avian	virulent

Table 1. --Continued

Group	Source	Strain	Hypersensitivity in guinea pigs	Infectivity for guinea pigs
IV	man	R-P380s	nondefinitive	virulent
	cattle	B117B-0	no sensitivity	avirulent
		B368D-1	no sensitivity	avirulent
		B292 E-1	no sensitivity	avirulent
		B28E-1	no sensitivity	avirulent
		M177F-0	no sensitivity	avirulent
Pseudo-chromes	swine	$152A_2$-1	avian	virulent
	cattle	63A-0	avian/battey	virulent
		58A-1	avian	virulent
		52H-1	avian	virulent
		65F-0	avian	virulent
		77F-1	non-definitive	virulent

Table 2. Known laboratory species from which culture filtrate antigens were prepared

Species	Hypersensitivity in guinea pigs	Infectivity for guinea pigs
M. bovis, Ravenel	mammalian	virulent
M. bovis, BCG	mammalian	virulent
M. tuberculosis, H37Ra	mammalian	virulent
M. tuberculosis, H37Rv	mammalian	virulent
M. avium	avian	virulent
M. fortuitum	no sensitivity	virulent
M. phlei	no sensitivity	avirulent
M. smegmatis	no sensitivity	avirulent

Table 3. Organisms used for the production of antibodies

Strain	Group	Hypersensitivity in guinea pigs	Infectivity for guinea pigs
M. bovis, Ravenel		mammalian	virulent
M. avium		avian	virulent
M. phlei		no sensitivity	avirulent
M. fortuitum		no sensitivity	virulent
R-P 8	I	mammalian	virulent
R-P15	II	nondefinitive	avirulent
R-P39	III	avian	virulent
107E-1	III	nondefinitive	virulent
$193C_2$-1	III	mammalian	virulent
R-P380s	IV	nondefinitive	virulent
58A-1	Pseudo-chrome	nondefinitive	virulent

Atypical strains are grouped according to the Runyon system of classification. In addition, the coding system of the tuberculosis research group of Michigan State University has been retained for culture designations.

The letter "R" preceding atypical strains of human origin refers to cultures originally identified by Dr. E. H. Runyon.

The letter "X" preceding a number indicates that the strain was isolated from soil.

Letters preceding numbers of cultures isolated from bovine and porcine tissues refer to the methods used for initial isolation: "B" refers to the pentane-enzyme method; "M" refers to the pentane method followed by a disinfectant treatment, and no letter preceding the culture number refers to a culture isolated by the sodium hydroxide method.

The letter designations following the numbers of atypicals of animal origin indicate the tissues from which the strains were isolated; A = cervical lymph nodes, B = thoracic lymph nodes, C = mesenteric lymph nodes, D = carcass lymph nodes, E = lung, F = skin, H = Peyer's patches, I = lesions.

The number following the letter refers to the year in which the culture was isolated; 0 = 1960, 1 = 1961, and 2 = 1962.

Tests[1] of the ability of ten strains of atypicals to infect calves showed that one strain, $152A_1$-1, was virulent. Strains 50B-0, 62D-0, 107E-0, 93C-0, $172C_1$-1, 186C-1, $193C_2$-1, B117B-0, and 52H-1 were avirulent for calves.

Antigens

Strains of mycobacteria used for antigen production were seeded into Dubos Broth Base (Difco) with 1% glucose to obtain actively growing cultures. These cultures were seeded into the medium of Wong and Weinzirl as modified by Fregnan, Smith, and Randall (1961). The latter medium was dispensed as follows: 2 ml and 5 ml into 30 ml screw-cap culture tubes, 10 ml in to 50 ml screw-cap culture tubes, 50 ml into 250 ml Erlenmeyer flasks, and 500 ml into 1 gal glass bottles.

Tubes containing 2 ml of modified Wong and Weinzirl's medium were seeded with 2 ml of Dubos Broth cultures. When abundant growth developed, the 4 ml culture was seeded into a tube containing 5 ml of modified Wong and Weinzirl's medium. After heavy growth appeared in this tube, the 9 ml culture

[1]Calves were injected with 1.0 to 10.0 mg wet wt of culture. Only those producing progressive disease as determined by histopathological studies were considered virulent.

was added to 10 ml of Wong-Weinzirl's medium. Similar stepwise increases in amounts of inoculum were employed until four bottles, each containing 500 ml of this medium were seeded with each strain.

The cultures were grown as pellicle cultures in the bottles for 8-20 weeks. Büchner funnels with Reeve Angel filter paper no. 202 were used to remove clumps of cells, and the filtrate was sterilized by Berkefeld filtration under vacuum. The filtrate was stored at 4 C and sterility tests were conducted by seeding duplicate 1 ml quantities of the filtrate into tubes of Brain Heart Infusion Broth and modified Wong and Weinzirl's medium. Tubes containing the latter medium were incubated at 37 C for 3 weeks, and the tubes with Brain Heart Infusion Broth were incubated at 37 C for 3 days. When necessary, the filtrate was refiltered through Berkefeld filters.

The sterile filtrates were concentrated about 10X at room temperature by the pervaporation method of Jennings (1953). Dialysis tubing with a flat width of 2.88 to 3.14 inches and a wall thickness of 0.0016 inches was sterilized by autoclaving at 121 C for 15 min. The filtrate was poured aseptically into the tubing and the filled sac was placed in front of an electric fan set at medium speed. When

concentrated, the filtrate was stored at 4 C and sterility tests were conducted as above.

Antisera

A reference antiserum, lyophilized M. tuberculosis (H37Ra) antiserum (rabbit origin) was obtained from Difco Laboratories, Detroit, Michigan. For use in precipitation tests, the lyophilized antiserum was reconstituted with physiological saline. Antibodies were elicited from culture filtrates of eleven strains of mycobacteria: M. avium, M. phlei, M. fortuitum, M. bovis (Ravenel), R-P8, R-P15, R-P39, R-P380s, 58A, 107E-1, and $193C_2$-1. Three Dutch Belted rabbits were used for each of the first nine strains listed, and two rabbits each for strains 107E-1 and $193C_2$-1.

The method employed for stimulating antibody production was essentially the same as that used by Parlett and Youmans (1956). Falba and paraffin oil (viscosity 125/135) were placed individually in screw-cap tubes and sterilized in the hot air oven at 180 F for 2 hrs. A water-in-oil emulsion was prepared by mixing with a syringe two parts of concentrated culture filtrate, two parts paraffin oil and one part melted Falba (Freund, 1951; Freund and Bonanto, 1944). At intervals during mixing, one drop of the mixture was

placed on water (at room temperature) to test its stability (Crowle, 1961). When the drop remained intact, mixing was discontinued and the emulsion stored at 4 C.

One week preceding inoculations, 15 ml of blood were drawn from each rabbit by cardiac puncture. Aliquots of each serum specimen were stored at 4 C and -20 C to be used subsequently as controls.

Subcutaneous inoculations of 0.75 ml of the water-in-oil emulsions were made into one site once weekly for six weeks. Ten days after the last injection, blood was collected, and aliquots of each serum speciment were stored at 4 C and -20 C.

Electrolytes Used for Preparing Diffusion Media

The electrolyte used to prepare the medium employed for all final studies was phosphate-saline, 0.15 M, pH 7.2. The formula for this buffer and those for others used in preliminary studies follow:

Phosphate-Saline, 0.15 M, pH 7.2

Monopotassium Phosphate Solution, 0.15 M	150 ml
Disodium Phosphate Solution, 0.15 M	350 ml
Sodium Chloride Solution, 0.15 M	500 ml

Barbital, ionicity 0.15,* pH 7.4

Sodium Barbital		6.98 g
Sodium Chloride		6.00 g
1 N Hydrochloric Acid		2.70 ml
Distilled Water	q.s.	1000.00 ml

TRIS, ionicity 0.15,* pH 7.4

2-Amino-2-(Hydroxymethyl)-1, 3-Propanediol		9.30 g
1 N Hydrochloric Acid		74.00 ml
Sodium Chloride		7.00 g
Distilled Water	q.s.	1000.00 ml

Ethylenediamine-Acetic Acid (ETDA), ionicity 0.15,* pH 7.4

Ethylenediamine		15.80 g
Glacial Acetic Acid		23.80 g
Distilled Water	q.s.	1000.00 ml

Phosphate, ionicity 0.15,* pH 7.4

Disodium Phosphate		12.80 g
Monosodium Phosphate Monohydrate		2.62 g
Distilled Water	q.s.	1000.00 ml

Phosphate, 0.01 M, pH 7.5

1 part of 0.01 M Monopotassium Phosphate

5 parts of 0.01 M Dipotassium Phosphate

Isotonic Sodium Chloride, ionicity 0.15,* pH variable

Sodium Chloride	8.80 g
Distilled Water	q.s. 1000.00 ml

Media for Diffusion Studies

Different diffusion media were used in preliminary tests conducted by the glass-tube, petri dish, and glass-slide methods. Purified agar, Difco certified, was the gelling agent and the various media were prepared in the following manner: To 100 ml of an electrolyte solution contained in a flask, 1 ml of 1-100 merthiolate and a weighed quantity of agar were added, and the flask placed in a flowing steam. When the agar had dissolved, the solution was filtered through Reeve Angel filter paper no. 202 and immediately used for the preparation of diffusion tubes or plates.

Gels for Preliminary Studies by the Tube and Petri Dish Methods. Gels containing 1% and 2% agar were prepared as above in 0.01 M phosphate buffer, pH 7.5 for studying antigen-antibody systems by the glass-tube and Petri dish methods.

*Ionicity values (based on electric conductivity tests) taken from p. 302 in Immunodiffusion by A. J. Crowle (1961).

Gels for Preliminary Studies by the Agar Slide Method. Preliminary studies to determine the best diffusion medium for the development of precipitation bands were conducted on agar slides by varying the agar concentration, pH, ionic strength, and the buffering system. Electrolytes used to prepare 1% and 1.5% agar media, pH 7.4, with an ionic strength of 0.15 were: Sodium chloride, barbital, tris (hydroxymethyl) aminomethane (TRIS), ethylenediamine-acetic acid (ETDA), phosphate, and phosphate-saline. Other media employed in studies by the slide method were: 0.7% agar gels prepared in 0.01 M phosphate buffers of pH 7.2 and 7.4, respectively; 0.7% agar gel prepared in 0.01 M phosphate-saline, pH 7.2; and 1% agar gel prepared in 0.15 M phosphate-saline, pH 7.2.

Immunodiffusion Test Procedures

In preliminary studies the glass tube, Petri dish and agar slide methods were employed. The latter method was used in the final studies of precipitinogenic relationships. Concentrated modified Wong and Weinzirl's medium and normal rabbit serum served as controls. The reactants were concentrated culture filtrates and rabbit antisera.

Tube Method. Soft-glass tubing with an inside diameter of 3 mm was cut into 10 cm lengths. The tubes were washed with Tide and put through one tap water rinse, five distilled water rinses, and a final rinse in 95% ethanol. Each tube was heat-sealed at one end and internally coated with a thin film of 0.1% agar prepared with ditilled water. The film was applied by inverting the tubes in a beaker containing sufficient agar to cover them, and autoclaving at 15 lbs pressure for about 15 min. When the temperature decreased to 75 C, excess agar was removed from the tubes by inverting and shaking them individually. The tubes were placed in plastic bags, dried overnight at 42 C, and stored at 4 C.

Before the tube immunodiffusion tests were performed, the other materials and culture filtrates were warmed to prevent premature solidification of the agar. Sterile Kahn tubes, culture filtrates and 2% agar medium were placed in a 50 C water bath. Graduated 0.2 ml pipettes and capillary pipettes were warmed in a 45 C incubator.

A 2:1 ratio of antigen to agar mixture was prepared in a Kahn tube and then added, by means of a capillary pipette, to a depth of 2.5 to 3.0 cm in the agar-coated tubes. When the antigen-agar layer had gelled, a 0.5 cm layer of 1% agar was added. When the second layer had solidified, undiluted

antiserum was added and the tube placed upright in a wooden rack.[1]

The tubes were covered and some were incubated at 37 C for 7 days, others at 25 C for 14 days. At 12-hr intervals, the reactions were observed by holding the tubes against a black background and passing a strong fluorescent light obliquely through them.[2]

Petri Dish Method. Glass Petri dishes were washed and rinsed as described above in the tube method. To form a 4 mm layer, 8 ml of diffusion medium were pipetted into each dish and allowed to solidify. The dishes were immediately placed in portable humidity chambers[3] at 4 C for 6-12 hr. Then, with paper templates as guides, circular reactant wells were cut with cork borers and the agar-discs removed with a 16-gauge needle. Wells employed were: 7 mm wells placed 8 mm apart, 6 mm wells placed 7 mm apart, and 4 mm wells

[1]Racks were made by drilling holes, 0.5 cm in diameter and 4.0 cm deep, in 2 x 4 x 8-inch softwood blocks.

[2]The light source was lamp model M209, made by the Dazor Mfg. Corp., St. Louis, Mo.

[3]Moistened gauze was placed in polyprolene pans with tightly fitting covers. The pans were made by the Dynalab Corp., 625 Goodman St., Rochester, N. Y.

placed 5 mm apart. The wells were sealed with 0.3% agar prepared with distilled water, and the dishes stored in humidity chambers for at least 1 hr before the reactants were added.

The wells were filled by means of tuberculin syringes. The dishes were incubated for 10 days in moist chambers at constant temperature of 28 C and 37 C. Reactions were observed daily by holding the dishes in front of the difffused blue-filtered light from a microscope illuminator.[1]

Agar Slide Method. Glass slides of two sizes, 3.25 x 4.0 in. and 2.75 x 2.75 in. were cleaned, rinsed and dried as in the previously described methods. They were then coated with 0.3% agar prepared with distilled water. All agar was removed from one side of each slide with a lintless cloth before drying at 37 C.

Each slide was placed with the coated-side up on a level surface, and covered with a uniform 2 mm layer formed by pipetting 10 ml of diffusion medium onto it. These agar blanks were stored in moist chambers at 4 C for 6-12 hr.

[1]Lamp Model 370, 100 watts, 115 volts, made by the American Optical Co., Brooklyn, N. Y.

Circular reactant wells were cut with cork borers; reactant trenches were cut with thin plastic rulers of the same lengths as the desired trenches. The most common size and arrangement of the reactant depots was one or more 86 mm x 3 mm antigen trenches extending the length of the 3.25 x 4.0 in slide, with circular antibody wells, 6 mm in diameter, placed parallel to and 7 mm from the trenches.

All reactant depots were sealed with 0.3% agar prepared with distilled water. The reactants were added by means of tuberculin syringes and the slides incubated in moist chambers at 28 C for 5 days. Observations were made at 8-hr intervals by holding the slides so that diffused blue light from a microscope illuminator[1] passed obliquely through the medium.

The Concentration of Reactants

Small quantities of M. bovis (Ravenel) and M. avium antisera R-P39 and 107E-0 antisera were placed in sterile dialysis tubing. They were concentrated 2X by two methods: (1) a filled-tube was placed in a concentrated solution of Carbowax 4000[2] at room temperature for about 2 hr, and (2) a similarly

[1]See Footnote 1. P. 40

[2]Obtained from the Union Carbide Co., Charleston 30, W. Va.

filled-tube was concentrated by pervaporation at 4 C until the volume decreased one-half.

The antisera that had been concentrated by dialysis against Carbowax were employed in immunodiffusion tests with and without being further dialyzed against phosphate buffer, pH 7.2. The antisera concentrated by pervaporation were used in the tests without further treatment.

Portions of the culture filtrates that had been formerly concentrated 10X were further concentrated to 20X by pervaporation at 4 C. Preliminary tests were performed by the agar slide method with concentrated antisera and culture filtrates concentrated 20X.

Immunodiffusion tests were conducted employing numerous combinations of filtrate-antiserum concentrations. Anti-sera concentrated two-fold, undiluted, and diluted 1:2 and 1:4 with physiological saline were diffused against culture filtrates that were concentrated 20X, 10X, and 5X.

Staining of Precipitiation Lines

The reactant depots on the agar slides were rinsed and filled with distilled water. Moistened filter paper was immediately placed on top of the agar, and the slide dried at room temperature. The filter paper was carefully removed

and the slide soaked overnight in phosphate-saline buffer, pH 7.2. It was then placed in distilled water for 15 min and finally stained for 10 min in 0.1% thiazine red R prepared with 1% acetic acid.

The agar background of the precipitation lines was destained by dipping the slide in 70% ethanol containing 1% acetic acid. After drying at room temperature, the slide was examined for stained precipitates.

Method for Calculating Antigenic Relatedness of Reference Strains

By modifying an equation employed by Colwell (1963), the degree to which the reference strains were antigenically interrelated could be determined. In taxonomic studies by Adansonian analysis, Colwell used this equation:

$$S = \frac{N_s}{N_s + N_d} \text{ ; where}$$

S = the similarity of two organisms; N_s = the number of positive features shared; and N_d = the number of features positive for one organism and negative for the other.

In adapting the equation to this study, the following symbols are employed:

S_{xy} = the percent of the total antigens in strains X and Y that are related.

X_r = the number of antigens in X related to those in Y.

Y_r = the number of antigens in Y related to those in X.

X_t, Y_t = the total detectable antigens in strains X and Y , respectively.

The antigenic relatedness of two strains, then, may be represented by the percent obtained when the following equation is employed:

$$S_{xy} = \frac{X_r + Y_r}{X_t + Y_t} \cdot 100$$

and since $X_r = Y_r$,

$$S_{xy} = \frac{2X_r}{X_t + Y_t} \cdot 100$$

For example, by letting X = M. avium and Y = R-P15, and substituting values for the related and total antigens as listed in Table 6, it is seen that:

$$S_{xy} = \frac{2 \cdot 1}{6 + 4} \cdot 100$$

$$= 20$$

Or 20% of the total antigens found in M. avium and R-P15 are related.

RESULTS AND DISCUSSION OF PRELIMINARY OBSERVATIONS

Relative Sensitivities of Methods

Table 4 presents a comparison of the relative sensitivities of the tube, Petri dish, and agar slide methods for studying the precipitation lines formed by the homologous antigen-antibody systems of M. avium and M. tuberculosis, H37Ra.

In repeated tests performed by the agar slide method, six precipitation lines were detected in the reaction area between the M. avium culture filtrate and M. avium antiserum, and five lines were distinguishable in reactions of M. tuberculosis, H37Ra, filtrate and the homologous antiserum (Difco).

In tests conducted by the tube and Petri dish methods, the homologous systems of M. avium formed five precipitation lines, and M. tuberculosis, H37Ra, formed four.

Varying the methods by replenishing the antiserum layer of the tubes and refilling the reactant depots of the agar slides and Petri dishes served to intensify the lines of precipitation that developed, but did not increase the number of lines.

Table 4. The number of precipitation lines detectable in double-diffusion tests conducted by the tube, petri dish, and agar slide methods

Method	Lines formed by the homologous antigen - antibody systems of:	
	M. avium	*M. tuberculosis* H37Ra
Tube, 0.7% Agar*	5	4
1.0% Agar	5	4
Petri Dish, 0.7% Agar	5	4
1.0% Agar	5	4
2.0% Agar	5	4
Agar Slide, 0.7% Agar	6	5
1% Agar	6	5
2.0% Agar	6	5

*The diffusion media for tests conducted by all methods were prepared with 0.01 M phosphate buffer at pH 7.4.

In addition to the sensitivity of the agar slide method it was the simplest method to perform, and the reactions on slides were most easily observed and photographed. The agar slide method was therefore employed for the study itself.

Reactions in Various Media

The number of precipitation lines that developed in various diffusion media on agar slides is summarized in Table 5. An identical number of lines formed in all media: 6 lines from the homologous antigen-antibody systems of M. avium and 5 lines from M. tuberculosis, H37Ra.

A 1% agar gel prepared with 0.15 M phosphate-saline, pH 7.2, was selected as the medium for studying the antigenic relationships of the mycobacteria.

The Influence of Reactant Concentrations

After selecting the diffusion medium and the method for studying the precipitinogenic relationships, tests were performed to determine the necessity of concentrating the antisera and further concentrating the culture filtrates beyond 10X.

At various concentrations of the antigens of M. avium, M. bovis (Ravenel), R-P39, and 107E-0 with their homologous antibodies it was found that:

Table 5. The number of precipitation lines detectable in various diffusion media in tests conducted by the agar slide method

Diffusion medium	Homologous antigen-antibody systems of:	
	M. avium	M. tuberculosis, H37Ra
Physiological Saline, pH variable, Agar 1%	6	5
Physiological Saline, pH variable, Agar 1.5%	6	5
Barbital, $\mu = 0.15$, pH 7.4, Agar 1%	6	5
Barbital, $\mu = 0.15$, pH 7.4, Agar 1.5%	6	5
TRIS, $\mu = 0.15$, pH 7.4, Agar 1%	6	5
TRIS, $\mu = 0.15$, pH 7.4, Agar 1.5%	6	5
ETDA, $\mu = 0.15$, pH 7.4, Agar 1%	6	5
ETDA, $\mu = 0.15$, pH 7.4, Agar 1.5%	6	5
Phosphate, 0.01 M, pH 7.4, Agar 0.7%	6	5
Phosphate, $\mu = 0.15$, pH 7.4, Agar 1%	6	5
Phosphate, $\mu = 0.15$, pH 7.4, Agar 1.5%	6	5
Phosphate-Saline, 0.01 M, pH 7.2, Agar 0.7%	6	5
Phosphate-Saline, 0.15 M, pH 7.2, Agar 1%	6	5
Phosphate-Saline, 0.15 M, pH 7.4, Agar 1%	6	5
Phosphate-Saline, 0.15 M, pH 7.4, Agar 1.5%	6	5

(1) The two-fold concentration of antisera diffused against the 10X and 20X concentrated filtrates enhanced the intensity and broadness of precipitate bands. It did not increase the number of bands.

(2) No precipitation lines developed when 1:2 and 1:4 dilutions of the antisera from R-P39 and 107E-0 were diffused against 5X, 10X, or 20X concentrations of their homologous filtrates.

(3) In contrast to the six lines that developed in homologous reactions employing undiluted or 2X concentrations of antisera containing antibodies elicited by M. avium and M. bovis (Ravenel), only four precipitation lines formed when antisera diluted 1:2 were used. Further dilution of the M. bovis (Ravenel) and M. avium antisera to 1:4 resulted in the formation of one and two precipitation lines, respectively.

(4) Except in those instances in which the antigen wells were replenished during the immunodiffusion tests, fewer numbers of precipitation lines formed from the homologous reactions of the 5X concentrated filtrates than the 10X.

(5) Filtrates concentrated 20X did not increase the number of precipitation lines that had been observed when the 10X concentrated filtrates were used.

On the basis of the above results undiluted antisera and culture filtrates that were concentrated a maximum of 10X were used for studies of the precipitinogenic relationships of the mycobacteria. However, when comparing the reactions of two antisera with one filtrate, or two filtrates with one antiserum, dilutions of the reactants were sometimes employed.

Antibody Response in Individual Rabbits

Tests of the reactivity of antisera collected from rabbits showed wide variations in the precipitin response of individual rabbits to injections of the same antigenic preparation. The results of these tests are presented in Table 6.

Of nine culture filtrates that were each injected into three rabbits, only the filtrate prepared from M. avium elicited identical precipitin responses in all. Six of the nine filtrates elicited identical responses in two rabbits only, and two filtrates elicited a different precipitin response in each of the three rabbits injected.

Of two filtrates that were injected into only two

Table 6. The number of precipitins detected in individual rabbit antiserum by homologous antigen-antibody reactions*

Culture filtrate	No. rabbits injected	Maximum no. of precipitins detected in individual rabbit antiserum		
M. bovis, Ravenel	3	6	6	5
M. avium	3	6	6	6
M. phlei	3	6	4	6
M. fortuitum	3	6	6	5
R-P8	3	2	4	4
R-P15	3	3	4	2
R-P39	3	2	2	1
107E-0	2	2	1	
$193C_2$-1	2	3	3	
R-P380s	3	6	5	4
58A-1	3	1	1	2

*All tests were conducted on agar slides in 1.0% agar prepared with 0.15 M phosphate-saline buffer, pH 7.2 (incubated at 28 C).

rabbits each, only one elicited identical precipitin responses in both rabbits.

The antisera employed in the study of precipitinogenic relationships were those that contained the greatest number of detectable precipitins in the preliminary tests of homologous antigen-antibody systems.

RESULTS OF
PRECIPITINOGENIC STUDIES

Homologous Antigen-Antibody Reactions

In diffusion tests of 12 mycobacterial strains employing homologous culture filtrate antigens and antisera, from 2 to 6 precipitation lines were observed (Table 7).

Reactions of the homologous antigen-antibody systems of *M. bovis*, Ravenel (Fig. 1), *M. avium*, *M. phlei* (Fig. 2), *M. fortuitum* (Fig. 3), and R-P380s each developed six precipitation lines. This was evidence that each organism possessed a minimum of six precipitinogens.

The culture filtrate of *M. tuberculosis* (H37Ra) reacted with the homologous commercial antiserum (Difco) to produce five lines of precipitation. Yet, the same culture filtrate produced six precipitation lines when reacting with the heterologous *M. bovis* (Ravenel) antiserum. Repeated immunodiffusion tests designed to demonstrate that six or more antibodies were present in the Difco-prepared *M. tuberculosis* (H37Ra) antiserum were conducted. The dialysis of the culture filtrate and its use in tests in varying proportions and concentrations of both reactants, failed to demonstrate more than five antibodies existing in the Difco antiserum.

Since the presence of six precipitinogens in M. tuberculosis (H37Ra) was established in tests with M. bovis (Ravenel) antiserum, the detection of only five with the reconstituted Difco antiserum indicated that the former was a somewhat superior reactant for immunodiffusion studies. This could possibly be due to variations in the methods of preparing antiserum or to individual differences in the response of rabbits immunized. Nevertheless, the Difco antiserum was employed as one of the reference antisera and was diffused against all culture filtrates.

Two strains of human origin, R-P8 (Group I) and R-P15 (Group II) formed four lines of precipitation with the homologous antiserum. With each of the strains, two of the four lines were of moderate intensity and the other two were of low intensity.

Three lines, all of moderate intensity (Fig. 6) were produced by the homologous filtrate-antiserum systems of $193C_2$-1, a Group III strain originally isolated from mesenteric lymph node lesions of a pig.

The least reactive homologous antigen-antibody systems produced but two lines of precipitation. These were the systems of a Runyon Group III strain of human origin, R-P39; a Group III strain of bovine (lung) origin, 107E-0; and a

Table 7. The number of precipitation lines formed by homologous and heterologous antigen-antibody reactions

	antiserum											
	M. bovis, Ravenel	*M. tuberculosis*, H37Ra (Difco)	*M. avium*	*M. phlei*	*M. fortuitum*	R-P8 (Grp I)	R-P15 (Grp II)	R-P39 (Grp III)	107E-0 (Grp III)	$193C_2$-1 (Grp III)	R-P380s (Grp IV)	58A-1 (pseudochrome)
M. bovis, Ravenel	6	5	1	0	2	2	2	0	0	1	2	1
M. tuberculosis, H37Ra	6	5	1	0	2	2	2	0	0	1	2	1
M. avium	1	1	6	1	2	1	1	1	1	3	1	0
M. phlei	0	0	1	6	2	1	1	0	0	1	0	0
M. fortuitum	2	2	2	2	6	1	1	0	0	2	2	1
R-P8 (Grp I)	2	2	1	1	1	4	2	0	0	2	1	0
R-P15 (Grp II)	2	2	1	1	1	2	4	0	1	2	1	1
R-P39 (Grp III)	0	0	1	0	0	0	0	2	0	0	0	0
107E-0 (Grp III)	0	0	1	0	0	0	1	0	2	1	0	0
$193C_2$-1 (Grp III)	1	1	3	1	2	2	2	0	1	3	1	1
R-P380s (Grp IV)	2	2	1	0	2	1	1	0	0	1	6	0
58A-1 (pseudochrome)	1	1	0	0	1	0	1	0	0	1	0	2
M. tuberculosis, H37Rv	6	5	1	0	2	2	2	0	0	1	2	1
M. bovis, BCG	3	3	1	0	1	1	1	0	0	1	1	1
R-P4 (Grp I)	0	0	0	0	0	2	0	0	0	0	0	0
M. smegmatis	0	0	2	2	2	0	1	0	0	0	0	0

pseudochrome, 58A-1, originally isolated from a bovine cervical lymph node.

The two lines of precipitation that developed from the homologous reactions of these three strains were of rather low intensity. Replenishing reactant wells enhanced the intensity but did not increase the number of bands observed.

Precipitinogenic Relationships of the 12 Reference Strains

The number of precipitation lines formed in the homologous and heterologous reactions of 12 strains of mycobacteria are summarized in Table 7. Included are classical strains, two saprophytes, members of the four Runyon groups, and a pseudochrome.

Filtrates of the Ravenel strain of M. bovis and the H37Ra and H37Rv strains of M. tuberculosis gave identical antigenic patterns when diffused against the 12 antisera. There were no cross-reactions of the three filtrates with the antiserum prepared from M. phlei, R-P39, or 107E-O. Each of the filtrates of M. bovis (Ravenel) and M. tuberculosis (H37Ra and H37Rv) reacted to produce one line of precipitation with the antisera of M. avium, $193C_2$-1, and the pseudochrome, 58A-1; two lines each were produced in reactions with M. fortuitum, R-P8, R-P15, and R-P380s; six lines

developed with the M. bovis (Ravenel) antiserum, and five lines with the Difco M. tuberculosis (H37Ra) antiserum.

The reactions of the six antigens in the filtrates of M. bovis (Ravenel) and M. tuberculosis (H37Ra and H37Rv) were compared; when M. bovis (Ravenel) antiserum was placed in the center well and diffused against the three filtrates contained in three equidistant depots surrounding the antiserum well, six continuous lines of precipitation developed. This indicated that a minimum of six identical antigens were present in the Ravenel strain of M. bovis, and the H37Ra and H37Rv strains of M. tuberculosis (Fig. 4).

One of the six precipitating antigens in the above three strains was identical to an antigen found in M. avium; and two of the six antigens were identical to antigens found in the atypicals of human origin, R-P8 (Group I) and R-P15 (Group II).

Mycobacterium tuberculosis (H37Ra and H37Rv) and M. bovis (Ravenel) shared one antigen of identity and one antigen of partial identity with M. fortuitum and R-P380s (Fig. 5).

The culture filtrate of M. phlei possessed two antigens that were related to those found in M. fortuitum (Fig. 7),

Fig. 1. Homologous antigen-antibody reactions of M. bovis (Ravenel)

1 = Conc. culture medium
3 = Conc. M. bovis culture filtrate
B = M. bovis antiserum
N = Normal rabbit serum

Fig. 2. Homologous antigen-antibody reactions of M. phlei

1, 2, 3, = M. phlei antisera
Ag = Conc. M. phlei culture filtrate

Fig. 3. Homologous antigen-antibody reactions of M. fortuitum

1 = Conc. culture medium
2 = Conc. M. fortuitum culture filtrate
A = M. fortuitum antiserum
N = Normal rabbit serum

Fig. 4. Reactions of culture filtrates of classical mammalian species with M. bovis (Ravenel) antiserum

1 = M. bovis (Ravenel) antiserum
A = Conc. culture medium
B = Conc. M. bovis (Ravenel) culture filtrate
C = Conc. M. tuberculosis (H37Ra) culture filtrate
D = Conc. M. tuberculosis (H37Rv) culture filtrate

Fig. 5. Homologous and heterologous antigen-antibody reactions

C = M. bovis (Ravenel) antiserum
A, D = Conc. M. bovis culture filtrate
B = Conc. M. fortuitum culture filtrate
E = Conc. R-P380s culture filtrate

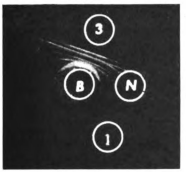

Fig. 1

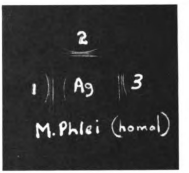

Fig. 2

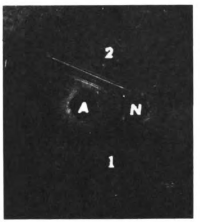

Fig. 3

Fig. 4

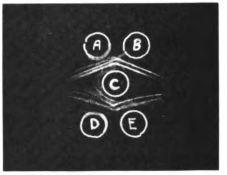

Fig. 5

Fig. 6. Homologous antigen-antibody reactions of $193C_2$-1 (Group III, Swine Origin)

B = Antibodies elicited by $193C_2$-1
N = Normal rabbit serum
1 = Concentrated culture medium
3 = Concentrated culture filtrate of $193C_2$-1

Fig. 7. Homologous and heterologous reactions of culture filtrates of M. phlei and M. fortuitum with reference antisera

Antigens:
4, 6 = Concentrated culture filtrate of M. fortuitum
5 = Concentrated culture filtrate of M. phlei

Antisera:
A = R-P 8
B = R-P 15
C = R-380s
D = 58A-1
E = M. bovis, Ravenel
F = M. avium
G = M. phlei
H = M. fortuitum
I = R-P 39
J = 107E-0
K = $193C_2$-1
L = M. tuberculosis, H37Ra ("Difco")

Fig. 8. Precipitates formed by cultures of three Group III strains in reactions with reference antisera

Reference antisera:
P 8 = R-P 8 (Group I, human origin)
P15 = R-P15 (Group II, human origin)
P 380s = R-P 380s (Group IV, human origin)
58A-1 = 58 A-1 (Pseudochrome, bovine origin)
Rav = M. bovis, Ravenel Av = M. avium
Phl = M. phlei For = M. fortuitum
P 39 = R-P 39 (Group III, human origin)
107 = 107E-0 (Group III, bovine origin)
193 = $193C_2$-1 (Group III, swine origin)
Ra = M. tuberculosis, H37Ra ("Difco")

Antigens:
Conc. 186C-1 culture filtrate Conc. 198C-1 culture filtrate
Conc. $172C_1$-1 culture filtrate

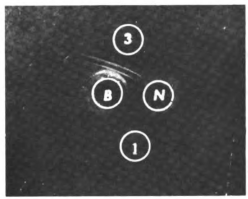

Fig. 6

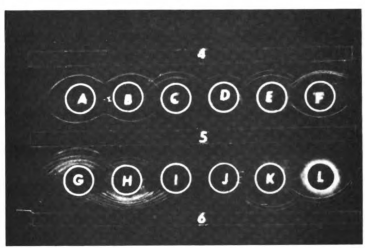

Fig. 7

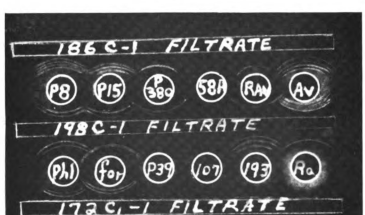

Fig. 8

both giving reactions of partial identity when compared with antigens of the latter species. One antigen of M. phlei also gave reactions of partial identity with one of the six antigens possessed by M. avium and with one of the three antigens possessed by $193C_2$-1. One antigen of M. phlei was related to antigens found in R-P8 (Group I) and R-P15 (Group III); when this antigen was compared with each of the antigens in the latter strains, reactions of partial identity were observed for both.

The antigens of M. avium reacted with one or more of the antibodies elicited by each of the reference strains except the pseudochrome, 58A-1. Unlike M. tuberculosis and M. bovis, M. avium was related to M. phlei, R-P39, and 107E-0.

Strain $193C_2$-1, a Group III of swine origin, was related to all of the reference strains except R-P39, the Group III strain of human origin, and was most closely related to M. avium. The three antigens detected in the homologous antigen-antibody reactions of $193C_2$-1 were all related to M. avium. A reaction of identity was observed for one, and a reaction of partial identity for the other two.

The Group III strains, R-P39 (human origin) and 107E-0 (bovine origin), were relatively non-reactive. Each produced but two precipitation bands in their homologous

antigen-antibody reactions. One antigen of each strain was related to an antigen possessed by M. avium. Strain 107E-0 also possessed one antigen that was related to R-P15, and one that was related to $193C_2$-1.

The atypical strain of human origin, R-P4, contained two antigens that were related to antigens in the other Group I strain isolated from man, R-P8, but was not related to the other 11 reference strains. In contrast, R-P8 was related to 8 reference strains, and not related to strains R-P39, 107E-0, and 58A-1.

The filtrate of BCG, though reacting with the same reference antisera as did the filtrate of the Ravenel strain of M. bovis, was far less reactive then the latter. The filtrate of BCG reacted with the Ravenel antiserum to form but three precipitation bands, whereas, six bands were developed by reactions of the homologous antigen-antibody systems of M. bovis, Ravenel. The BCG strain was also less reactive with other reference antisera than was Ravenel, and had only one antigen related to M. avium, M. fortuitum, R-P8, R-P15, $193C_2$-1, R-P380s, or 58A-1.

Mycobacterium smegmatis, contained two precipitating antigens that were related to antigens in filtrates of M. phlei, M. avium, and M. fortuitum, and one antigen related

to an antigen in R-P15. M. smegmatis was not related antigenically to these reference strains: M. bovis (Ravenel), M. tuberculosis (H37Ra), R-P8, R-P39, 107E-0, $193C_2$-1, R-P380s and 58A-1.

The antigenic "relatedness" of 11 selected mycobacteria is shown in Table 8. The values listed in the table were determined as previously described. Of the total precipitating antigens found in M. bovis (Ravenel) and R-P8, as well as, in M. bovis (Ravenel) and R-P15, 40% were related. Mycobacterium bovis (Ravenel) was found to be more closely related to these two strains than to the other 10 selected strains in Table 8.

Mycobacterium avium and $193C_2$-1 were more closely related than were any other two strains of the group with 67% relatedness. Mycobacterium phlei was more closely related to M. fortuitum than to other strains; 33% of their total antigens were related. On the other hand, M. fortuitum was related most closely to $193C_2$-1. The strains, R-P8, R-P15, 107E-0 and 58A-1, also were more closely related to $193C_2$-1 than to other strains. The Group IV strain, R-P380s (human origin), was related to the same extent to M. bovis (Ravenel) and M. fortuitum; 33% of the total antigens of R-P380s and each of the latter two strains were related.

Table 8. The antigenic "relatedness" of selected mycobacteria reported in percent of their total antigens that are related

	M. bovis, Ravenel	*M. avium*	*M. phlei*	*M. fortuitum*	R-P8	R-P15	R-P38	107E-0	$193C_2$-1	R-P380s	58A-1
M. bovis, Ravenel	X*	17	0	33	40	40	0	0	22	33	25
M. avium	17	X	17	33	20	20	25	25	67	17	0
M. phlei	0	17	X	33	20	20	0	0	22	0	0
M. fortuitum	33	33	33	X	20	20	0	0	44	33	25
R-P8 (Grp I)	40	20	20	20	X	50	0	0	57	20	0
R-P15 (Grp II)	40	20	20	20	50	X	0	33	57	20	33
R-P39 (Grp III)	0	25	0	0	0	0	X	0	0	0	0
107E-0 (Grp III)	0	25	0	0	0	33	0	X	40	0	0
$193C_2$-1 (Grp III)	22	67	22	44	57	57	0	0	X	22	40
R-P380s (Grp IV)	33	17	0	33	20	20	0	0	22	X	0
58A (Pseudochrome)	25	0	0	25	0	33	0	0	40	0	X

X* = 100% and represents the homologous reaction

Table 9. The number of precipitation lines formed by culture filtrates of Group II strains in reactions with reference antisera

Culture filtrate	antiserum											
	M. bovis, Ravenel	M. tuberculosis, H37Ra (Difco)	M. avium	M. phlei	M. fortuitum	R-P8 (Grp I)	R-P15 (Grp II)	R-P39 (Grp III)	107E-0 (Grp III)	$193C_2$-1 (Grp III)	R-P380s (Grp IV)	58A-1 (Pseudochrome)
R-P15 (human origin)	2	2	1	1	1	2	4	0	0	2	1	1
368E-1 (bovine origin)	0	0	2	4	3	1	1	0	0	0	1	0
X 27-1 (soil)	0	0	0	3	0	0	0	0	0	0	0	0
X 28-1 (soil)	0	0	0	1	0	0	0	0	0	0	0	0
X 37-1 (soil)	0	0	1	4	2	2	2	0	0	0	0	0

Precipitinogenic Relationships of Mycobacteria of Human, Bovine, Porcine and Soil Origins Presented by Groups

Group II. The number of precipitation lines formed by culture filtrates of Group II strains in reactions with the reference antisera is shown in Table 9. All Group II strains possessed one or more antigens related to those of M. phlei. The antigens of X27-1 and X28-1 reacted only with antibodies elicited by M. phlei and produced 3 and 1 precipitation bands, respectively.

Reactions of the filtrate of 368E-1 with the reference antisera resulted in the development of the following precipitation bands: 4 with M. phlei, 3 with M. fortuitum, 2 with M. avium, one each with R-P8, R-P15 and R-P380s, and no bands with the remaining reference antisera.

Strain R-P15, one of the reference strains of human origin, was the only member in Group II that was related antigenically to M. bovis (Ravenel), M. tuberculosis (H37Ra), $193C_2$-1, or 58A-1.

Group III. The results of immunodiffusion tests in which the filtrates of 22 Group III strains were diffused against the antisera prepared from the 12 reference mycobacteria are shown in Table 10.

One of the 22 strains, that failed to sensitize or cause an intradermal lesion in guinea pigs, B66F-1, was the only member of the group antigenically related to 58A-1.

Seven strains of swine origin ($152A_1$-1, 93C-0, 151C-1, 186C-1, $193C_2$-1, 198C-1, and 228C-1) and one strain of bovine origin (B102E-0) were related to all but three of the reference strains; the non-related reference strains were R-P39, 107E-0, and 58A-1.

Three strains of swine origin that were avirulent for calves, 93C-0, 186C-1 and $193C_2$-1, and a strain of the same origin that was virulent for calves, $152A_1$-1, were related to the same 9 reference strains. These four strains also developed similar antigenic patterns in reactions with the reference antisera, showing that the antigens present in the virulent strain were not distinct from those possessed by the avirulent strains of similar origin.

Two strains of cattle origin, B102E-0 and 83F-0, were the only strains in Group III that were more closely related to R-P380s, a Group III of human origin, than to one of the other reference strains.

Most of the strains of cattle and swine origins in Group III did not possess antigens that were related to

Table 10. The number of precipitation lines formed by culture filtrates of Group III strains in reactions with reference antisera

Culture filtrate	antiserum											
	M. bovis, Ravenel	M. tuberculosis, H37Ra (Difco)	M. avium	M. phlei	M. fortuitum	R-P8 (Grp I)	R-P15 (Grp II)	R-P39 (Grp III)	107E-0 (Grp III)	$193C_2$-1 (Grp III)	R-P380s (Grp IV)	58A-1 (pseudochrome)
Human origin:												
R-P39	0	0	1	0	0	0	0	2	0	0	0	0
Bovine origin:												
50B-0	1	1	0	0	0	0	0	0	1	1	0	0
62D-0	2	2	5	2	2	2	2	1	0	2	2	0
B102E-0	2	2	2	1	3	3	3	0	0	1	4	0
107E-0	1	1	1	0	0	0	1	0	2	1	0	0
83F-0	0	0	1	1	2	0	0	0	0	0	3	0
94F-0	0	0	1	0	2	1	1	0	0	0	1	0
98F-0	0	0	1	2	1	1	0	0	1	1	1	0
B124F-0	0	0	1	2	1	1	1	0	0	0	1	0
B66F-1	0	0	1	0	0	1	0	0	0	1	0	1
Porcine origin:												
$152A_1$-1	1	1	4	1	2	2	2	0	0	3	1	0
93C-0	1	1	3	1	1	2	2	0	0	2	1	0
151C-1	1	1	3	1	2	2	2	0	0	2	1	0
$172C_1$-1	0	0	3	2	2	2	1	0	0	0	1	0
186C-1	1	1	4	1	2	4	3	0	0	2	1	0
$193C_2$-1	1	1	3	1	2	2	2	0	1	3	2	0
198C-1	1	1	4	2	2	2	2	0	0	3	2	0
228C-1	1	1	4	2	3	2	1	0	0	2	2	0
350-1	0	0	1	0	0	0	0	0	0	0	0	0
352-1	1	1	3	0	0	2	1	1	0	0	0	0
242I-1	0	0	2	0	1	1	0	0	2	1	0	0
244I-1	0	0	2	0	1	0	2	0	0	3	0	0

the Group III of human origin, R-P39, the Group III of bovine origin, 107E-0, or the pseudochrome of porcine origin, 58A-1 (Fig. 8).

The strains originally isolated from cattle varied considerably in their antigenic relationships. Though eight of the nine strains contained at least one antigen related to those possessed by M. avium, only 62D-0 was related more closely to M. avium than to one of the other reference strains.

Six of the isolants from cattle were related to some extent to one or more of the strains, M. fortuitum, R-P8, $193C_2$-1 and R-P380s; five strains were related to M. phlei, and five to R-P15.

All of the Group III isolants from swine were closely related but not identical to M. avium. The relatively non-reactive filtrate of 350-1 reacted only with the avian antiserum, and formed one precipitation band. The antigens of 186C-1 developed four bands with M. avium antiserum, four with R-P8, three with R-P15, two with 107E-0, and one band each with M. bovis (Ravenel), M. tuberculosis (H37Ra) and R-P380s.

Strain $193C_2$-1 possessed three precipitating antigens. As described in the results of homologous and heterologous

reactions of selected mycobacteria, two of these antigens gave reactions of partial identity, and one antigen, a reaction of identity with antigens of M. avium.

Unlike the Group III strains of bovine origin, the Group III swine isolants contained either the same number of antigens related to M. avium as to other reference strains, or they contained a greater number related to M. avium. Seven swine isolants were more closely related to M. avium than to the other 11 representative strains. One strain, 186C-1 was related equally to M. avium and R-P8; strain 244 I-1 was related equally to M. avium and 107E-0; and 242I-1 was related more closely to another Group III swine isolant ($193C_2$-1) than to M. avium.

In general, the 22 Group III strains of both swine and bovine origin contained antigens that were more related to antigens of M. avium than to those of other representative strains. Only the filtrate from the relatively non-reactive strain, 50B-0, failed to react with M. avium antiserum.

Group IV. Reactions of seven Group IV strains (a reference strain of human origin, R-P380s, and six strains of bovine origin) are shown in Table 11. Antigens of these strains failed to react with the antibodies elicited by

107E-0 or 58A-1, and only some of the antigens of R-P380s formed precipitates with the antibodies elicited by M. tuberculosis (H37Ra), M. bovis (Ravenel), or M. fortuitum. The filtrate of R-P380s also developed one precipitation band in reactions with the antisera of M. avium, R-P8, R-P15 and $193C_2$-1.

Only two Group IV isolants of bovine origin reacted with the antibodies elicited by R-P380s, and these two, B117B-0 and B368D-1, each had but one antigen related to antigens possessed by R-P380s.

Strain 141F-1, the only bovine isolant in Group IV capable of inducing avian hypersensitivity or intradermal lesions in guinea pigs, formed three bands with M. avium antiserum and failed to react with other antisera. Even though M. avium was the only strain among the 12 representative mycobacteria to which 141F-1 was related, 3 of the 7 strains in Group IV were not related to M. avium.

Only one strain in the Group was related more closely to M. fortuitum than to the other reference strains. The filtrate prepared from this strain, B368D-1, developed three lines of precipitation with the antiserum of M. fortuitum, and one line each with the antibodies induced by M. phlei, R-P8, R-P15, and R-P380s.

Table 11. The number of precipitation lines formed by culture filtrates of Group IV strains in reactions with reference antisera

Culture filtrates	antiserum: *M. bovis*, Ravenel	*M. tuberculosis*, H37Ra (Difco)	*M. avium*	*M. phlei*	*M. fortuitum*	R-P8 (Grp I)	R-P15 (Grp II)	R-P39 (Grp III)	107E-0 (Grp III)	$193C_2$-1 (Grp III)	R-P380s (Grp IV)	58A-1 (pseudochrome)
Human origin:												
R-P380s	2	2	1	0	2	1	1	0	0	1	6	0
Bovine origin:												
B117B-0	0	0	0	1	0	0	0	0	0	0	1	0
B368D-1	0	0	0	1	3	1	1	0	0	0	1	0
B292E-1	0	0	1	0	0	1	1	1	0	0	0	0
B28E-2	0	0	1	1	2	1	2	0	0	0	0	0
B177F-0	0	0	0	1	0	1	0	0	0	0	0	0
141F-0	0	0	3	0	0	0	0	0	0	0	0	0

Though B368D-1 was closely related to M. fortuitum, four of the seven isolants were not related to the latter strain. The Group IV isolants are quite heterogeneous in their antigenic components.

The Pseudochromes. The reactions of filtrates prepared from the pseudochromes with the 12 reference antisera are shown in Table 12. In general, the precipitinogens of the pseudochromes were found to be related to those possessed by one or more of the strains: M. avium, M. phlei, M. fortuitum, and R-P8.

Four strains, $152A_2$-1, 52H-1, 65F-0, and 77F-1, were related to M. phlei. Of the four, all strains except 77F-1 were also related to M. fortuitum. Antigens of 77F-1 formed two bands with antibodies elicited by M. phlei and failed to react with those elicited by the remaining reference strains. The pseudochrome, 63A-0, was also relatively non-reactive; it was related only to R-P39, a Group IV strain of human origin.

The culture filtrate of $152A_2$-1, a swine isolant, reacted with M. avium and R-P15 antisera to form two precipitation bands with each. One band was formed with the antiserum of each of the strains, M. phlei and M. fortuitum, and

Table 12. The number of precipitation lines formed by culture filtrates of pseudochromes in reactions with reference antisera

Culture filtrate	antiserum											
	M. bovis, Ravenel	*M. tuberculosis*, H37Ra (Difco)	*M. avium*	*M. phlei*	*M. fortuitum*	R-P8 (Grp I)	R-P15 (Grp II)	R-P39 (Grp III)	107E-0 (Grp III)	$193C_2$-1 (Grp III)	R-P380s (Grp IV)	58A-1 (pseudochrome)
Porcine origin:												
$152A_2$-1	0	0	2	1	1	0	2	0	0	0	0	0
Bovine origin:												
58A-1	1	1	0	0	1	0	0	0	0	1	0	2
63A-0	0	0	0	0	0	0	0	1	0	0	0	0
52H-1	0	0	2	2	1	1	2	0	0	1	0	0
65F-0	0	0	0	2	1	2	1	0	0	0	1	0
77F-1	0	0	0	2	0	0	0	0	0	0	0	0

no bands in immunodiffusion tests with the eight remaining reference antisera.

The most reactive pseudochrome was 52H-1. This strain had two precipitating antigens related to M. avium, M. phlei, and R-P15, and one antigen related to M. fortuitum, R-P8, and $193C_2$-1.

No two pseudochromes reacted to the same degree with all of the various antisera, and as a group, the pseudochromes were also heterogeneous. The antibodies elicited by 58A-1 (a pseudochrome) which reacted to form two precipitation lines with the homologous antigenic preparation failed to react with the antigens of the other five pseudochromes. Strain 58A-1 was also the only one in the group related to M. tuberculosis (H37Ra).

Of the 12 reference mycobacterial strains employed to elicit antibodies, all except 107E-0 were related to one or more pseudochromes.

Bovine-skin Isolants. The results of the precipitation reactions of nine isolants from skin lesions of cattle are presented in the results of the respective Runyon groups. However, these strains have been regrouped in Table 13 to compare their antigenic relationships with the 12 representative mycobacteria.

Table 13. A comparison of the number of precipitation lines formed by culture filtrates prepared from various isolants of bovine-skin lesions in reactions with reference antisera

Culture filtrate	antiserum											
	M. bovis, Ravenel	*M. tuberculosis*, H37Ra (Difco)	*M. avium*	*M. phlei*	*M. fortuitum*	R-P8 (Grp I)	R-P15 (Grp II)	R-P39 (Grp III)	107E-0 (Grp III)	$193C_2$-1 (Grp III)	R-P380s (Grp IV)	58A-1 (pseudochrome)
Group III:												
83F-0	0	0	1	1	2	0	0	0	0	0	0	0
94F-0	0	0	1	0	2	1	1	0	0	0	1	0
98F-0	0	0	1	2	1	1	0	0	1	1	1	0
B124F-0	0	0	1	2	1	1	1	0	0	0	1	0
B66F-1	0	0	1	0	0	1	0	0	0	1	0	1
Group IV:												
M177F-0	0	0	0	1	0	1	0	0	0	0	0	0
141F-1	0	0	3	0	0	0	0	0	0	0	0	0
Pseudochromes:												
65F-0	0	0	0	2	1	2	1	0	0	0	1	0
77F-1	0	0	0	2	0	0	0	0	0	0	0	0

None of the skin isolants were related to M. bovis, M. tuberculosis, or R-P39 (a Group III of human origin); one strain, 98F-0, was related to $193C_2$-1; and only B66F-1 was related to the pseudochrome, 58A-1.

Six of the skin isolants and the reference strains with which they were related most closely were: 83F-0 with the Group IV strain, R-P380s; 94F-0 with M. fortuitum; 98F-0, B124F-0, and 77F-0 with M. phlei; and 141F-1 with M. avium.

The skin isolants were quite heterogeneous. Though three strains were not related to M. phlei, as a group, these isolants were more closely related to M. phlei than to any single reference strain.

A Comparison of the Relationships of "Atypicals" of All Runyon Groups

The precipitinogens of atypical strains of the various groups vary considerably in their relationships. Table 14 presents a summary of the number of atypical strains, listed by Runyon groups, that had one or more related antigens in common with each of the 12 representative mycobacteria.

This table was compiled on the basis of one or more precipitation lines developing in specific reactions of filtrates with antisera prepared from each of the representative strains. No distinction was made between antigens of

Table 14. The number of strains within each Runyon Group that possessed one or more precipitating antigens which were related to antigens found in 12 reference strains

		The number of strains that reacted with the antiserum prepared from:											
	No. of strains	M. bovis, Ravenel	M. tuberculosis, H37Ra	M. avium	M. phlei	M. fortuitum	R-P8 (Grp I)	R-P15 (Grp II)	R-P39 (Grp III)	107 E-O (Grp III)	$193C_2$-1 (Grp III)	R-P380s (Grp IV)	58A-1 (pseudochrome)
Group I													
From man	2	1	1	1	1	1	2	1	0	0	1	1	0
Group II													
From man	1	1	1	1	1	1	1	1	0	0	1	1	1
From cattle	1	0	0	1	1	1	1	1	0	0	0	1	0
From soil	3	0	0	1	3	1	1	1	0	0	0	0	0
TOTALS	5	1	1	3	5	3	3	3	0	0	1	2	1
Group III													
From man	1	0	0	1	0	0	0	0	1	0	0	0	0
From cattle	9	4	4	8	5	6	6	5	1	3	6	6	1
From swine	12	8	8	12	8	10	10	10	1	1	9	8	0
TOTALS	22	12	12	21	13	16	16	15	3	4	15	14	1
Group IV													
From man	1	1	1	1	0	1	1	1	0	0	1	1	0
From cattle	6	0	0	3	4	2	4	3	1	0	0	2	0
TOTALS	7	1	1	4	4	3	5	4	1	0	1	3	0
Pseudochromes													
From cattle	5	1	1	1	3	3	2	2	1	0	2	1	1
From swine	1	0	0	1	1	1	0	1	0	0	0	0	0
TOTALS	6	1	1	2	4	4	2	3	1	0	2	1	1
TOTALS OF ALL ATYPICALS	41	16	16	31	27	27	28	26	5	4	20	21	3

identity or partial identity, nor was any consideration given to the number of precipitation lines formed in a reaction. The table presents then, an estimate of the relationships of the groups of "atypicals" with selected mycobacterial strains. It does not show the degree of the relationships, yet it accurately reflects the wide-range of antigens possessed by strains within each Runyon group.

A number of striking comparisons are summarized in the Table: Of 41 atypicals of all groups and sources, 31 or 76% were related to the classical avian bacillus and but 16, or 39% were related to the mammalian strains.

A surprisingly large number (27 to 28 strains) were related to M. phlei, M. fortuitum, R-P8, and R-P15.

Only 3 to 5 "atypicals" were related to the Group III strains, R-P39 (of human origin), and the pseudochrome, 58A-1.

In Table 15 the "atypicals" have been grouped with known mycobacterial strains with which they were most closely related antigenically.

Table 15. A regrouping of atypical strains based on their antigenic relationships among known mycobacterial species

Original Runyon Group	Known strains to which the atypicals were closely related					
	A. *M. bovis* and *M. tuberculosis*	B. *M. avium*	C. *M. phlei*	D. *M. fortuitum*	E.[1] Non-definitive	F. Non-related
I	R-P8 (m)[2]					R-P4 (m)
II	R-P15 (n)		368E-1(-) X27-1(-) X28-1(-) X37-1(-)			
III	50B-0(a/b)	R-P39(a) 62D-0(a/b) B66F-1(-) 152A_1-(a) 93C-0(a) 151C-1(a) 172C_1-1(m) 186C-1(a) 193C_2-1(m) 198C-1(a) 228C-1(m) 350-1(a) 352-1(a) 242I-1(a) 244I-1(a)	98-0(a) B124F-0(n)	B102E-0(a) 83F-0(n) 94F-0(n)	107E-0(n)	

Table 15. -- Continued

Original Runyon Group	Known strains to which the atypicals were closely related					
	A. *M. bovis* and *M. tuberculosis*	B. *M. avium*	C. *M. phlei*	D. *M. fortuitum*	E.[1] Non-definitive	F. Non-related
IV		B292E-1(-) 141F-1(a)	B117B-0(-) M177F-0(-)	B368D-1(-) B28E-2(-)	R-P380s(n)	
Pseudo-chromes		$152A_2$-1(a)	65F-0(a) 77F-1(n)		52H-1(a)	63A-0(a/b)

[1]Group E was composed of strains that were equally related to two or more known strains.

[2]The type of hypersenstitivity developed in guinea pigs: m = mammalian, a = avian, n = non-definitive, a/b = avian/battey, (-) = no sensitivity.

DISCUSSION

It is well to bear in mind that the results observed could have been tempered by many factors. Employing concentrated culture filtrates in diffusion reactions and for eliciting antibodies in rabbits is hardly ideal. Not only should one expect the various antigens present in a filtrate to exist in varying concentrations, but also, the filtrate preparation itself to contain a wide variety of metabolic and autolytic products of bacterial cells. These products may have varying affinities for combining with the determinant groups of different antigens, thereby altering antigenic specificity or completely blocking the antigen.

In this study no attempt was made to purify the filtrates used to elicit antibodies in rabbits. However, partial purification of M. tuberculosis (H37Ra) filtrate by dialysis, and diffusion of the dialyzed product against Difco M. tuberculosis (H37Ra) antiserum showed that dialysis of this filtrate had little effect. Slightly sharper precipitation lines were developed by the dialyzed filtrate, but the number of lines forming remained the same as before dialysis. Since no added information was gathered other culture filtrates were not dialyzed.

Another factor that may alter immunodiffusion results is the method employed to elicit antibodies. The low antigenicity of the mycobacteria prompted the adoption of a procedure, for this study, that is a form of hyperimmunization. Crowle (1961) reported that " . . . hyperimmunization tends to induce production of a range of antibodies which make the antiserum likely to cross-react even with distantly related antigens."

No direct evidence was sought in this study to show that the form of hyperimmunization employed did not cause non-specificty of the antibodies. However, each of the 49 filtrates of mycobacterial strains reacted with at least one antiserum, and no single antiserum reacted with all of the 49 filtrates. The results, then, suggest that the antibodies possessed at least moderate specificities.

Another factor influencing the quality of antisera--and thereby, immunodiffusion results--is the variation of individual animals in response to injections of antigens. In this study, there was much variation in the number of antigens that were detectable when the same filtrate was diffused against antisera obtained from different rabbits that had been injected with identical filtrates. The antiserum for each rabbit was tested individually; only those

which developed the largest number of precipitation bands in homologous reactions were used for studying the precipitinogenic relationships.

A final limitation of immunodiffusion techniques is that during periods of incubation of a plate, precipitation lines are formed at varying rates and have different relative intensities; a dense line developing at a rapid rate may sometimes mask the development of a slower-developing line. To minimize such effects, reactants were employed at varying relative concentrations, and observations were made at 8 hr intervals during incubation of the agar diffusion slides.

When interpreting results, it is well to realize that shortcomings of the aforementioned sort are inherent in immunodiffusion studies.

In this study, the results of immunodiffusion tests of 49 concentrated culture filtrates with the 12 reference antisera suggest that marked antigenic differences exist among various strains of mycobacteria. These variances not only exist among strains of different origins and different Runyon groups, but also among strains isolated from similar sources with similar morphological and growth characteristics. However, certain definite patterns of antigenic relationships were noted with many strains.

The observation of six identical precipitinogens in the Ravenel strain of M. bovis, and the H37Ra and H37Rv strains of M. tuberculosis, substantiates, in part, an earlier finding of Parlett and Youmans (1958). These authors found six identical precipitating antigens in H37Ra and H37Rv strains, but reported that only four of these antigens were possessed by the four strains of M. bovis which they studied. However, their study did not include the Ravenel strain.

The non-pathogenic species, M. smegmatis and M. phlei, and the bovine and soil isolants that were avirulent for guinea pigs were not related to M. bovis (Ravenel) or M. tuberculosis (H37Ra). If this pattern were consistent with avirulent strains only, the relationship would immediately have useful applications. But it was observed that 15 strains, virulent for guinea pigs (of bovine, swine, and cattle origins) were also unrelated to H37Ra and Ravenel. Yet, the criterion for determining virulence in guinea pigs had been the production of any size intradermal lesion at the site of inoculation, and not necessarily, progressive disease. If one assumed that the production of an intradermal lesion alone is an inadequate test for virulence, it would be of interest to set a more rigid criterion for

virulence and compare the relationships of the avirulent vs. the virulent atypical strains with the classical human and bovine tubercle bacilli.

The atypical strains of soil, bovine, and porcine origins were, with but one exception, related more closely to one or more of the strains, M. avium, M. phlei, and M. fortuitum, than to M. bovis or M. tuberculosis. The strains in Runyon Group II and the majority of the pseudochromes were more closely related to M. phlei; the Group III strains of swine origin were invariably more closely related to M. avium; and the strains in Groups III and IV of bovine origin were related more closely to either M. avium, M. phlei, or M. fortuitum.

The atypical isolants from man, however, were quite different in their antigenic relationships. In general, they too, were related to M. avium, M. phlei, and M. fortuitum, but in addition, three of the five atypical human isolants each had two antigens related to M. bovis and M. tuberculosis. This finding gives added support to the conviction that the atypicals of animal and soil origins markedly differ from the atypical human isolants.

The nine bovine-skin isolants were either members of Runyon Groups III and IV, or they were pseudochromes. Though

somewhat related to M. avium, M. phlei, and M. fortuitum, and R-P8, these strains were relatively non-reactive with the 12 reference antisera. Whether this could be due to a general lack of antigenicity of the skin isolants or to a lack of relationship to the reference strains, cannot be assessed as there was no antiserum prepared from a skin isolant or from M. ulcerans, a species associated with skin infections of man.

It is noteworthy that this study, like a previous study by Parlett and Youmans (1958), failed to reveal that a common precipitinogen was possessed by all strains of mycobacteria. This finding, then, precludes the grouping of the strains on the basis of antigens possessed in common by more than one species of a certain genus.

The shortcomings of other systems for classifying atypical mycobacteria, in part, prompted this study. From the results presented, it is clear that the many variations in the biochemical, morphological, and growth characteristics of the "atypicals" are accompanied by a similar lack of harmony in the precipitinogenic relationships. The extreme adaptability and mutability of the mycobacteria continue to be apparent from all approaches of study. It is probable that a knowledge of the antigenic relationships will never,

in itself, solve all of the problems of classifying the mycobacteria. Such information, nevertheless, when evaluated with data obtained from other approaches of study, may well provide for a more meaningful classification system than presently exists.

The results reported in this study showed that "atypicals" of soil, porcine and bovine origins were related to M. phlei, M. fortuitum, and to the classical mammalian and avian strains. However, with few exceptions, each "atypical" was more closely related, antigenically, to one known strain than to others. On this basis alone, it has been possible to place the "atypicals" in six new groups (Table 13).

The "mammalian-like" strains were placed in Group A; the "avian-like" strains, in Group B; the "phlei-like" strains, in Group C; the "fortuitum-like" strains, in Group D; the strains related equally to two or more known strains, in Group E; and the strains non-related antigenically to the known strains, in Group F.

Group A consisted of but three strains, all of mammalian origin (one bovine and two human), and each was from a different Runyon Group (I, II, and III). Two strains were virulent and the other, avirulent for guinea pigs. The

three isolants each induced a different type of hypersensitivity in guinea pigs (mammalian, avian/battey, and non-definitive).

Group B consisted of 18 strains: a single Group III human isolant, 2 Group III bovine isolants, 12 Group III swine isolants, 2 Group IV bovine isolants, and a pseudochrome of porcine origin. Of the 18 strains, 16 were virulent and induced hypersensitivity in guinea pigs; 12 strains induced avian, 3 mammalian, and 1 induced avian/battey sensitivity.

Group C consisted of 10 strains: 3 soil isolants and a bovine isolant from Runyon Group II; 2 bovine isolants from each of the Runyon Groups, III and IV; and 2 pseudochromes of bovine origin. Four strains in this group were virulent and caused hypersensitivity in guinea pigs, (2 induced avian and 2, non-definitive sensitivity).

Group D consisted of 5 bovine isolants: 3 from Runyon Group III and 2 from Group IV. Only the 3 Group III strains were virulent and induced hypersensitivity in guinea pigs; one strain induced avian and the other two induced non-definitive sensitivity.

Group E consisted of 3 strains: a Group III of bovine origin, a Group IV of human origin and a pseudochrome of

bovine origin. The 3 strains were virulent and induced hypersensitivity in guinea pigs; the pseudochrome induced avian and the other two, non-definitive sensitivity.

Group F consisted of R-P4, a Group I strain of human origin, and 63A-0, a pseudochrome of bovine origin. Both strains were virulent and induced hypersensitivity in guinea pigs; the Group I isolant induced mammalian sensitivity, and the pseudochrome induced avian/battey sensitivity. The two strains were relatively non-reactive with the reference antisera; R-P4 was related only to R-P8, and 63A-0 was related only to R-P39.

This grouping is not presented to suggest that it would be either a practical or a meaningful system for classifying mycobacteria, but rather, to show the heterogeneity that exists within the atypical strains of varied origins. The strains placed in the various groups in Table 14 shows definite patterns of similarities in their morphological (Runyon Grouping), virulence, and hypersensitivity characteristics, but no clear-cut positive correlation of these characteristics with the new grouping is noted.

It is clearly shown here that atypical isolants of cattle, swine, and soil origins have greater antigenic similarities with a known saprophyte (M. phlei), than has been

reported for atypical human isolants. In a study of 98 strains, primarily of human origin, Parlett and Youmans (1958) reported, "The clear-cut lack of antigenic similarity between the 'atypicals' and the saprophytic mycobacteria demonstrates that this simple double-diffusion method [Agar plate] will provide an easy method for the differentiation between these two groups."

It is clear, and to be expected, that the above does not apply to "atypicals" of bovine, porcine, and soil origins. In soil one should expect to find some saprophytes having characteristics that are identical to those of M. phlei, and others with characteristics that are dissimilar in varying extents to those of M. phlei. Also, pathogenicity and virulence are such relative factors in infection, and are interrelated with host resistance and the size of inoculum. It is highly conceiveable, then, that cattle and swine, in their natural habitats, could be exposed to such huge numbers of saprophyte-like mycobacteria that these could develop lesions.

SUMMARY

The precipitinogenic relationships of 49 representative mycobacteria of human, bovine, porcine, and soil origins were compared by use of an agar slide immunodiffusion technique. Concentrated culture filtrate antigens from 49 strains were employed; 12 of them were used to prepare reference antisera. The 12 strains included the classical pathogens and saprophytes, and "atypicals" of human and animal origins.

Antigenic dissimilarities exist between species and groups, and in addition, between strains within a group. No antigenic differences were observed between the six precipitinogens possessed by each M. bovis (Ravenel) and M. tuberculosis (strains H37Rv and H37Ra). They were related to other known species as follows: one was identical to an antigen of M. avium; one was related and one identical to antigens of M. fortuitum; and none were related to M. phlei and M. smegmatis.

In all tests, the homologous antigenic preparations and antisera reacted to give equal or greater numbers of precipitate bands than did heterologous systems.

The Group II strains of bovine and soil origins were closely related to M. phlei, but had no antigens in common

with M. tuberculosis or M. bovis. In contrast, a Group II strain of human origin possessed two antigens identical to those in the latter strains, and one antigen related to M. phlei.

Very few of 22 strains in Group III (predominantly of bovine and porcine origins) were related antigenically to either of the Group III reference strains of human and bovine origins (58A-1). Eight of nine strains of bovine origin contained at least one antigen related to M. avium. Only one of these eight, however, was more closely related to M. avium than to other reference strains. In contrast, all Group III strains of swine origin were closely related, but not identical, to M. avium.

The pseudochromes and the strains in Group IV were even more heterogeneous antigenically than the Group III organisms. Few Group IV strains and pseudochromes were related to M. bovis and M. tuberculosis; most of them were closely related to M. phlei.

A new grouping of "atypicals," on the basis of their antigenic relationships to known mycobacteria, has been presented. This grouping does not, however, solve the problem of classifying these organisms.

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