CLASSIFICATION OF MYCOBACTERIA BY THE AID OF SKIN REACTIONS

by

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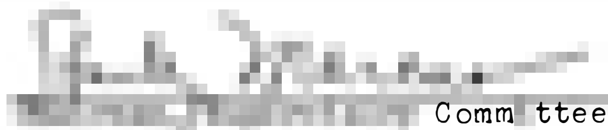

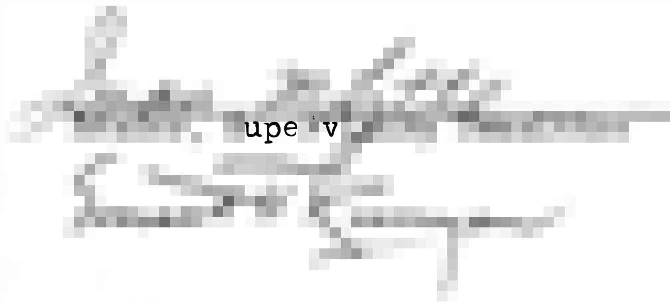

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INTRODUCTION

Reports of studies in several laboratories concerning mycobacterial isolates have made it apparent that mycobacteria other than human and bovine tubercle bacilli are the causative agents of human disease. These published reports have dealt mainly with the cultural and morphological characteristics of these strains.

In order to give species names to previously undescribed mycobacteria, it is necessary to study and relate similar characteristic properties. Investigation of these properties presently includes studies such as fermentation, serologic typing (precipitation, agglutination), phage susceptibility, nutrient requirements, drug sensitivity, cultural, and morphological characteristics.

The study of skin reactions elicited by purified protein derivatives (PPD) prepared from strains of mycobacteria, when used in animal experiments, may aid in identifying the antigenic properties of these mycobacteria. The method consists briefly of injection of guinea pigs with the organisms under investigation. Six weeks later the animals are skin tested with a battery of PPDs prepared from various known strains of mycobacteria. Identification of the unknown strain is made by the observation that the homologous antigen usually produces a larger reaction than the heterologous antigen.

The study of the metabolic activities of mycobacteria has yielded inconsistent results. These results may be due to the slow metabolic rate of most acid-fast strains. It has been proposed that these difficulties may be overcome by the use of an extremely heavy inoculum of actively growing organisms.

Although these recently recognized mycobacteria are being investigated in many laboratories, very little is known about their source in nature. No person-to-person transmission has ever been reported. The widespread occurrence of both disease and hypersensitivity due to more than one species of mycobacteria suggests that these organisms may be able to survive outside of the hosts. Studies of strains isolated from soil and water indicate that mycobacteria do exist in the environment. The isolation of mycobacteria from the sputum or saliva of a portion of a healthy adult population indicates a possible "carrier" state. The possibility that infection from lower animals might occur should not be overlooked.

The purpose of this thesis is to report the cutaneous reactions to multiple PPD antigens in guinea pigs injected with various strains of mycobacteria. Also reported are various metabolic reactions of mycobacteria using a heavy-inoculum technique. The relationship of organisms found in nature with those isolated from disease is also reported.

LITERATURE REVIEW

I. EARLY HISTORY

During the seventeenth century tuberculosis was referred to as the "White Plague." John Bunyon described it as "The Captain of all these Men of Death." From Topley and Wilson (1955) we get the following sequence of early discoveries with acid-fast bacteria. In 1868 Hansen described the first member of this group, the leprosy bacillus. Koch, in 1882, discovered the mammalian tubercle bacillus. Theobald Smith, in 1898, demonstrated that the mammalian tubercle bacilli could be divided into two types, human and bovine. The discovery of the avian tubercle bacilli was credited to Rivolta, Maffuci, Cadiot, Gilbert, and Roger prior to 1900. Although Smith had clearly demonstrated the two types of mammalian tubercle bacilli, there was much controversy concerning the infectious nature of these types.

John Francis (1959), reviewing the work of the British Royal Commission on Tuberculosis, sets apart the reason for the controversy by quoting Robert Koch.

> . . . Though the important question whether man is susceptible to bovine tuberculosis at all is not yet absolutely decided, and will not admit of absolute decision today or tomorrow, one is nevertheless already at liberty to say that if such a susceptibility really exists, the infection of human beings is but a very rare occurrence. I should estimate the extent of infection by the milk and flesh of tuberculous cattle

> and the butter made of their milk as hardly greater than that of hereditary transmission, and I, therefore, do not deem it advisable to take any measures against it.

This statement from the discoverer of the tubercle bacillus caused much concern by the medical people of that time. A royal commission was created to study the problem concerning the transfer of infection from animals to man and from man to animals. It was the opinion of the commission after ten years' research that these transmissions did occur and were the causes of many deaths, especially to infants and children.

Shortly after this investigation, legislation enforcing inspection and testing of cattle was passed. This along with other public health precautions caused the rate of tuberculosis to decline. These reports are not intended to stress the eradication of tuberculosis but rather to emphasize that early workers recognized that mycobacteria other than *M. tuberculosis* might cause disease in man.

During the following years there was little work done on acid-fast strains other than human and bovine bacilli. Cobbett (1918) reported an atypical acid-fast organism from an injured soldier. Rabinowitsch-Kempner (1927) at a session on the *Biology of the Tubercle Bacillus*, presented the following ideas: Human and bovine strains of tubercle bacilli cause pulmonary disease in man. Occasionally human

tuberculosis is caused by strains of tubercle bacilli resembling the avian type. Atypical strains occur frequently in cases of tuberculosis of the skin (Lupus). She recommended a further study of the atypical strains. Freeman (1938) reported the isolation of mycobacteria from abscesses of two patients. Neither microorganism fits the description of a previously recorded *Mycobacterium*.

II. RECENT INVESTIGATIONS

Acid-fast organisms other than the human and bovine strains of tubercle bacilli have been demonstrated to be the cause of pulmonary disease. Feldman and associates (1943) described an organism isolated from a patient with a long history of pulmonary disease. This strain of mycobacteria was similar to *Mycobacterium avium* morphologically but was not typical of *Mycobacterium tuberculosis* and *Mycobacterium bovis*. It differed from *M. avium* antigenically. This organism, not of the human or bovine type, may have been the first described which was a cause of human pulmonary disease. It is probable that this was the first description of what we now refer to as the Group III strains.

Buhler and Pollak (1953) described 2 cases in which pulmonary diseases resembling tuberculosis were caused by an atypical, yellow, acid-fast organism. They reported that the intensity of the pigment became deeper when the culture

was incubated at room temperature.

Chofnas and Newton (1955) reported isolation of chromogenic acid-fast bacteria from 10 patients. The strains were avirulent for guinea pigs and more resistant to chemotherapeutic agents than tubercle bacilli.

Crow and co-workers (1957), at the Battey State Hospital in Rome, Georgia, studied 60 patients with pulmonary disease (Battey disease) who produced sputum containing "atypical" acid-fast bacilli. The patients were admitted over a five-year period and comprised about 1 per cent of the hospital admissions. By using cultural morphology, 90 per cent of these organisms were classified as nonphotochromogens. These organisms were more resistant to antituberculosis drugs than tubercle bacilli. In all cases no more than one person in a family had the disease. This would indicate the mode of transmission is not person-to-person contact; however, the study was rather small to warrant such a conclusion.

Engback *et al*. (1957) reported a fatal case of lung disease indistinguishable from pulmonary tuberculosis. They concluded from various bacteriological tests that the organism belonged to the ever-growing assembly of unclassified mycobacteria. Engback *et al*. (1959) reported another case of lung disease caused by a similar atypical acid-fast organism.

Timpe and Runyon (1954) studied atypical strains from 88 patients. Some of these strains appeared to be associated with pulmonary disease, although none was virulent for guinea pigs. They also showed that strains which produced pigment only at room temperature were more often associated with pulmonary disease than other unclassified mycobacteria.

Hardy et al. (1958) reported the bacteriological findings of "atypical" acid-fast organisms for a period of two years. During this time these bacilli were isolated from 108 patients with pulmonary disease. The organisms were distinguished from tubercle bacilli by the catalase reaction, growth characteristics, or pigmentation. Of the hospitalized cases 67 per cent were males. The presence of disease in the Negro was relatively rare. These workers could not demonstrate person-to-person transmission nor could they find organisms from the soil which could be implicated as the cause of disease.

Runyon (1959) classified these anonymous mycobacteria into four groups. The data for this classification were gathered by a VA-NTA survey. The summary of this paper follows:

1. The guinea pig test is insufficient to indicate the pathogenic potential of acid-fast bacilli for man.

2. Clinical, radiological, and histological studies are insufficient to differentiate pulmonary disease due to

various acid-fast organisms.

3. Four groups of these anonymous organisms can be distinguished from tubercle bacilli by drug resistance, catalase activity and capacity to grow at room temperature.

4. Groups I and III are more pathogenic. They can be distinguished from each other by the presence (Group I) or absence (Group III) of light-conditioned pigmentation; i.e., photochromogenicity.

5. Group II bacilli, the yellow-orange scotochromogens, occur occasionally in specimens from any source but are rarely associated with pulmonary disease.

The classification system as proposed by Runyon was not meant to be permanent, but rather to serve until species names should become available.

In Group III we probably have multiple species, one of which is *M. avium*. It is controversial whether the strains that cause disease in man and strains isolated from various animals are or are not distinct species. Strains from soil or water, included in Group III, have also been questioned as possible sources of Battey disease.

Froman *et al*. (1961), using bacteriophages, investigated the relationship of strains isolated from animals and man. They believed that man and swine were infected by the organisms. These organisms are *M. avium* or closely related Group III strains. These workers did not solve the problem

of whether man and swine are infected from the same source or if swine serve as a source of infection for man.

Scammon and associates (1963) examined mycobacterial strains isolated from swine, avian and human sources. These organisms were identified as Group III, and all were closely related as to growth characteristics, niacin content, resistance to antituberculosis drugs, and in eliciting delayed hypersensitivity. They appeared to be different in virulence for chickens although the difference was only a matter of degree.

A pleomorphic organism, tentatively called *Mycobacterium X* isolated from mouse brain was characterized by Vischer and co-workers (1955). It was suggested that *Mycobacterium X* represents an attenuated type of avian tubercle bacillus. Suter and Strain (1959) investigated the immunogenic properties of this strain. This organism induced infection immunity which was slightly inferior, to the state of resistance induced by vaccination with BCG.

The antigenicity of mycobacterial strains isolated from patients and dogs in Japan was investigated by Takeya *et al*. (1961). Guinea pigs were injected with 6 mg of heat-killed bacilli of each test organism. The cells were suspended in liquid paraffin oil and injected intramuscularly. About 6 weeks after injection, the guinea pigs were tested with a purified protein fraction (TT) of each organism. As expected

the animals reacted most strongly to the purified protein of the homologous strain used for sensitization. There were, however, considerable cross reactions with other mycobacterial antigens. The reaction to human Tr by guinea pigs sensitized with nonphotochromagens isolated from dogs was on the average about 7 mm of induration. The average reaction to human Tr of guinea pigs sensitized with nonphotochromogens isolated from patients was about 11 mm of induration.

Kubica *et al*. (1961) sampled soil and water for the presence of acid-fast bacilli in two areas of Georgia where: (1) tuberculin surveys of the area had been carried out, or (2) a large percentage of tuberculin positive cattle brought to slaughter fell into the "no gross lesion" category. More than 1,200 samples of soil and water were collected. Almost 50 per cent of the samples studied yielded acid-fast bacilli; most of these organisms were of the unclassified group. No tubercle bacilli were found. The concluded,

> . . . From all indications, it appears that many of the unclassified mycobacteria may reside in the soil, being carried from there to water supplies, and thence to man or animals, either by inhalation or ingestion.

Edwards and Palmer (1959) reported that certain strains of mycobacteria could be isolated from the saliva of healthy people. This raised the question of a "carrier" state in man which may be either transitory or permanent.

Takeya and associates (1962) investigated the

epidemiology of the unclassified mycobacteria in Fukuoka Prefecture, Japan. They found a very low incidence of sensitivity to the skin test antigens (π) prepared from the unclassified mycobacteria. It was also found that in a population which had been kept in contact with a patient suffering from unclassified mycobacterial infection, the positive rate for "unclassified" π was much higher than the normal population, thus indicating a possible mode of transmission, at least of hypersensitivity. The protein component π and its potency per unit weight was uniform irrespective of the strain of mycobacteria from which it was prepared (Zinnaka et al., 1962).

The possible transmission of photochromogen disease by person-to-person contact was investigated by Eckman et al. (1961). Although the study was small, it indicated that family contacts of patients with M. kansasii infections show a significantly higher incidence of skin sensitivity to both tuberculin antigen (PPD-S) and to photochromogenic antigen (O.T. type). The incidence of reactors to M. kansasii antigen among family contacts correlated with the incidence of reactors from contacts of patients with active tuberculosis. They conclude, "It would seem that the spread of typical tuberculosis and infections due to photochromogenic bacteria may occur in the same manner." Crow and co-workers (1961) determined the skin sensitivities of spouses and household

contacts of 158 patients admitted to the hospital with demonstrable pulmonary lesions due to nonphotochromogenic mycobacteria. Among 458 household contacts, 27 per cent were positive to OT or PPD. Among 269 household contacts tested with PPD-S and PPD-B (the skin test antigen prepared from nonphotochromogenic mycobacteria) only 15 per cent were positive to PPD-S and 35 per cent were positive to PPD-B. From this evidence they concluded that the source of infection might be environmental, and that the organisms may be avirulent to man except under certain circumstances or predisposing conditions.

Further testing of household contacts of patients with pulmonary disease caused by mycobacteria other than *M. tuberculosis* by Chapman *et al*. (1962), revealed that evidence was not conclusive in regard to person-to-person transmission. Chapman (1962) reported in an editorial, "Communicability of mycobacterial infection has not been established."

Edwards *et al*. (1959) reported, concerning dose of tuberculin, "Reactions to the 5 TU dose of tuberculin are largely a result of tuberculous infection but most reactions to a large dose are cross reactions caused by infection with other organisms." Edwards *et al*. (1960) reported that guinea pigs infected with different mycobacteria often show cross sensitivity to heterologous mycobacterial antigens; usually the cross reactions are smaller than the homologous reaction.

Edwards *et al.* (1961) further reported on the skin sensitivity of guinea pigs infected with different mycobacteria. Most strains of any particular group induced like skin sensitivity to the PPD antigens used in the testing. They called these reactions sensitivity profiles.

III. BIOCHEMICAL TESTS

Tsukamura (1960) demonstrated that picric acid can be reduced enzymatically to picramic acid. Later (1961) he demonstrated that mycobacteria could be divided into two groups with respect to their activity in reducing picric acid. The first group consisted of *M. tuberculosis*, including drug resistant strains, and *M. phlei*. The second group reduced picric acid at a higher rate than did the first.

The urease activity of 65 strains of mycobacteria was investigated by Singer and Cysner (1952). All except one of the strains including 11 true tubercle bacilli, 3 cold blooded types, and 44 paratubercle types hydrolized urea; *M. pelligrino* did not. They suggested for diagnostic purposes that strains which show ammonia production in six hours or less not be considered true tubercle bacilli. However, the slow hydrolysis of urea was not proof that the strain concerned was M. tuberculosis. Toda *et al.* (1961) tested 151 strains of mycobacteria for the presence of urease. The urease positive strains were human, bovine, so-called "avian,"

and photochromogens. Some of the scotochromogens and most of the saprophytes were also positive. On the other hand, all avian strains, nonchromogens, some scotochromogens, and a few saprophytes were negative in the test. Bonicke (1961) using a low concentration of urea substrate investigated the urease activity of many mycobacterial strains and species. He was able to divide them into three distinct groups. A group with high enzymatic activity included the rapidly growing mycobacteria of the species *M. smegmatis* and *M. phlei*. The second group with moderate urease activity includes *M. fortuitum*, *M. tuberculosis*, *M. bovis*, *M. marinum*, *M. balnei*, *M. thamnopheos*, Group I and Group II. The third group included the strains which were not capable of enzymatic urea breakdown, viz.: *M. avium*, *M. ulcerans*, and the avium-like mycobacteria (Group III nonphotochromogens) and some of the Group II strains. Bonicke subdivided the Group II scotochromogens into Group IIa (urease-positive) and Group IIb (urease-negative).

Whitehead and associates (1953) studied an enzyme, arylsulfatase, that is capable of splitting the bond between the sulphate group and the aromatic ring in compounds having the general formula $ROSO_3H$ where R represents the aromatic ring structure. The substrate potassium phenolphthalein disulfate is hydrolyzed. The phenolphthalein liberated by this enzymic hydrolysis can be detected by the addition of

alkali. Evidence was presented that arylsulfatase is widely distributed among mycobacteria. One hundred fifty-four strains were studied. It was possible to divide them into two groups. All the strains of M. tuberculosis, most of the strains of M. bovis, together with one murine strain and a few saprophytes failed to hydrolyze the substrate. All of the other pathogenic strains (cold-blooded, murine, avian, and bovine types) hydrolyzed the substrate. Wayne and co-workers (1958) studied the arylsulfatase activity of some mycobacteria. They found that almost all species of mycobacteria hydrolyzed demonstrable amounts of phenolphthalein disulfate if sufficient inoculum was used. Kubica and Vestal (1961) investigated the arylsulfatase activity of 73 strains of acid-fast bacilli. The data indicated that avian and mammaliam tubercle bacilli could be differentiated from other mycobacteria. Kubica and Beam (1961) further investigated the arylsulfatase activity of M. avium and Group III strains. The M. avium strains lacked arylsulfatase activity as contrasted to variable but definite enzmatic activity of other Group III strains. In a study of 24 unknown cultures they correctly typed the strains with an accuracy of 96 per cent. Wayne (1961) found that the rate of phenolphthalein sulfatase activity of M. fortuitum on a solid medium was greater than that of other rapidly growing mycobacteria. All the strains of M. fortuitum tested (68) were positive for

enzyme activity in 3 days. Strains of M. smegmatis, M. phlei, M. rhodochrous, and miscellaneous rapid growing strains were all negative at 3 days.

Wayne (1962) reported a simple qualitative test to determine the degradation of Tween 80 by mycobacteria. He tested 172 strains including Group II scotochromogen, Group III battey, "Radish," swine, and avian subgroups. About half of the Group II strains became positive in 2 weeks, none of the Group III battey, avian or swine strains became positive; however, the "radish" strains became positive in 48 hours. He concluded that this test might be incorporated into a simple scheme to aid in the identification of mycobacteria.

In Bergey's Manual of Determinative Bacteriology (1957) we find data on formation of nitrite from nitrate in 5 mycobacterial species, M. phlei, M. smegmatis, M. fortuitum, M. thamnopheos, and M. marinum. No data is available from this source concerning clinically important strains.

Virtanen (1960) investigated the nitrate reducing activity of 842 strains of mycobacteria. He found that strains of the human type show a distinct reducing effect on nitrate. There was no difference in this respect between isoniazid sensitive and isoniazed resistant strains. The attenuated-virulent strain R1Rv and the avirulent strains R1Ra and H37Ra, behaved like virulent strains. M. bovis,

BCG strains and BCG-like human strains either slowly reduced nitrate or did not reduce it. The avian strains and some of the strains of "atypical" mycobacteria, demonstrated distinct nitrate reductase activity. Bonicke (1961) confirmed the work of Virtanen. Bonicke also further studied the nitrate reductase activity of the Runyon Groups (studied by Virtanen only as a single group). The strains of Group II were wirh-out reductase activity, all avian strains were negative as were the strains of Group III. The Group I strains were positive.

Sweeny and Jann (1961) investigated the carbohydrate utilization of mycobacteria. They used a massive inoculum technique and found that Group III "Battey" strains utilized trehalose but were xylose negative, in contrast to avian strains which were xylose positive and trehalose negative. Bojalil and Cerbon (1960) studied carbohydrate utilization of *M. avium* and nonphotochromogens using growth, and the change of bromcresol purple indicator to represent positive reactions with some strains of each type of organism. The majority of each strain failed to utilize any of the carbo-hydrates tested.

Schweiger *et al*. (1958) tested virulent and avirulent strains of tubercle bacilli for the presence of catalase under varying temperatures and pH. They found that virulent organisms still produced catalase at pH 1 and temperatures

greater than 60°C. The avirulent strains (except M. phlei) did not produce catalase at pH 1 or at temperatures above 60°C. They suggested that the rate of catalase activity at pH 1 and 60° might be useful as a measure of mycobacterial virulence. Kubica and Gleason (1960) investigated the catalase activity of 100 stock and recently isolated wild strains of mycobacteria at room temperature and 60°C. With the exception of 4 isoniazid-resistant strains, all cultures exhibited catalase activity at room temperature. It was found that the catalase enzyme of human and bovine strains (regardless of virulence) was inactivated by suspending the cultures in a phosphate buffer pH 7 which was then held in a 68°C. water bath for twenty minutes. All other cultures retained their catalase activity under these conditions. They suggested that further studies of this enzymatic activity might prove useful as a means of subgrouping the acid-fast bacilli.

Pope and Smith (1946) studied one strain each of M. tuberculosis and M. bovis. They found that nine vitamins including nicotinic acid were synthesized by both strains. Appreciable differences were observed in the amount of nicotinic acid synthesized by human and bovine strains. Konno et al. (1957) further reported that there was a striking difference in the nicotinic acid synthesis between human and other types of mycobacteria. Human tubercle

bacilli produced more than 13.5 gamma per ml of culture filtrate while the other mycobacteria did not exceed 2.0 gamma per ml. Konno *et al*. (1958a) compared nicotinic acid production by a bioassay technique and a direct chemical method. The two methods gave similar results. The niacin production of human tubercle bacilli was ten times that of atypical acid-fast bacilli. Konno *et al*. (1958b) studied 26 unknown strains. But the use of the niacin test all were correctly reported. These workers also reported the value of the niacin test in a study of 663 routine cultures from 9 hospitals. All of the strains but 6 were classified as tubercle bacilli. Konno (1956) devised a simple modification of the test for the presence of niacin. It consists briefly of adding a few colonies of the organism to 1 ml of 10 per cent cyanogen bromide. A positive test shows the development of an intensive canary-yellow color. Konno (1959) modified the niacin test so routine testing of mycobacteria could take place. The organisms were transplanted from solid slants to Dubos tween-albumin liquid medium. The aniline cyanogen bromide test for niacin was positive after ten days' growth. Runyon *et al*. (1959) described a modified niacin test applicable to mycobacterial cultures on solid medium. Niacin was extracted from the cultures within 5 minutes by layering with water. The extract was then tested for the presence of niacin. The results were comparable with those reported by

Konno (1958b) and false-negative results appeared to be eliminated.

Konno et al. (1960) reported that bovine tubercle bacilli reveal significantly lower nicotinamidase activity than other mycobacteria.

Nagayama et al. (1961) found an enzyme, formamidase, which occurs only in saprophytic mycobacteria. This enzyme catalyses the formation of ammonia from formamide.

Bonicke (1962) in a review on the biochemistry of mycobacteria reported a classification scheme based upon the deamination of 9 amides and urease activity. The Group I photochromogens deaminated nicotinamide and hydrolyzed urea. None of the 36 strains tested deaminated the other amides. Forty Group II scotochromogens were tested, 24 of them were capable of deaminating only urea. Sixteen strains were incapable of deaminating any of the 10 amides. Twenty-one strains of M. avium and 38 strains of Group III nonphotochromogens were tested. The results were similar. Both were able to deaminate nicotinamide and pyrazinamide. They were unable to deaminate the other amides. In view of the results Bonicke regarded only Group I (M. kansasii) as a new species. Because of their uniform enzymatic equipment, the Group III nonphotochromogens were grouped as varieties of M. avium.

IV. BACTERIOPHAGES OF THE MYCOBACTERIA

Cater and Redmond (1961) isolated more than forty bacteriophages lytic for saprophytic mycobacterial strains. The authors reported a need for bacteriophages with specific activity for the unclassified mycobacteria.

> . . . Although the DS_2 phage per se has little practical significance the method used in the adapting and isolating of it could lead to the isolation of phages specific for more important mycobacteria, such as the unclassified groups, and to better understanding of the factors involved in adaptation of phage.

Ward and Redmond (1962) observed, in studies of mycobacteria, examples of a clearing reaction not due to true phage lysis. This false positive reaction was due to heavy concentration of phage rather than to lytic activity. They concluded

> . . . In view of these results it appears that there is considerable question regarding the value of many of the results reported from tests of phage-typing mycobacteria, especially when only heavy concentration of phage produced clearing and no production of plaques was observed.

Redmond (1963) reported mycobacterial phages isolated from soil, from the digestive tract of mosquito larvae, from mycobacteria (lysogenic), and from human stool specimens. Many of the phage strains have wide host ranges. Bacteriophages have also been found to be active upon both pathogenic and nonpathogenic strains of mycobacteria. Redmond and Carter (1962) isolated four bacteriophages from stool specimens of patients. They used these and three other strains in a phage

typing scheme to identify mycobacteria important in human disease. *M. kansasii* was lysed by one strain making it possible to differentiate this species from other mycobacteria. One phage lysed only the human but not the bovine species. In general phage typing has not been successful in the classification of mycobacteria.

V. VIRULENCE TESTING

Nearly every report of isolations of previously unnamed mycobacterial species included data concerning the virulence of these organisms. As previously noted the unclassified mycobacteria do not cause disease in guinea pigs unless given in extremely high concentration. The work of Kubica *et al*. (1960) is of particular interest. They compared intracutaneous, subcutaneous, and intracardial routes of challenge for guinea pigs. The intracutaneous route appeared to be the more adequate measure of the apparent virulence of isoniazid resistant and "atypical" acid-fast bacilli for man. The degree of virulence was measured according to the severity of the lesion formed at the injection site. These results might indicate a possible test for a classification of mycobacteria.

VI. COLONY MORPHOLOGY

Runyon and Johnson (1961) reported the formation of

multibacillary structures in agar gel beneath the colonies of *Mycobacterium* species (*avium*, *smegmatis*, *fortuitum*, *kansasii*) and of the groups of the unclassified mycobacteria. These structures appeared to be homologous with the submerged mycelium of *Nocardia* but not with the rhizoids of other organisms. They proposed the shortened form rhizode for these multibacillary formations. These structures appeared an untrustworthy means of distinguishing *Nocardia* and *Mycobacterium* species. Fregnan *et al*. (1961) investigated the colony types of *M*. *kansasii* and *M*. *fortuitum*. They demonstrated a relationship between colony morphology and mycoside content of these 2 species.

VII. SEROLOGIC TESTS

Parlett and Youmans (1959) reported a gel double diffusion (GDD) test for the detection of antibody to mycobacterial antigens in the serum of human beings. Parlett and Rehr (1959) reported on further studies to standardize the gel diffusion tests for the detection of mycobacterial antibody. Parlett (1962) used a GDD test to determine the antibody titer of patients with tuberculosis. A total of 936 serum specimens were studied. Of this series 84 per cent of the sera were found to be GDD test positive. In regard to the specificity of the procedure, depending on the age and history of the normal populations studied, the average range

of error was between 5 and 15 per cent. In hospital employees 24 to 50 per cent gave "false positive" results. This raised a question of whether or not antibody might be present in serum from healthy people as a result of naturally acquired immunity.

Beck (1961) investigated the antigenic relationship of acid-fast bacteria. Antisera were prepared against the organ isms in rabbits, and their specificity was investigated by absorption and inhibition techniques. Atypical mycobacteria were found to differ antigenically from saprophytic mycobacteria such as _M. phlei_ and _M. smegmatis_, although they share an antigen with them. Further, the experiments suggested that human tubercle bacilli and photochromogenic (Group I) bacilli have a common antigen.

The agglutination reactions of avian and Group III strains have been investigated by Schaefer (1963). Thirty-seven strains of _M. avium_ isolated from birds, swine, or cattle were classified into 3 serotypes. Among 54 battey strains, 17 strains were serologically indistinguishable or closely related to 2 _M. avium_ types. Nineteen strains belonged to 4 other serotypes. The remaining 18 strains belonged to 14 additional serotypes. The close relationship of Battey and avian strains was demonstrated. Also demonstrated is the heterogeneity of the Group III strains.

Hemagglutinating antibodies present in the sera of

patients with active tuberculosis have been demonstrated by Middlebrook and Dubos (1948). The active extract appeared to be a component of a polysaccharide fraction of mammalian tubercle bacilli. Boyden (1951) described a hemagglutination technique in which tuberculin PPD was adsorbed into erythrocytes pretreated with tannic acid.

Takahashi and Ona (1961a) found a phosphatide fraction of M. tuberculosis capable of sensitizing normal sheep erythrocytes to hemagglutination in the presence of tuberculous serum. These workers (1961b) compared several methods of testing. They found an antigen to saline drop method for preparing the sensitizing emulsion gave the most stable suspension of phosphatide. A comparison of three different serologic tests; hemagglutination, complement fixation, and precipitation tests, using the same antigen revealed that the hemagglutination test was the most sensitive.

Takahashi et al. (1961c) found the level of production of three different kinds of antibody, antipolysaccharide, antiprotein, and antiphosphatide, differed considerably with the mode of infection and the virulence of the bacilli. They gave evidence that production of the antipolysaccharide, and antiprotein was stimulated without regard to the mode of infection or virulence of the organism. The antiphosphatide was produced chiefly under conditions where tubercle bacilli might have undergone destruction. The level of

antiphosphatide was found to reflect most faithfully the progression of experimental tuberculosis infection. Takahashi *et al*. (1961d) confirmed the above results in a study of patients with tuberculosis.

Takahasi (1962) sensitized a kaolin particle suspension with a methanol-extracted phosphatide fraction of *M. tuberculosis*. He compared kaolin agglutination with hemagglutination and found the phosphatide kaolin-agglutination test a reliable quantitative method in the diagnosis of polmonary tuberculosis in man. Freedman *et al*. (1963) demonstrated that washed peripheral leucocytes of all adult human subjects studied contained hemagglutinating antibodies to PPD. Subjects with positive tuberculin skin tests had significantly higher titers of these "cell fixed" hemagglutinating antibodies to tuberculin PPD than did tuberculin skin test negative subjects. They indicated that these findings reflected minor degrees of tuberculin hypersensitivity not detected by skin testing. This sensitivity might be due to cross reactivity with mycobacteria other than *M. tuberculosis*.

VIII. TUBERCULIN TEST

In an address delivered before the *Tenth International Medical Congress* held in Berlin, August 4, 1890, Robert Koch (1890a) announced that he had found the substance by means of

which the tubercle bacillus could be rendered inert, not only when growing in artificial media, but also when developing in guinea pigs. Koch (1890b) later described the fluid and the method of injection. He also described the effect of the substance in the human subject. In the tuberculous patient a marked general and local reaction occurs. The substance had little effect on the healthy subjects. The general reaction consists of an attack of fever, articular pains, cough, great prostration, often nausea and vomiting, and sometimes icterus. Koch (1891) disclosed that the fluid was a broth culture filtrate of tubercle bacilli to which he gave the name tuberculin. In this report he also described the process which has later been called Koch's phenomenon.

> . . . If a healthy guinea pig is inoculated with the pure cultivation of the tubercle bacilli, the inoculation wound is generally closed by a viscid exudation, and at first appears to heal up. In from ten to fourteen days a hard nodule presents itself, and this soon breaks down and forms an ulcerating sore, which becomes deeper and deeper until the death of the guinea pig occurs. On the other hand, if a guinea pig already suffering from tubercle is inoculated, quite a different condition of things will result. In such animals the exudation at the seat of inoculation assumes the same viscid appearance to begin with, but no nodule forms. On the contrary, the part on the second day becomes hard and assumes a darker color, which spreads to the neighboring parts until a diameter of 0.5 cm to 1.0 cm is reached. Within a few days the skin at the affected part becomes more obviously necrotic, and then desquamates, leaving a shallow ulcer, which generally heals with rapidity without infecting the neighboring lymphatic glands.

Although the use of tuberculin as a cure for tuberculosis soon fell into disrepute, its use as a diagnostic agent

showed much promise. Edwards (1960) wrote, "The method of preparing old tuberculin, as Koch's fluid is known today, is still essentially the same as Koch described it in 1891."

The use of tuberculin as a diagnostic agent led to the development of new methods of administration. These methods include a cutaneous test (Moro), a conjunctival test (Calmette), a modification of Koch's original subcutaneous test (Hamburger) and an intracutaneous test (Mantoux). Three methods have survived: The Pirquet cutaneous test, the Moro patch (percutaneous), and the Mantoux intracutaneous test. The intracutaneous test is the most widely used and precise test.

Krause (1916) suggested that cutaneous hypersensitivity to tuberculo-protein is inaugurated by the establishment of infection and the development of the initial focus. That is, "A positive cutaneous test means infection or the presence of tubercle, although the disease tuberculosis does not of necessity exist."

Doubt about the specificity of the tuberculin test arose in the United States during the 1930's. Pulmonary calcification was often accompanied by a negative tuberculin test. This led to the investigations which showed *Histoplasma capsulatum* to be a common cause of pulmonary calcification. This organism was found in the regions where the tuberculin test had seemingly failed.

Calmette (1925) submitted evidence to show that non-toxic paratubercle bacilli are capable of sensitizing the body. Crawford (1926) injected a variety of cultures of acid-fast bacteria including the Moller timothy-hay bacillus; hog-skin bacillus, Friedman turtle bacillus, smegma bacillus and others introabdominally in guinea pigs. Thirty days later the animals were skin tested with mammalian and avian tuberculin. Although the reactions were not consistent, Crawford concluded that in general the skin was sensitized more frequently to mammalian tuberculin than to avian tuberculin, in one group, however, the sensitization was practically the same for both. Crawford (1927) later reported a technique for differentiating atypical and avirulent strains of tubercle bacilli. This he termed the sensitization method of differentiation. He injected human, bovine, BGG, and four avian strains of tubercle bacilli. He then tested the guinea pigs with antigens made from these organisms. He found the reactions to homologous antigen were invariably well marked and constant, while reactions to heterologous antigens tended to be less marked and inconstant. Crawford stated:

> . . . It is common knowledge that guinea pigs affected with mammalian tuberculosis manifest a very marked hyper-sensitiveness to mammalian tuberculin, while a relatively slight sensitiveness is manifested toward avian tuberculin. With guinea pigs sensitized by avian tubercle bacilli, however, reactions are obtained at times with both kinds of tuberculin, but in every instance avian tuberculin produces the more marked reaction.

Other studies clearly demonstrated that sensitization to tuberculin can result from infection with various acid-fast strains. Hagan and Zeissig (1929) showed that guinea pigs infected with avian tubercle bacilli or *M. johnei* will react to intracutaneous injections of either avian tuberculin or johnin (the antigen prepared from Johne's bacillus). Frey and Hagan (1931) described a technique to demonstrate the presence of acid-fast bacteria in soil samples. The strains isolated from soil were injected into guinea pigs. The sensitization patterns supported groupings based on morphology, although there were group cross reactions. It was shown that the organisms of any one of the groups exhibited a closer relationship to each other than to organisms of the other groups. Hastings *et al*. (1930) reported sensitizing cattle with acid-fast bacilli other than tubercle bacilli. They found that in cattle a positive response to tuberculin was not absolute proof of infection with tubercle bacilli. Other mycobacteria may invade the tissues and sensitize to tuberculin.

Edwards *et al*. (1958) and Affronti (1959) have shown that skin test antigens prepared from various mycobacteria possess a certain specificity. This means that when man or animals are infected with a mycobacterium they elicit a stronger reaction to the skin test antigen prepared from the homologous mycobacterium than from an antigen prepared from

other mycobacterial species.

Magnusson (1961) suggested the term sensitin for a nonantigenic substance, prepared from a microorganism, capable of revealing sensitivity of the delayed type evoked by the organism. He showed the sensitins prepared from 16 strains of acid-fast bacilli were specific for the species from which they were prepared. However, there was no difference in the specificity of tuberculins prepared brom BCG and from 4 strains of human virulent bacilli. Magnusson (1962) further investigated sensitins prepared from other mycobacteria. In the majority of cultures, classification of the strains on the basis of sensitin specificity was in agreement with classification by other methods. Magnusson and co-workers (1961) prepared purified tuberculin from 37 unclassified strains of acid-fast bacteria and on the basis of their specificity were able to correlate groupings made by skin test reactions and those made by cultural and biochemical methods.

Palmer and Edwards (1962) reported some results of a U.S. Public Health Service--U.S. Navy cooperative study. Navy recruits were tested with PPD skin test antigens (Affronti, 1959) prepared from tubercle (PPD-S) and battey bacilli (PPD-B). More than 250,000 recruits were tested. The prevalence of sensitivity to PPD-B was shown to be much higher than to PPD-S: about 30 per cent of all recruits

reacted to PPD-B, while about 5 per cent reacted to PPD-S. The variation in prevalence of sensitivity to PPD-B reflected a strong geographic influence; the percentages increased from 20 per cent in the northwest to more than 80 per cent in the southeast. The highest reaction to PPD-S occurred in the southwest.

MATERIALS AND METHODS

I. METHODS OF SKIN TESTING

Sources of organisms. Sixty-six strains of mycobacteria obtained from a variety of sources were used in this study. Table I lists these strains and their source. The number assigned each strain is the accession number of the mycobacterial culture bank (Dr. E. H. Runyon, Veterans Administration Hospital, Salt Lake City, Utah). The majority of strains were acquired from one of four sources: patients, healthy humans, animals, soil and water.

Source of PPDs. Table II, page 39, contains the source and designation of the purified protein derivatives (PPDs) used for testing guinea pig skin sensitivity. The PPDs were obtained from the United States Public Health Service, through the courtesy of Dr. Lydia Edwards, where they were standardized as to protein nitrogen content.

Preparation of organisms for guinea pig sensitization. Cultures of each strain which had been grown in Dubos liquid medium (BBL) were injected into five albino Hartley strain guines pigs. One injection of 0.5 ml liquid medium containing 1 mg live bacilli (wet weight as determined by the Hopkins tube method, Kubica and Vestal, 1959) was made subcutaneously in the back of the neck. Control animals

TABLE I

STRAINS USED FOR SENSITIVITY TESTING

Strain number	Source	Location	Disease	Group*
8	One gastric lavage	Kansas	-	II
50	One gastric lavage	Kansas	-	II
210	Distilled water	Georgia		II
251	Sputa Lung tissue	Ohio	+	II
379	Multiple sputa	Georgia	+	III
514	Multiple sputa	Florida	+	IIIy

*Groups - As classified by mycobacterial culture bank, Dr. E. H. Runyon, Veterans Administration Hospital, Salt Lake City, Utah:

Group II: Yellow-orange scotochromogen

Group III: Nonphotochromogen minimal pigmentation

Group IIIav: Probably *M. avium* as indicated by growth at 45°C and/or pathogenicity for chickens

Group IIIR: Rough colony, nonphotochromogen

Group IIIy: Yellow, nonphotochromogen

Group IIx) Differing markedly from the usual strain of the group
Group IIIx)

TABLE I (continued)

Strain number	Source	Location	Disease	Group
584	Sputa lung tissue	Florida	+	III
585	Lung tissue	Florida	+	III
629	Sputum	N. Y. City		III
736	Sputa lung tissue	Georgia	+	III
769	Cervical lymph node	Montreal	Adenitis	II
791	Cervical lymph node	Minnesota	Adenitis	II
792	Cervical lymph node	Alabama	Adenitis	II
886	Bone marrow, cervical lymph node	N. Y. City	+	III
924	Bone marrow	Netherlands	+	III
940	Bone marrow	Pennsylvania	+	III
942	Pig lymph nodes	California	-	IIIav
943	Pig lymph nodes	California	-	IIIav
944	Pig lymph nodes	California	-	IIIav
945	Pig lymph nodes	California	-	IIIav
947	Sputa, lung tissue	Florida	+	IIIy
968	Sputum	Rome, Georgia	+	III

TABLE I (continued)

Strain Number	Source	Location	Disease	Group
974	Cervical lymph node	Minnesota	adenitis	II
1082	Gastric	Rome, Georgia	-	III
1083	Normal sputum	Georgia	-	III
1088	Normal sputum	Georgia	-	III
1134	Normal sputum	Georgia	-	IIIR
1175	Normal sputum	Georgia	-	IIIR
1187	Normal sputum	Georgia	-	IIIR
1201	Sputum, lung tissue	Australia	+	III
1227	Mouse tissue (brain)	Florida	(Myco "X")	III
1344	Sputum, gastric lavage	Ontario	+	III
1349	Pig lymph nodes	Australia	-	III
1350	Pig lymph nodes	Australia	-	III
1351	Pig lymph nodes	Australia	-	III
1352	Pig lymph nodes	Australia	-	III
1353	Pig lymph nodes	Australia	-	III
1354	Pig lymph nodes	Australia	-	III

TABLE I (continued)

Strain number	Source	Location	Disease	Group
1382	Sputum	Australia	+	III
1499	Gastric lavage	Germany	+	III
1514	Tracheal lavage	Netherlands	+	III
1581	Partridge	California	+	IIIR
1590	Dog lymph node	Japan	-	III
1591	Dog lymph node	Japan	-	III
1592	Dog lymph node	Japan	-	III
1593	Dog lymph node	Japan	-	III
1594	Dog lymph node	Japan	-	III
1611	Fowl	Minnesota	+	IIIy av
1621	Teal duck	California	+	IIIy
1639	Water vat	Georgia		IIIx
1642	County road	Georgia		III
1645	Water vat	Georgia		IIx
1646	Pig farm (soil)	Georgia		IIIx
1648	Pig farm (soil)	Georgia		IIIx

TABLE I (continued)

Strain number	Source	Location	Disease	Group
1652	Pig	Georgia	-	IIIav
1817	Lettuce	Florida	-	III
1818	Carrot	Tampa, Florida		III
1819	Radish	Florida	-	III
1911	Radish	Georgia	-	IIIR
1931	Lymph node jawline	Utah	+	IIIav

TABLE II

SOURCE AND DESIGNATION OF PURIFIED PROTEIN DERIVATIVES USED FOR TESTING GUINEA PIG SKIN SENSITIVITY

PHS designation	Our designation		Source
PPD-S	Tuberculin	(TBN)	Tubercle bacillus
PPD-A	Aviin	(AVN)	Avian bacillus (wey bridge)
PPD-B	Batteyin	(BTN)	Battey strain
PPD-210	Scotochromin	(SCTN)	Group II scoto-chromogen

received 0.5 ml of the liquid medium.

Skin tests in guinea pigs. Six weeks after initial inoculation, skin sensitivities were determined by the intradermal injection of 0.1 ml of 25 TU (0.005 mg) strength of each of the PPDs (Table II). The reactions were recorded as the diameter of erythema in millimeters 48 hours after the intradermal skin test. The PPDs were randomized as to injection site so that the reader of the tests did not know their identity.

Statistical methods. An analysis of variance technique was used to analyze the data. The experiments were carried out using a completely randomized design. If a significant "F" value was obtained,

$$F = \frac{\text{between samples mean square}}{\text{within samples mean square}}$$

the significance between the sample means selected at random was tested by a "T" test

$$T = \frac{\bar{X}_1 - \bar{X}_2}{\sqrt{S^2(1/n_1 + 1/n_2)}}$$

Table III indicates the equations used in computing the analysis of variance. The probability factor used to indicate significant differences was $^{P}.05$. Techniques used in the analysis of variance may be found in Woolf's

TABLE III

EQUATIONS USED IN ANALYSIS OF VARIANCE

Source of variation	Degrees of freedom	Sums of squares	Mean square
Total	$\sum_{1}^{K} n_i - 1$	$\sum_{1}^{nK} X^2 - \frac{(\sum_{1}^{nK} X)^2}{\sum_{1}^{K} n_i}$	
Between samples	K-1	$\sum_{1}^{K} \frac{(\sum_{1}^{n} X)^2}{n_i} - \frac{(\sum_{1}^{nK} X)^2}{\sum_{1}^{K} n_i}$	$\frac{\text{Sums of squares}}{\text{degrees of freedom}}$
Within samples (S^2)	$(n_i - 1)$	$\sum_{1}^{K} \left[X^2 - \frac{(\sum_{1}^{n} X)^2}{n_i} \right]$	$\frac{\text{Sums of squares}}{\text{degrees of freedom}}$

$$\text{Standard error} = \sqrt{\frac{\text{Within samples mean square}}{n_i}}$$

Syllabus of Biometry.

Arylsulfatase (liquid medium) test. A working solution containing 0.003 M tripotassium phenolphthalein disulfate[1] was made in the following way:

Dubos broth base[2]	7.2 gms
5 per cent bovine serum albumin[3] .	100 ml
0.04 M substrate	77 ml
Distilled water to make	1000 ml

This solution was then sterilized by Seitz filtration and dispensed into sterile 20 x 125 mm screw cap culture tubes. Actively growing cultures on solid American Trudeau Society (ATS) medium were tested. A cotton swab was used to remove organisms from the solid slant, the swab was then twisted in the liquid test medium to disperse the organisms. A concentrated, dispersed suspension was obtained. This "large inoculum" technique was used for other biochemical tests. The resulting suspensions were placed at 37°C, and small portions (2 to 3 drops) removed and tested for the presence of phenolphthalein by the addition of equal quantities of

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[1]L. Light & Co., Colnbrook, England.

[2]Baltimore Biological Laboratory, Baltimore, Maryland.

[3]Armour Pharmaceutical Company, Division of Armour & Co., Kankakee, Illinois.

1 M $NaCO_3$. If phenolphthalein was present, a pink-red color developed throughout the solution.

Arylsulfatase (solid medium) test. Wayne phenolphthalein sulfatase agar[4] was dissolved in boiling water and dispensed into 20 x 125 mm screw cap culture tubes according to the manufacturer's instructions. The surface of the agar was inoculated with the strains to be tested. The presence of free phenolphthalein was demonstrated by means of adding 0.5 ml of 1 M $NaCO_3$ to the culture; production of a pink to red color is a positive test.

Test of urease activity. Three grams of urea were added to 100 ml of phosphate buffer pH 6.7 containing 2 ml of 0.1 per cent phenol red. The solution was sterilized by filtration. Bacilli were added to the urea buffer in the manner previously described for the liquid medium arylysulfatase test. The suspensions were incubated at 37°C. Observations were made at 24-hour intervals; urease positive strains produce a red color.

Nitrate reduction test. A 0.01M solution of sodium nitrate was inoculated with actively growing mycobacteria in the usual manner. The suspension was then incubated at 37°C

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[4]Baltimore Biological Laboratory (BBL), Baltimore, Maryland.

for 2 hours after which 1 drop of hydrochloric acid diluted 1:1, 2 drops of 0.2 per cent sulfanilamide, and 2 drops of 0.1 per cent N-(1-napthyl) ethylendiamine dihydrochloride aqueous solution were added to the suspension. Readings were made by comparing the color of the tests with a positive and negative control. The positive control consisted of the nitrate solution and elemental zinc. The negative control consisted of reagent only. If nitrite has been formed, a purple color will develop in the test suspension. Results were read 5 minutes after the color developing reagents were added.

<u>Test for picric acid reduction</u>. Dubos broth base (BBL) was dissolved in phosphate buffer pH 7.8. Bovine serum albumin was added to final concentration of 0.5 per cent. Picric acid was added to a final concentration of 0.05 per cent. The medium was inoculated in the manner described previously and incubated at 37°C. Formation of red picramic acid indicates a positive test.

<u>Carbohydrate fermentation test</u>. Basal medium was composed of:

Phenol red	8 mg
Yeast extract	200 mg
K_2HPO_4	70 mg
Agar (BBL)	2 mg
Distilled water	320 mg

The pH was adjusted to 7.0, and the medium was dispensed in 3.2 ml quantities in screw cap tubes and autoclaved at 121°C for 15 minutes. The tubes were cooled in vertical position. Ten per cent carbohydrate solutions were prepared, brought to a pH of 7.2, and sterilized by filtration. By overlaying 0.8 ml of carbohydrate solution on a 3.2 ml of agar base medium the final concentration of carbohydrate is 2 per cent. The carbohydrates used in this study were trehalose and xylose. A heavy inoculum was made as described above in the carbohydrate solution layered on top of the basal medium. The tubes were incubated at 37°C and observed at 24-hour intervals. Results were interpreted as a change of color of the phenol red indicator. Yellow (indicating acid production) was a positive result, red to purple was negative, orange was interpreted as doubtful.

EXPERIMENTAL RESULTS

I. SKIN TEST RESULTS

A summary of the average skin reactions induced by the mycobacteria tested may be found in Table IV. A single asterisk indicates that the reaction deviated significantly from the next largest reaction when tested using the statistical analysis as outlined in the methods section.

Skin Sensitivity Induced by Patient Strains

The skin reactions elicited by Group III strains isolated from 18 patients having tuberculosis-like disease may be found in Table V, page 49. No *M. tuberculosis* was isolated in any case.

Two of the strains (947, 2061) were weakly or not at all allergenic as measured. Strain 947, a yellow-pigmented battey strain elicited no reaction to any of the PPDs used. Strains 2061, 2062, and 886 appeared to be *M. avium* by growth at 45° and Schaefer's agglutination testing. These 3 strains elicited larger reactions to aviin than to batteyin.

Sensitivity induced by the rest of the battey strains was to both aviin and batteyin (average 10.3 and 9.8 mm). Some strains consistently stimulated reactions more to batteyin, some more to aviin, but none to only one of these PPD antigens. It is of interest to note that cross reactions

TABLE IV

AVERAGE GUINEA PIG SENSITIVITY FROM ALL STRAINS USED IN STUDY

Strain number	Number of pigs	TBN	AVN	BTN	SCTN
8	5	0	0	0	0
50	5	0	1.0	4.6	3.8
210	5	0	4.6	7.2	12.6
251	5	0	5.0*	7.0	13.6*
379	5	0	10.8	17.4	6.6*
514	9	0.8	9.4*	6.7*	3.0*
584	4	2.5	7.8	11.0	2.5
585	5	1.2	6.8	12.0*	4.6
629	3	0	5.3	0	19.7*
736	5	3.8	10.6*	12.2	3.8
769	5	0	6.6	3.6*	9.8
791	5	0	6.8	3.4	5.8
792	5	2.4	4.6	3.2	13.2*
886	5	7.2	17.1	15.8	11.8
924	4	2.0	11.8	10.8	6.0
940	5	0.8	11.0*	11.6	
942	10	0.6	11.6*	8.4*	2.6
943	3	0	15.7	12.7*	5.4
944	8	0.3	11.0*	5.3	4.8*
945	4	0	15.5*	5.5	2.3
947	5	0	0	0	0
968	4	3.0	7.3	10.0	6.3
974	5	1.2	2.4	2.0	9.8*
1032	5	0	6.4	5.4	3.4
1083	3	1.7	9.0	8.3	4.7
1088	4	3.0	8.5	6.8	5.8
1134	5	0	0	0	0
1175	5	0	2.4	0	0
1187	5	0.4	0.4	0.4	0
1201	5	0.8	10.8*	11.6	5.6*
1227	4	0.8	10.0*	10.5	
1344	5	1.2	19.4*	14.4*	8.0*
1349	7	1.0	8.4	11.4	4.1
1350	3	2.3	14.0	17.3	9.0*
1351	4	0	9.0*	14.8*	2.0
1352	3	0	9.3	12.3	5.6

*See text for meaning.

TABLE IV (continued)

Strain number	Number of pigs	TBN	AVN	BTN	SCTN
1353	4	0	10.8*	14.5*	3.8*
1354	4	0	11.8	11.8*	2.4
1382	7	0.7	10.9	9.6	4.3
1499	3	0.7	11.3	5.7	4.7
1514	7	1.1	9.6	10.7	5.9*
1581	5	0.4	11.4*	6.2	2.8
1590	4	1.5	10.0*	11.8	
1591	5	10.0	9.6	5.2	3.2
1592	4	0	13.3	12.8*	
1593	5	3.4	8.8	12.8	1.4
1594	4	0.5	10.0	8.5*	
1611	5	0	5.8	7.8	3.0
1621	9	0	15.9	14.3*	6.2
1639	3	0	4.7	6.3	
1642	4	3.0	3.0	3.0	
1645	5	0	4.2	1.8	6.8
1646	5	0	4.8	2.2	4.0
1648	5	2.4	4.6	4.0	8.4
1652	5	3.2	16.0*	10.8	9.4*
1817	3	4.3	8.0	10.0	5.3
1818	5	1.6	4.6	0	3.8
1819	5	2.2	8.0	6.2	5.2*
1911	5	0.8	3.6	1.6	1.6
1931	4	7.0	18.5*	15.5*	11.3*
2061	5	0	5.4	2.0	2.0
2062	5	0	9.8	5.6	3.0
2064	5	0.8	7.8	4.2	3.4
2123	5	2.4	4.8	2.6	6.6
2124	4	3.8	7.0	4.3	3.3
2125	5	4.0	5.6	4.8	4.4
control	20	0	0	0	0
66	341				

TABLE V

GUINEA PIG SKIN REACTIONS FROM GROUP III STRAINS ISOLATED FROM HUMAN DISEASE

Strain number	Number of pigs	TBN	AVN	BTN	SCTN
379	5	0	10.8	17.4*	6.6*
514	9	0.8	9.4*	6.7*	3.0*
584	4	2.5	7.8	11.0	2.5
585	5	1.2	6.8	12.0*	4.6
736	5	3.8	10.6*	12.2	3.8
886	5	7.2	17.1	15.8	11.8
924	4	2.0	11.8	10.8	6.0
940	5	0.8	11.0*	11.6	
947	5	0	0	0	0
968	4	3.0	7.3	10.0	6.3
1201	5	0.8	10.8*	11.6	5.6
1344	5	1.2	19.4*	14.4*	8.0*
1382	7	0.7	10.9	9.6	4.3
1499	3	0.7	11.3	5.7	4.7
1514	7	1.1	9.6	10.7	5.9*
1931	4	7.0	18.5*	15.5*	11.3*
2061	5	0	5.4	2.0	2.0
2062	5	0	9.8	5.6	3.0
Total 18	92 Ave.	1.7	10.3	9.8	5.3

*See text for meaning.

to scotochromin occurred with 8 strains giving an average of 5.3 mm of erythema, while the cross reactions to PPD-S tuberculin occurred in only 2 of the 18 strains (886, 1931).

Skin Sensitivity Induced by *M. avium*

The skin reactions induced by 8 strains identified as *M. avium* are shown in Table VI. These strains were all derived from birds or swine. All except one induced maximum sensitivity to aviin. The one exceptional strain (1611) produced only little sensitivity, the maximum being to betteyin (average 7.8 mm). This strain is reported always to have been yellow (Karlson *et al.*, 1962). Originally it was virulent for fowl, but it is not so now. Five of the strains induced reactions to aviin which was significantly larger than the reactions to batteyin.

Skin Sensitivity Induced by Animal Strains

Six strains isolated from Australian swine were investigated (Table VII). These strains resulted in maximum sensitivity to batteyin; however, in only 2 cases were the batteyin reactions significantly greater than the aviin reactions.

Five strains isolated from the lymph nodes of Japanese dogs were studied. The skin sensitivities induced by these strains resulted in sensitivity to aviin and

TABLE VI

GUINEA PIG SENSITIVITY FROM AVIAN STRAINS

Strain number	Number of pigs	TBN	AVN	BTN	SCTN
942	10	0.6	11.6*	8.4*	2.6
943	3	0	15.7	12.7*	5.4
944	8	0.3	11.0*	5.3	4.8*
945	4	0	15.5*	5.5	2.3
1581	5	0.4	11.4*	6.2	2.8
1611	5	0	5.8	7.8	3.0
1621	9	0	15.9	14.8*	6.2
1652	5	3.2	16.0*	10.8	9.4*
Total	49 Ave.	0.5	12.7	9.0	4.3

*See text for meaning.

TABLE VII

GUINEA PIG SENSITIVITY FROM AUSTRALIAN SWINE STRAINS

Strain number	Number of pigs	TBN	AVN	BTN	SCTN
1349	7	1.0	8.4	11.4	4.1
1350	3	2.3	14.0	17.3	9.0*
1351	4	0	9.0*	14.8*	2.0
1352	3	0	9.3	12.3	5.6
1353	4	0	10.8*	14.5*	3.8*
1354	4	0	11.8	11.8*	2.4
Total	25 Ave.	0.6	10.2	13.3	4.2

*See text for meaning.

batteyin. One of the strains (1591) stimulated a maximum reaction to tuberculin.

Skin Sensitivity Induced by Soil and Water Strains

Strains were received from Dr. G. Kubica and F. Dunbar. These cultures were isolated in Georgia and Florida, the geographic areas of this country where Battey disease is most prevalent (Table IX). The 13 strains studied included 2 group II scotochromogens (210, 1645) and 11 Group II nonphotochromogens. Four of the Group III strains stimulated positive reactions to batteyin or aviin. The other strains were conspicuously less allergenic to batteyin or aviin than the patient strains. The yellow strains and some of the nonpigmented strains induced positive reactions to scotochromogen.

Skin Sensitivity Induced by Strains Isolated from Healthy People

Six strains derived from a study of mycobacteria isolated from healthy people were investigated (Edwards and Palmer, 1959). Three of these strains (1134, 1175, 1187) appeared to be nonsensitizing as tested, while 2 other strains (1083, 1088) stimulated reactions to aviin and batteyin as did Battey Hospital patient strains (Table X, page 55). Strain 1032 appeared to be on the borderline resulting in

TABLE VIII

GUINEA PIG SENSITIVITY FROM JAPANESE DOG STRAINS

Strain number	Number of pigs	TBN	AVN	BTN	SCTN
1590	4	1.0	10.0*	11.8	
1591	5	10.0	9.6	5.2	3.2
1592	4	0	13.3	12.8*	
1593	5	3.4	8.8	12.8	1.4
1594	4	0.5	10.0	8.5*	
Total	22 Ave.	3.4	10.2	10.1	2.3

*See text for meaning

TABLE IX

GUINEA PIG SENSITIVITY FROM SOIL AND WATER STRAINS

Strain number	Number of pigs	TNB	AVN	BTN	SCTN
210	5	0	4.6	7.2	12.6
1639	3	0	4.7	6.3	
1642	4	3.0	3.0	3.0	
1645	5	0	4.2	1.8	6.8
1646	5	0	4.8	2.2	4.0
1648	5	2.4	4.6	4.0	8.4
1817	3	4.3	8.0	10.0	5.3
1818	5	1.6	4.6	0	3.8
1819	5	2.2	7.8	6.2	5.2
1911	5	0.8	3.6	1.6	1.6
2123	5	2.4	4.8	2.6	6.5
2124	4	3.8	7.0	4.3	3.3
Total	59 Ave.	2.0	4.1	4.6	6.8

slightly positive reactions to aviin and batteyin.

Skin Sensitivity Induced by Pigmented Strains

Twelve pigmented strains were studied. None of these resulted in more than minimal reactions to aviin or batteyin. Included in this series were 5 Group II scotochromogens isolated from patients with disease (usually cervical lymph adenitis), 4 group II scotochromogens of nonpathological origin, and 3 strains classified as Group III yellow, i.e., pigmented nonphotochromogens. The skin sensitivities of these groups are presented in Table XI.

The scotochromin reactions were significantly greater from the strains which were related to disease then from strains of nonpathological origin. The strains classified as Group III-yellow failed to sensitize the animals maximally to scotochromin, thus supporting their separation as a distinct group.

Skin Sensitivity Induced by Miscellaneous Strains

The results of skin tests of 2 strains (629, 1227) were not placed in any of the groups. Their reactions may be found in Table IV, page 47. Strain 629 was sent to us as isolated from sputum; we had no knowledge of whether disease was present. It is interesting that this nonpigmented strain induced strong sensitivity to scotochromin, although other

TABLE X

GUINEA PIG SENSITIVITY OF STRAINS FROM HEALTHY PEOPLE

Strain number	Number of pigs	TBN	AVN	BTN	SCTN
1032	5	0	6.4	5.4	3.4
1083	3	1.0	9.0	8.3	4.7
1088	4	3.0	8.5	6.8	5.8
1134	5	0	0	0	0
1175	5	0	2.4	0	0
1187	5	0.4	0.4	0.4	0
Total	27 Ave.	0.6	4.0	3.0	2.0

TABLE XI

GUINEA PIG SENSITIVITY FROM TWELVE PIGMENTED STRAINS

	Number of strains	TBN	AVN	BTN	SCTN
Group II Disease	5	0.7	5.1	3.6	10.4
Group II No disease	4	0.0	2.4	3.4	5.8
Group III Yellow	3	0.4	6.0	3.6	2.0

nonpigmented strains have sensitized to scotochromin, none of these were maximum reactions. Strain 1227 is known as Mycobacterium X, and was isolated from the brain of a mouse. Its sensitivity pattern is very similar to the strains isolated from patients with disease.

II. BIOCHEMICAL RESULTS

Arylsulfatase (Liquid Medium) Test

Liquid medium arylsulfatase tests were performed on the cultures as previously outlined. The presence of free phenolphthalein was noted at 1, 3, 7, and 14 days. The results are listed (Table XII) for strains classified according to conventional bacteriologic groupings and for strains grouped as to source. At the end of 14 days all the strains except one (1611) were positive. Strains classified as typical Group III appeared to break down the substrate faster than the atypical Group III strains.

Arylsulfatase (Solid Medium) Test

Table XIII, page 58, indicates the arylsulfatase activity of the mycobacterial groups tested. As shown, no definitive conclusions regarding the grouping can be made. It appears that the Group III strains from disease have less arylsulfatase activity than Group III strains derived from other sources. However, it is unlikely that this generality

TABLE XII

ENZYMATIC BREAKDOWN OF PHENOLPHTHALEIN DISULFATE
(LIQUID MEDIUM TEST)

Groups of mycobacteria	Number of strains	Number of strains positive Reaction time in days 1	3	7	14
II	9	0	0	8	9
IIIy	3	0	0	2	3
IIIx	3	0	0	3	3
IIIR	5	1	1	3	5
III	36	17	20	33	36
IIIav	10	0	0	7	9
Group III disease	18	4	5	15	18
M. avium	8	0	0	3	7
Animal strains	11	6	10	11	11
Soil and water strains	13	0	1	12	13
Group III healthy people	6	1	2	6	6
Group II disease	5	0	0	5	5
Group II no disease	4	0	0	4	4

TABLE XIII

ENZYMATIC BREAKDOWN OF PHENOLPHTHALEIN DISULFATE
(SOLID MEDIUM TEST)

Groups of mycobacteria	Number of strains	Activity at 2 weeks positive	slightly positive	negative
II	9	4	2	3
IIIy	3	1	0	2
IIIx	3	1	0	2
III_R	5	3	0	2
IIIav	10	2	0	8
III	36	16	2	18
Group III from disease	18	6	1	11
M. avium	8	2	0	6
Group III animal strains	11	10	0	1
Soil and water strains	13	10	0	3
Group III healthy people	6	2	0	4
Group II disease	5	2	2	1
Group II no disease	4	2	0	2

is valid.

Urease Test

The urease activity of the designated groups of mycobacteria may be found in Table XIV. Subdivisions of Group III again appear. Fifteen of the Group III strains had definite urease activity; the majority of the strains (77 per cent) failed to hydrolyze urea. The saprophytic Group II strains failed to break down urea, while all of the pathogenic Group II strains hydrolyzed the substrate. A difference between Group II strains isolated from disease and those isolated from a nonpathological source may be seen when comparing the urease activity of those strains.

Nitrate Reduction Test

The nitrate reducing ability of various strains of mycobacteria may be found in Table XV. These results again are not clear cut for each group. The Group III strains appear to be heterogeneous with a majority of the strains reducing nitrate. All of the Group III yellow strains failed to reduce nitrate, while the Group III rough strains all were positive for the presence of the reducing enzyme. The Group III strains isolated from disease also appeared to be heterogeneous with regard to nitrate reducing activity.

Picric Acid Reduction Test

The enzymatic reduction of picric acid to picramic

TABLE XIV

ENZYMATIC BREAKDOWN OF UREA

Groups of mycobacteria	Number of strains	Number of strains positive Reaction time in days				
		1	3	7	14	Neg. 14 days
II	9	5	5	6	6	3
IIIy	3	0	0	0	0	3
IIIx	3	0	0	0	0	3
III_R	5	1	1	1	1	4
IIIav	10	1	1	1	1	9
III	36	4	5	6	7	29
Group III from disease	18	1	1	1	2	16
M. avium	8	1	1	1	1	7
Group III animal strains	11	4	4	4	4	7
Soil and water strains	13	0	0	1	1	12
Group III healthy people	6	1	1	1	1	5
Group II disease	5	5	5	5	5	0
Group II no disease	4	0	0	1	1	3

TABLE XV

NITRATE REDUCING ACTIVITY IN MYCOBACTERIA

Groups of mycobacteria	Number of strains	Activity at 2 weeks positive	slightly positive	negative
II	9	3	0	6
IIIy	3	0	0	3
IIIx	3	1	1	1
III_R	5	5	0	0
IIIav	10	6	0	0
III	36	27	2	7
Group III from disease	18	10	2	6
M. avium	8	5	0	3
Group III animal strains	11	9	0	2
Soil and water strains	13	7	0	6
Group III healthy people	6	6	0	0
Group II disease	5	1	0	4
Group II no disease	4	0	0	4

acid by various strains of mycobacteria is given in Table XVI. Group III strains were noted as giving variable results further indicating the heterogeneity of this group. The majority of the strains demonstrated this enzyme activity. The Group III strains isolated from disease gave a high percentage (83 per cent) of positive tests. The Group II strains from disease also demonstrated high enzymatic activity. The strains isolated from other sources were about evenly divided as to presence of the enzyme.

Carbohydrate Utilization

The utilization of xylose and trehalose by various mycobacterial strains may be found in Table XVII. The results were variable. Some strains from all of the groups (except Group III_x) were able to utilize either of the sugars. Some of the strains failed to utilize any of the carbohydrates. The results of these tests indicate great variability of the mycobacterial strains under investigation. It appears that no subdivisions of these groups may be determined by carbohydrate typing.

TABLE XVI

PICRIC ACID REDUCING ACTIVITY IN MYCOBACTERIA

Groups of mycobacteria	Number of strains	Reductase activity in 3 days			
		positive	slightly positive	negative	% positive
II	9	4	0	5	
IIIy	3	2	0	1	
IIIx	3	3	0	0	
III_R	5	5	0	0	
IIIav	10	4	0	6	
III	36	23	0	13	
Group III from disease	18	15	0	3	83
M. avium	8	2	0	6	
Group III animal strains	11	7	0	4	
Soil and water strains	13	6	0	7	
Group III healthy people	6	4	0	2	
Group II disease	5	5	0	0	
Group II no disease	4	2	0	2	

TABLE XVII

CARBOHYDRATE FERMENTATION TEST

Groups of Mycobacteria	Number of strains	Xylose Trehalose	Activity at 14 days + +	+ -	- +	- -
II	9		2	2	4	1
IIIy	3		2	0	0	1
IIIx	3		0	1	0	2
III_R	5		1	2	1	1
IIIav	10		4	2	2	2
III	36		4	2	2	2
Group III from disease	18		6	4	3	5
M. avium	8		6	4	3	5
Group III animal strains	11		3	4	2	2
Soil and water strains	13		2	5	2	4
Group III healthy people	6		2	1	2	1
Group II disease	5		1	1	2	1
Group II no disease	4		1	1	2	0

DISCUSSION

Edwards et al. (1958), Affronti (1959), Magnussen (1961), and Takeya (1961) have shown that skin test antigens prepared from various mycobacteria possess a certain specificity. Guinea pigs injected with a strain of mycobacteria often show cross sensitivity to heterologous mycobacterial antigens. The average size of the cross reactions, however, is generally smaller than the reaction to homologous antigen. Palmer and Edwards (1962) reported on skin test reactions of 250,000 navy recruits. Nearly 5 per cent of the recruits gave a reaction of 8 mm or greater when tested with a 5 TU dose of PPD-S (tuberculin). The prevalence of sensitivity to Battey PPD (batteyin) was nearly 30 per cent, much higher than the tuberculin reactors.

It has been postulated that a mycobacterium closely related to *M. tuberculosis* which can infect but is not pathogenic, might be the cause of naturally acquired nonspecific sensitivity in man. There is evidence that Group II scotochromogens are the most common acid-fast saprophytes found in man. Figure 1 illustrates the skin reactions induced by known strains of mycobacteria. It can be seen that scotochromogens sensitize maximally to scotochromin (the homologous PPD). The average skin reactions elicited by the heterologous antigens were less than 5 mm in diameter.

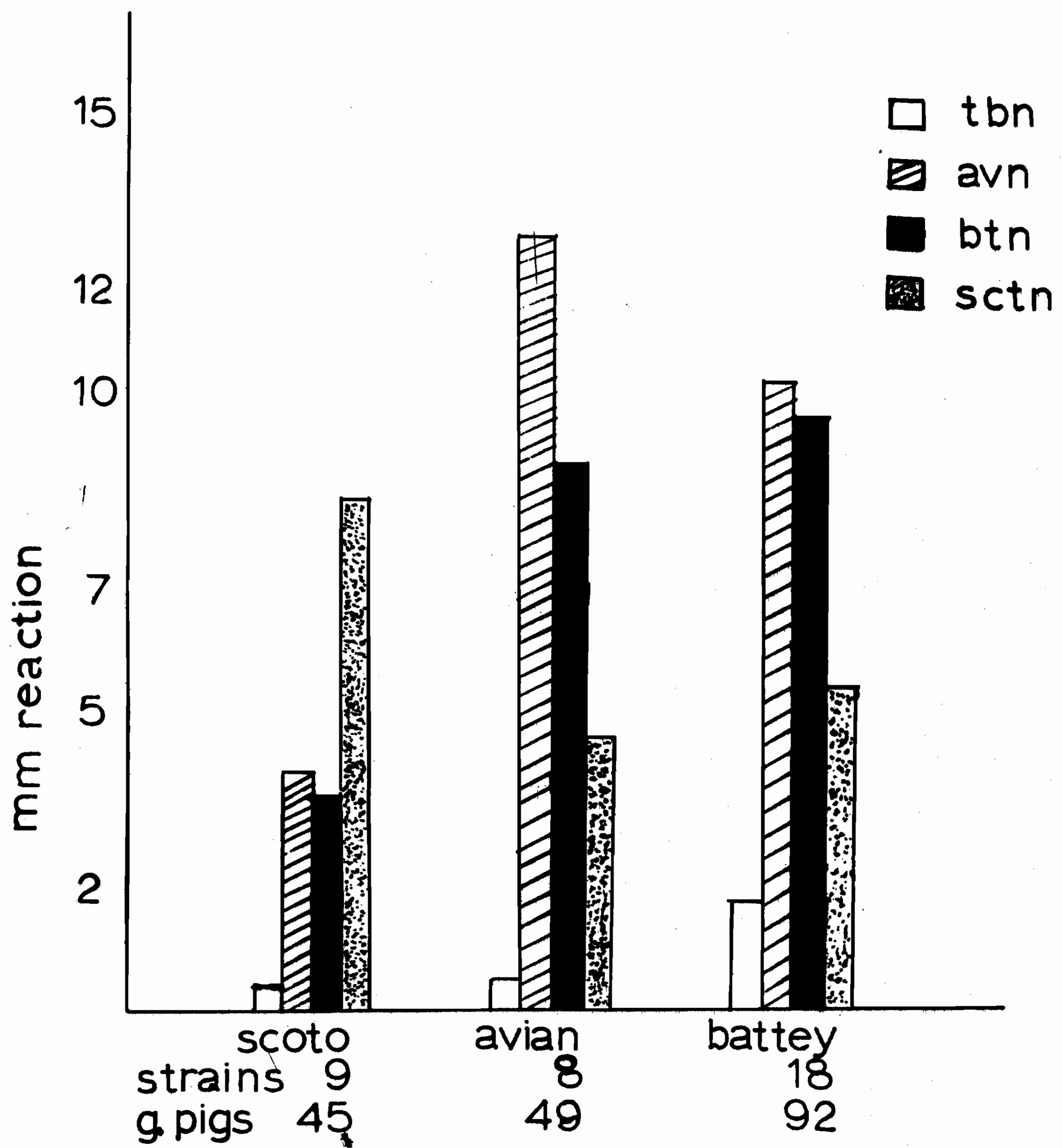

fig. 1 Average skin reactions of guinea pigs injected with known strains

According to our arbitrary designation these reactions were classified as negative. Thus animals injected with Group II scotochromogens elicited little if any cross sensitivity. These results fail to support some early work of Edwards *et al*. (1960). These workers injected 142 guinea pigs with 3 strains of Group II organisms. They found cross reactions greater than 5 mm to tuberculin, aviin, and batteyin. The animals were not tested with scotochromin. Later Edwards *et al*. (1962) found that injection of a Group II strain ("Gause") resulted in sensitivity of less than 5 mm erythema to tuberculin; however, cross reactions to aviin and batteyin were reported. The sensitivity profiles of navy recruits, i.e., the skin reactions elicited by a battery of skin test antigens, were very similar to the sensitivity profile of guinea pigs injected with Group II scotochromogens; however, the reactions of the navy recruits were smaller.

The results presented in Figure 1 indicate that although Group II scotochromogens may be infecting vast numbers of people in many areas of the country, they are probably not responsible for the cross sensitivity to tuberculin. The skin sensitivity induced in guinea pigs compared with the skin sensitivity of navy recruits indicates that guinea pigs and man react similarly to infection with Group II scotochromogens.

That the homologous antigen gives the largest reaction

can be further demonstrated in Figure 1. The guinea pigs injected with _M. avium_ strains gave greater reactions to the homologous PPD (aviin). The avian strains induced cross reactions to the Battey PPD. The close relationship of _M. avium_ and Battey bacilli is illustrated graphically when the skin reactions elicited by aviin and batteyin are compared. In the case of sensitization with Battey bacilli the homologous reaction is not maximum. The cross reactions induced by _M. avium_ and Battey bacilli indicate that a heterogeneous group of acid-fast bacilli might be infecting man, causing disease or skin sensitivity. This Group II-avian complex includes strains from a variety of sources including animals, healthy people, soil and water.

While the mode of transmission of tuberculosis has been well documented, that of Battey disease has not. No person-to-person transmission has been reported. Acid-fast strains morphologically similar to Battey bacilli may be isolated from a variety of sources. Figure 2 illustrates graphically the skin sensitivity induced in guinea pigs by strains isolated from patients with pulmonary disease due to infection with Battey bacilli, animals, soil, and healthy people. The animal strains used in this graph were isolated from stray Japanese dogs and Australian swine. No disease was present in either of these groups. Strains which were identified as _M. avium_ were not included even though many

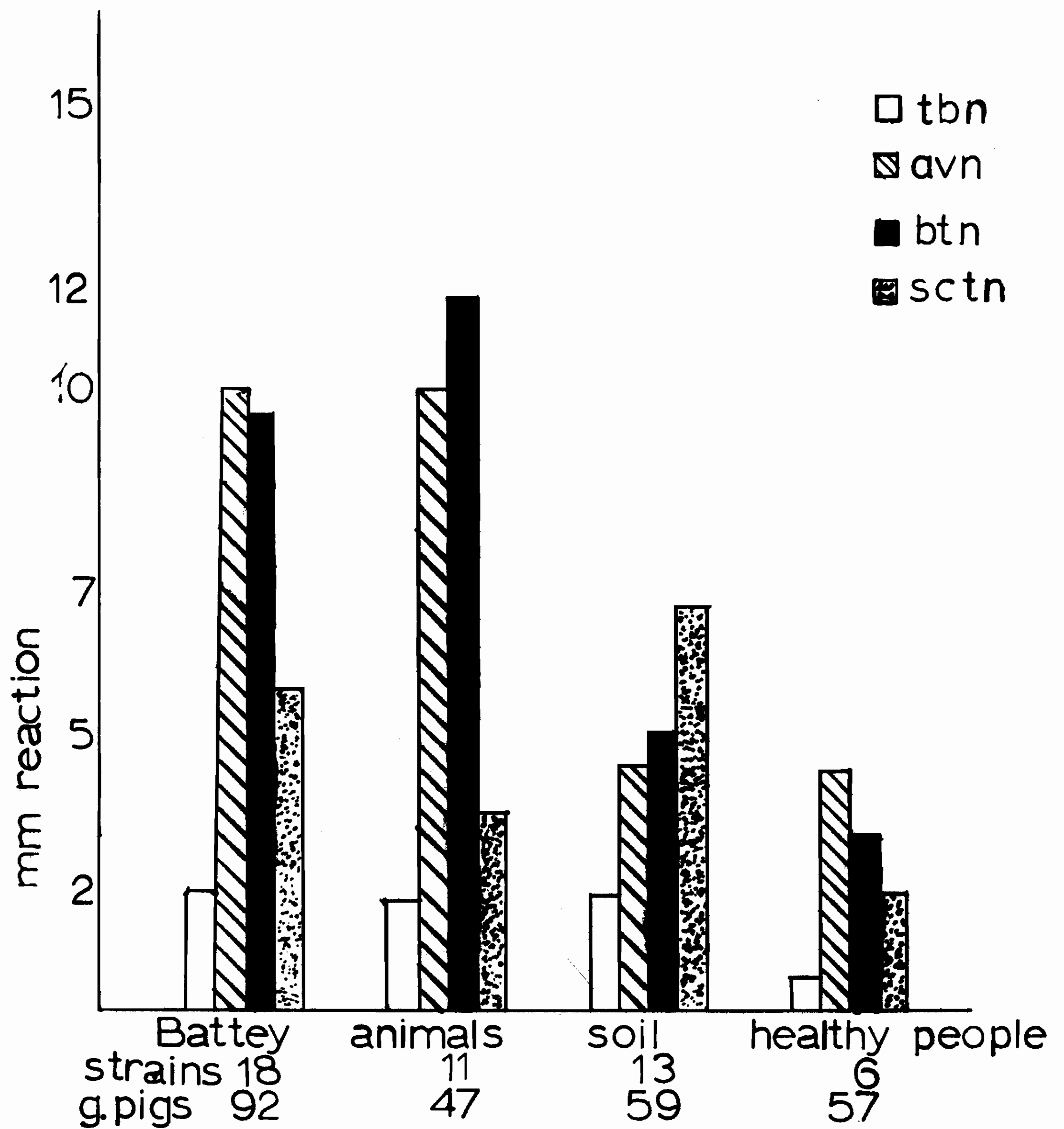

fig.2 Skin reactions induced by strains isolated from various sources.

were isolated from animals. The results with 11 strains indicate a sensitivity pattern similar to that of patient strains. Tuberculin sensitivity induced by animal strains is about equal with that of the patient strains, both being about 2 mm of reaction. Thus it appears possible that household pets such as dogs might be a source of Battey infection in man. Farm animals such as swine and fowl should also be considered as possible reservoirs of infection.

Thirteen strains isolated from soil and water sources were studied. These isolations were from the area of the United States where Battey disease is most prevalent and included farmyard soil, vegetables, and water from drinking troughs. The 13 strains included 2 Group II scotochromogens. On the average these strains failed to induce sensitivity to aviin and batteyin; however, some strains did induce sensitivity to these skin test antigens. The sensitivity pattern induced by strains isolated from soil is similar to the skin sensitivities of navy recruits mentioned previously. It is possible that strains found in soil and water might be causing sensitization of man; however, most of these strains appear to be different from strains which cause disease.

Six strains isolated from the sputum of healthy people were injected into guinea pigs. While on the average the results indicate that these strains are antigenically dissimilar from the patient strains, 3 of these strains

induced reactions very similar to the patient strains. Thus it may be that a possible "carrier" state exists in man and person-to-person transmission of Battey bacilli might be the source of Battey disease even though other evidence is to the contrary.

The skin sensitivity induced by pigmented strains was of interest. Group II scotochromogens, while often found and reported as saprophytic, may also cause severe cervical lymphadenitis. Skin reactions induced by Group II scotochromogens isolated from disease were compared with reactions induced by strains isolated from situations apart from disease. Figure 3 illustrates these comparisons graphically. The strains isolated from disease induced a much stronger reaction to scotochromin than the other strains. This difference was significant when tested with the "T" test at the .05 level of significance. The PPD (scotochromin) used was prepared from a strain isolated from a patient with cervical lymphadenitis.

Pigmented Group III strains failed to sensitize maximally to scotochromin whether isolated from disease or not. These unusual Group III strains do not correlate or fit in with any particular pattern of sensitivity; one strain failed to sensitize, another sensitized similarly to patient strains.

Although the title of this paper indicates

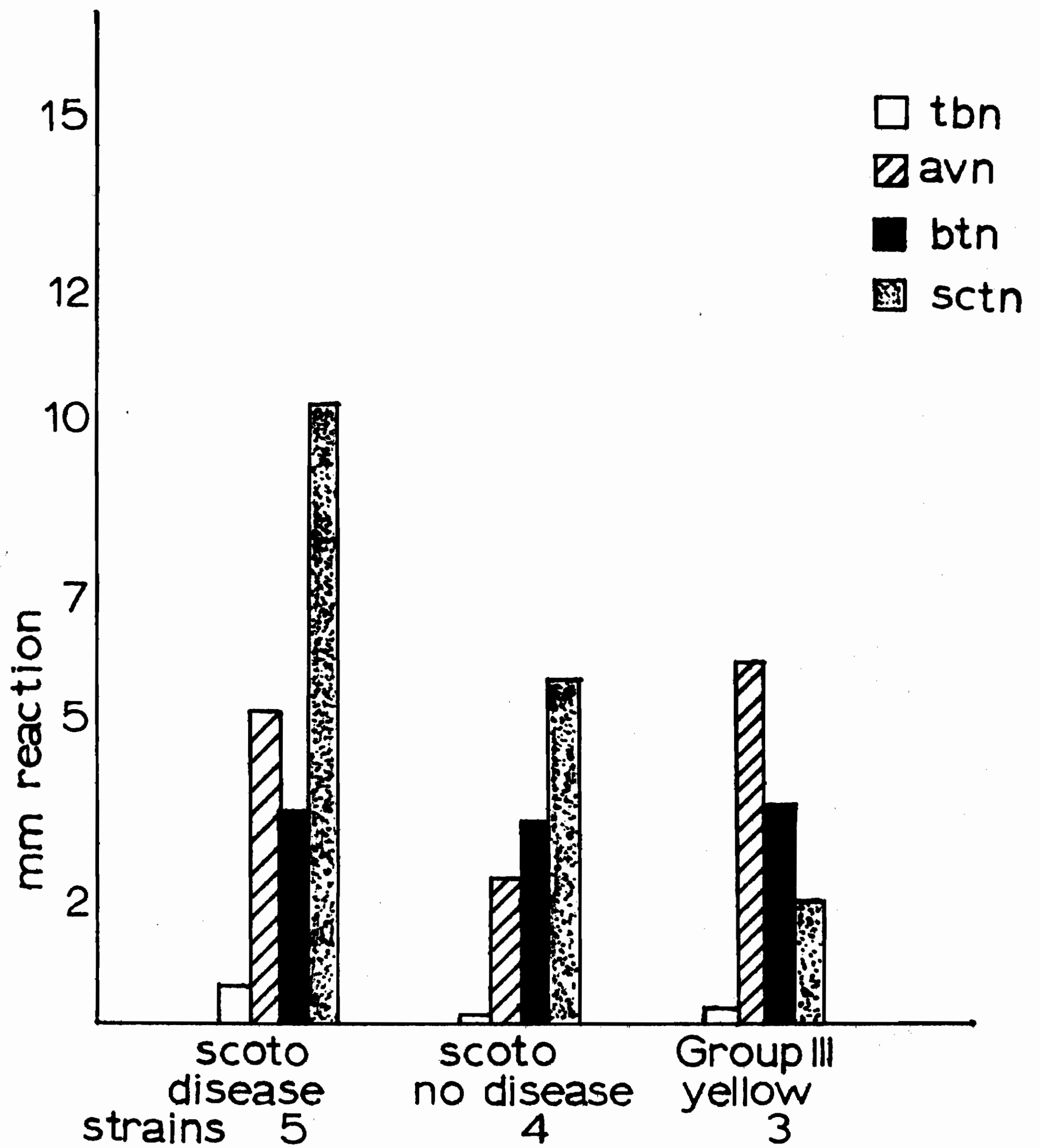

fig.3 Skin reactions induced by pigmented strains.

classification by means of skin reactions, species names can not be given without much further work and collaboration of other workers. Strains isolated from various sources induce sensitivity which on the average may be characteristic of that group. However, strains within the group may vary, and this variance may be statistically significant.

Guinea pig skin reactions induced by patient strains demonstrate the variability of reactions. While on the average these reactions are nearly equal, some strains induced reactions which were significantly greater to batteyin and some which were significantly greater to aviin. Thus, while the classification or grouping of mycobacteria by the aid of skin reactions could serve as a useful tool in the study of epidemiology and certain antigenic relationships of mycobacteria, it alone can not be substituted for use in the designation of species.

Even greater heterogeneity is shown by biochemical reactions. No group, whether based upon pigmentation, morphology, or skin reactions, gave completely homogeneous results. This would indicate the subgrouping would be appropriate. The work reported failed to support previously reported biochemical properties of mycobacteria. Sweeney and Jann (1961) reported that 4 strains of _M. avium_ utilized xylose and failed to utilize trehalose, while 4 Battey strains utilized trehalose and failed to utilize xylose.

These differences were not observed in the work here reported.

Using liquid medium containing substrate Kubica *et al.* (1961) found a lack of arylsulfatase activity in avian strains, while Battey strains demonstrated variable but definite activity. Our results using a massive inoculum technique indicate that all the strain tested (one exception) were arylsulfatase positive. Wayne *et al.* (1958) also found that all mycobacteria (except *M. rhodochrous*) produced arylsulfatase and that apparent differences in activity of different species were due in part to differences in growth rate.

Bonicke (1961) found that Group II scotochromogens include both urease-positive and urease-negative strains. He then subdivided the Group II strains into Group II_a (urease-positive and II_b (urease-negative). Toda *et al.* (1961) also reported some scotochromogens to be urease-positive and some to be urease-negative. The work reported in this paper correlates well with previous reports. In addition, the urease-positive strains in our investigation were isolated from disease, while the negative strains were isolated from situations apart from disease.

Virtanen (1960) reported on the nitrate activity of mycobacteria; however, he studied the atypical strains only as a single group. Bonicke (1962) reported that all Group III, Group II, and *M. avium* strains tested to be nitrate

reductase negative. The work reported (Table XV, page 61) in this paper indicates high enzymatic activity of many of the strains tested.

Picric acid reductase activity has not been reported to be of practical value in classifying mycobacteria. Subdivisions of Group II and Group III strains based upon this enzymatic activity are indicated (Table XVI, page 63). Strains isolated from disease exhibited high enzymatic activity.

SUMMARY

It has been shown by Edwards _et_ _al_., Magnusson, Takeya, and others that delayed skin reactions engendered in guinea pigs are a valuable means of differentiating mycobacterial species and types. In the studies here reported the close relationship of avian and Battey bacilli is confirmed. Nevertheless, skin sensitivity of guinea pigs inoculated with various strains of this GroupII-avian complex, clearly segregate these bacteria into 3 subgroups: (1) _M. avium_ strains, which regularly give a significantly greater reaction to aviin; (2) soil and water strains which sensitize to a minimal degree if at all; and (3) the Battey strains which sensitize about equally to aviin and batteyin. It appears highly improbable that the strains from soil or water, here tested, could be the source of so-called Battey disease or of batteyin skin sensitivity in man. The same is true of some strains from healthy people. But some strains from each of these sources, including people, Australian swine, and Japanese dogs yielded bacteria of the same sensitizing capacity as strains from patients.

Twelve pigmented strains became segregated by our studies into 3 subgroups: (1) strongly, (2) weakly, and (3) nonsensitizing to scotochromin, the PPD prepared from a lymph node strain. Only the first subgroup, the strongly

sensitizing, was related to disease.

Biochemical properties of the mycobacteria investigated proved variable and failed to provide a basis for further subdivisions. However, urease activity may be helpful as Group II scotochromogens isolated from disease were urease-positive and those isolated independent of disease were urease-negative.

REFERENCES

Affronti, L. P., 1959. Purified Protein Derivatives (PPD) and Other Antigens Prepared from Atypical Acid-Fast Bacilli and Nocardia asteroides. Am. Rev. Tuberc. and Pul. Dis. 79:284-295.

Beck, A., 1961. Serological Investigations of Atypical Acid-Fast Bacilli. J. Path. Bact. 82:45-51.

Bergey's Manual of Determinative Bacteriology 7th Ed. 1957. William & Wilkins Co.

Bojalil, L. F., and Cerbon, J., 1960. A Comparative Study of Non-Photochromogenic Mycobacteria and Mycobacterium Avium. Am. Rev. Resp. Dis. 81:382-386.

Bonicke, R., 1961. Identification of Mycobacteria by Biochemical Methods. Bull. Int. Union against Tuberc. 32:13-68.

Boyden, S. V., 1951. The Adsorption of Proteins on Erythrocytes Treated with Tannic Acid and Subsequent Hemagglutination by Antiprotein Sera. J. Ex. Med. 93: 107-120.

Buhler, V. B., and Pollak, A., 1953. Human Infection with Atypical Acid-Fast Organisms. Report of Two Cases with Pathologic Findings. Am. J. Clin. Path. 23:363-374.

Cater, J. C., and Redmond, W. B., 1961. Isolation of Studies on Bacteriophage Active against Mycobacteria. Can. J. Microbiol. 7:697-703.

Chapman, J. S., 1962. The Present Status of the Unclassified Mycobacteria. Am. J. Med. 33:471-477.

Chapman, J. S., Dewlett, H. J., and Potts, W. E., 1962. Cutaneous Reactions to Unclassified Mycobacterial Antigens. A Study of Children in Household Contact with Patients Who Excrete Unclassified Mycobacteria. Am. Rev. Resp. Dis. 86:547-552.

Chofnas, I., and Newton, J., 1955. Pneumonitis Due to Chromogenic Acid-Fast Facilli. Tr. 14th VA-Armed Forces Conference on the Chemotherapy of Tuberculosis. p. 205.

Cobbett, L., 1918. An Acid-Fast Bacillus Obtained from a Pustular Eruption. Brit. Med. J. 2:158.

Crawford, A. B., 1926. Tuberculin Sensitization in Guinea Pigs Caused by Various Acid-Fast Organisms. J. Am. Vet. M.A. 69:579-588.

Crawford, A. B., 1927. A Sensitization Method of Differentiating Avian from Mammalian Tubercle Bacilli. Am. Rev. Tuberc. 15:111-126.

Crow, H. E., King, C. T., Smith, E. C., Coupe, R. F., and Stergus, I., 1957. A Limited Clinical, Pathologic, and Epidemiologic Study of Patients with Pulmonary Lesions Associated with Atypical Acid-Fast Bacilli in the sputum. Am. Rev. Tuberc. and Pul. Dis. 75:199-222.

Edwards, L. B., and Palmer, C. E., 1958. Epidemiologic Studies of Tuberculin Sensitivity. I. Preliminary Results with Purified Protein Derivatives Prepared from Atypical Acid-Fast Organisms. Am. J. Hygiene. 68: 213-257.

Edwards, L. B., and Palmer, E. E., 1959. Isolation of "Atypical" Mycobacteria from Healthy Persons. Am. Rev. Resp. Dis. 80:747-749.

Edwards, L. B., Edwards, P. Q., and Palmer, C. E., 1959. Sources of Tuberculin Sensitivity in Human Populations: A Summing Up of Recent Epidemiologic Research. Acts Tuberc. Scandinav., Suppl. 47:77-97.

Edwards, L. B., Palmer, C. E., Affronti, L. F., Hopwood, L., and Edwards, P. Q., 1960. Epidemiologic Studies of Tuberculin Sensitivity. II. Response to Experimental Infection with Mycobacteria Isolated from Human Sources. Am. J. Hygiene. 71:218-241.

Edwards, L. E., Hopwood, L., Affronti, L. F., and Palmer, C. E., 1961. Sensitivity Profiles of Mycobacterial Infection. Bull. Int. Union against Tuberc. 32:384-394.

Edwards, P. Q., and Edwards, L. B., 1960. Story of the Tuberculin Test. From an Epidemiologic Viewpoint. Am. Rev. Resp. Dis. 81:1-47.

Eckman, P. J., Bickers, J. N., and Buechner, H. A., 1961. The Potential Importance of Person-to-Person Contact in the Spread of Photochromogenic Mycobacterial Infections. Trans. 20th Res. Con. and Pul. Dis. 204-211.

Engbaek, H. C., Friis, Th., and Ohlsen, A. S., 1957. A Case of Lung Disease Caused by an Atypical Acid-Fast Organism. Acta Tuberc. Scandinav. 34:145-162.

Engbaek, H. C., Magnussen, M., and Neilson, J. A., 1959. A Further Case of Lung Disease Caused by Atypical Acid-Fast Organism. Acta Tuberc. Scandinav. 37:227-244.

Feldman, W. H., Davies, R., Moses, H. E., and Andberg, W., 1943. An Unusual Mycobacterium Isolated from Sputum of a Man Suffering from Pulmonary Disease of Long Duration. Am. Rev. Tuberc. 48:82-93.

Francis, J., 1959. The Work of the British Royal Commission on Tuberculosis, 1901-1911. Tubercle, 66:124.

Freedman, S. O., Turcotte, R., Fish, A. J., and Sehon, A. H., 1963. The In Vitro Detection of "Cell Fixed" Hemagglutinating Antibodies to Tuberculin Purified Protein Derivatives (PPD) in Humans. J. Immunol. 90:52-59.

Fregnan, G. B., Smith D. W., and Randall, H. M., 1961. Biological and Chemical Studies on Mycobacteria. Relationship of Colony Morphology to Mycoside Content for Mycobacterium kansasii and Mycobacterium fortuitum. J. Bact. 82:517-527.

Frey, C. A., and Hagan, W. A., 1931. The Distribution of Acid-Fast Bacteria in Soils. J. Infect. Dis. 49: 497-506.

Froman, S., Scammon, L., Hanson, D., Black, J. P., and Will, D. W., 1961. The Bacteriophage Susceptibility of the Battey Type of Unclassified (Atypical) Acid-Fast Bacillus and M. avium isolated from Fowl and Swine: An Epidemic Logic Study. Trans. 20th Res. Con. and Pul. Dis. 218-224.

Hardy, A. H., Dunbar, F. P., Jeffries, M. B., Bond, J. O., and Lewis, A. G., 1958. Bacteriological and Epidemiological Studies of Pulmonary Diseases Associated with Atypical Acid-Fast Bacilli. Am. J. Pub. Health. 48: 754-759.

Hastings, E., Beach, B. A., and Thompson, I., 1930. The Sensitization of Cattle to Tuberculin by Other than the Bacilli. Am. Rev. Tuberc. 22:218-225.

Karlson, A. G., Davis, C. L., Cohn, M. L., 1962. Scotochromogenic Mycobacterium avium from a Trumpeter Swan. Am. J. of Vet. Res. 23:575-579.

Koch, R., 1890. Uber Bacteriologische Forchung Deutsche. Med. Wchnschr., 16:756 (translated in Lancet, 1890. 2:673)

Koch, R., 1890. I. Weitne Mettheilungen uber ein Heilmittel gegen Tuberculoses. Deutsche Med. Wchnschr., 16:1029 (translated in Lancet 2:1085-1086, 1891)

Koch, R., 1891. Forsetzung der Mittheilungen uber ein Heilmittel gegen Tuberculose, Deutsche med. Wchnschr. 17:101 (translated in Lancet 1:168-169, 1891)

Konno, L., 1953. The Metabolism of Nicotinic Acid of Acid-Fast Bacilli. A New Method to Differentiate Human Tubercle Bacilli from other Acid-Fast Bacilli (Bovine, Avian and Nonpathogenic Acid-Fast Bacilli) by Determining Nicotinic Acid in the Culture Filtrate. Proc. Jap. Acad. 29:289-292.

Konno, K., 1956. New Chemical Method to Differentiate Human-Type Bacilli from Other Mycobacteria. Science 124:985.

Konno, K., Kurzmann, R., and Bird, K. T., 1957. The Metabolism of Nicotinic Acid in Mycobacteria. A Method for Differentiating Tubercle Bacilli of Human Origin from Other Mycobacteria. Am. Rev. Tuberc. 75:529-537.

Konno, K., Kurzmann, R., Bird, K. R., and Sbarra, A., 1958a. Differentiation of Human Tubercle Bacilli from Atypical Acid-Fast Bacilli. I. Niacin Production of Human Tubercle Bacilli and Atypical Acid-Fast Bacilli. Am. Rev. Tuberc. 77:669-674.

Konno, K., Kurzmann, R., Bird, K. T., and Sbarra, A., 1958b. Differentiation of Human Tubercle Bacilli from Atypical Acid-Fast Bacilli. II. Clinical Applications. Am. Rev. Tuberc. 77:675-680.

Konno, K., and Sbarra, A. J., 1959. Differentiation of Human Tubercle Bacilli from Atypical Acid-Fast Bacilli. III. Modification of the Niacin Test. Am. Rev. Tuberc. 79:810-813.

Konno, K., Nagayama, H., and Oka S., 1960. Differentiation of Bovine Tubercle Bacilli from Other Mycobacteria by the Determination of Nicotinamidase Activity. Nature. 184:1743-1744.

Krause, A. K., 1916. Studies in Immunity to Tuberculosis: Experimental Studies on the Cutaneous Reaction to Tuberculo-protein. J. Med. Res. 35:1-23.

Kubica, G. P., and Vestal, A. L., 1959. Tuberculosis Laboratory Methods in Diagnosis. U.S. Department of Health Education and Welfare, Communicable Disease Center; Atlanta, Georgia.

Kubica, G. P., and Gleason, L. P., 1960. Studies on the Catalase Activity of Acid-Fast Bacilli. I. An Attempt to Sub-group These Organisms on the Basis of Their Catalase Activities at Different Temperatures and pH. Am. Rev. Resp. Dis. 81:387-391.

Kubica, G. P., Beam, E. R., Vestal, A. L., and Pool, G. L., 1960. The Intracutaneous Inoculation of Guinea Pigs As a Means of Determining the Virulence of Acid-Fast Bacilli, with Special Emphasis on the Anonymous (Atypical) Acid-Fast Bacilli and Isoniazid-Resistant Tubercle Bacilli. I. Preliminary Report. Am. J. Med. Tech. July-August, 1960. pp. 241-246.

Kubica, G. P., Bean R. E., Palmer, J. W., and Rigdon, A. L., 1960. The Isolation of Unclassified (Atypical) Acid-Fast Bacilli from Soil and Water Samples Collected in the State of Georgia. Am. Rev. Resp. Dis. 84:135 (abstract)

Kubica, G. P., and Vestal, A. L., 1961a. The Arylsulfatase Activity of Acid-Fast Bacilli. I. Investigation of Stock Cultures of Acid-Fast Bacilli. Am. Rev. Resp. Dis. 83:728-732.

Kubica, G. P., and Beam, R. E., 1961. The Arylsulfatase Activity of Acid-Fast Bacilli. II. The Differentiation of Mycobacterium avium from the Unclassified Group III Nonphotochromogenic Mycobacteria. Am. Rev. Resp. Dis. 83:733-740.

Magnusson, M., 1961. Specificity of Mycobacterial Sensitins. I. Studies in Guinea Pigs with Purified "Tuberculin" Prepared from Mammalian and Avian Tubercle Bacilli, Mycobacterium balnei, and other Acid-Fast Bacilli. Am. Rev. Resp. Dis. 83:57-67.

Magnusson, M., Engbaek, H. C., and Bentzon, M. W., 1961. Specificity of Mycobacterial Sensitins. II. Studies in Guinea Pigs with Purified Sensitin Prepared from Unclassified Acid-Fast Bacilli. Am. Rev. Resp. Dis. 83:1

Magnusson, M., 1962. Specificity of Sensitins. III. Further Studies in Guinea Pigs with Sensitins of Various Species of Mycobacterium and Nocardia. Am. Rev. Resp. Dis. 87:395-404.

Middlebrook, G., and Dubos, R. J., 1948. Specific Serum Agglutination of Erythrocytes Sensitized with Extracts of Tubercle Bacilli. J. Ex. Med. 88:521-528.

Nagayama, H., Konno, K., and Oka, S., 1961. Formamidase in Mycobacteria and Its Use in Differentiating Saprophytic Mycobacteria from Other Mycobacteria. Nature. 190: 1219-1220.

Palmer, C. E., and Edwards, L. B., 1962. Geographic Variations in the Prevalence of Sensitivity to Tuberculin (PPD-S) and to the Battey Antigen (PPD-B) Throughout the United States. Bull. Int. Union against Tuberc. 32:373-383.

Parlett, R. C., and Youmans, G. P., 1959. An Evaluation of the Specificity and Sensitivity of a Gel Double-Diffusion Test for Tuberculosis. A Double Blind Study. Am. Rev. Resp. Dis. 80:153-166.

Parlett, R. C., and Rehr, C. A., 1959. Further Studies on Gel Diffusion Tests in Tuberculosis. I. Methods for Standardization of the Antigen and Detection of Small Amounts of Antibody. Am. Rev. Resp. Dis. 80:886-894

Parlett, R. C., 1962. Some Immunological Considerations of Mycobacteria. Ann. New York Acad. of Sciences 98: 637-646.

Pope, H., and Smith, D. T., 1946. Synthesis of B-Complex Vitamins by Tubercle Bacilli When Grown on Synthetic Media. Am. Rev. Tuberc. 54:559-563.

Rabinowitsch-Kempner, L., 1927. The Types of Tubercle Bacilli in Human Tuberculosis. Am. Rev. Tuberc. 15: 225.

Redmond, W. B., and Cater, J. C., 1962. Bacteriophage from Stool Specimens with Activity on Mycobacteria. Phage Typing of Pathogenic Mycobacteria. V. A. Med. Res. Con. 13:129 (abstract)

Redmond, W. B., 1963. Bacteriophages of the Mycobacteria--A Review. Adv. Tuberc. Res. 12:191-229 (Karger, Basel New York)

Runyon, E. H., 1959. Anonymous Mycobacteria in Pulmonary Disease. Med. Clin. No. America. 43:273-290.

Runyon, E. H., Selin, M. S., and Harris, H. W., 1959. Distinguishing Mycobacteria by the Niacin Test. A Modified Procedure. Am. Rev. Tuberc. 79:663-665.

Scammon, L. A., Pickett, W. J., Froman, S., and Will, D. W., 1963. Nonchromogenic Acid-Fast Bacilli Isolated from Tuberculosis Swine. Their Relation to M. avium and the "Battey" Type of Unclassified Mycobacteria. Am. Rev. Resp. Dis. 87:97-102.

Schaefer, W. B., 1962. Personal Communication.

Schaefer, W. B., 1963. Serological Identification and Classification of the Mycobacteria Other than M. tuberculosis Encountered in Human Disease. Med. Abstracts. NTA Ann. Meeting.

Schwieger, O., Roman, E., and Soos, I., 1958. Studies on the Catalase Enzyme of Mycobacteria. I. Correlation between Catalase Activity and Virulence of Mycobacteria under Certain Experimental Conditions. Am. Rev. Tuberc. 77:146-154.

Seibert, F. G., and Morley, N., 1933. The Relationship of the Tuberculin Proteins of Different Acid-Fast Bacilli to Sensitization As Indicated by Their Reactivity in Sensitized Animals. J. Immunol. 24:149-156.

Singer, J., and Cysner, E., 1952. Urease Activity in Mycobacteriaceae. Am. Rev. Tuberc. 65:779-782.

Suter, E., and Strain, A. K., 1959. Infectivity and Immunogenicity of Mycobacterium X in Mice. Am. Rev. Tuberc. 79:47-51.

Sweeney, E. E., and Jann, G. J., 1961. Carbohydrate Typing of Mycobacteria. Soc. Ex. Biol. Med. 108:671-674.

Takahashi, Y., and Ono, K., 1961a. Study on the Passive Hemagglutination Reaction by the Phosphatide of M. Tuberculosis. I. The Reaction and Its Specificity. Am. Rev. Resp. Dis. 83:381-385.

Takahashi, Y., and Ono, K., 1961b. Study on the Passive Hemagglutination Reaction by the Phosphatide of M. Tuberculosis. II. Conditions of the Reaction. Am. Rev. Resp. Dis. 83:386-393.

Takahashi, Y., Omedera, T., and Yamamota, K., 1961c. The Behavior of Three Different Kinds of Antibodies in Tuberculosis: Antiprotein, Antipolysaccharide, and Antiphosphatide. I. Experimental Tuberculosis. J. Ex. Med. 114:555-567.

Takahashi, Y., Mochizuki, K., Nagayama, Y., 1961d. The Behavior of Three Different Kinds of Antibodies in Tuberculosis: Antiprotein, Antipolysaccharide, and Antiphosphatide. II. Human Tuberculosis. J. Ex. Med. 114:569-579.

Takahashi, Y., 1962. Specific Serum Agglutination of Kaolin Particles Sensitized with Tubercle Phosphatide and Its Clinical Evaluation As a Serodiagnostic Test for Tuberculosis. Am. Rev. Resp. Dis. 85:708-719.

Takeya, K., Nakashima, K., Takehara, Y., Toda, T., 1961. Tuberculin-Specificity of Unclassified Mycobacteria Isolated in Japan. Jap. J. Microbiol. 5:51-59.

Takeya, K., Zinnaha, Y., Nakayama, H., Inoue, Y., Hamada, Y., and Toda, T., 1962. Epidemiological Study of Unclassified Mycobacteria Infection Using Purified Tuberculin Protein. Kyushu J. Med. Science. 13: 181-192.

Tarshis, M. S., 1962. A Combined Neutral Red. Serpentine Cord, and Differential Growth Test for Distinguishing Human and Bovine Tubercle Bacilli from Other Mycobacteria. Am. Rev. Resp. Dis. 86:749-754.

Timpe, A., and Runyon, E. H., 1954. The Relationship of "Atypical" Acid-Fast Bacteria to Human Disease. A Preliminary Report. J. Lab. & Clin. Med. 44:202.

Toda, T., Hagihara, Y., and Takeya, K., 1961. A Simple Urease Test for the Classification of Mycobacteria. Am. Rev. Resp. Dis. 83:757-761.

Topley and Wilson. Principles of Bacteriology and Immunity, 1955. 4th Ed. Williams & Wilkins Co., Baltimore, Maryland.

Tsukamura, M., 1961. Enzymatic Reduction of Picric Acid. J. Biochem. 48:662-671.

Tsukamura, M., 1961. Enzymatic Reduction of Picric Acid by Mycobacteria. Am. Rev. Resp. Dis. 84:87-89.

Virtanen, S., 1960. A Study of Nitrate Reduction by Mycobacteria. The Use of the Nitrate Reductase-test in the Identification of Mycobacteria. Acta Tuberc. Scand. Suppl. 48.

Vischer, W. A., Daniels, J., and Pappenheimer, A. M., 1955. Observations on a Pleomorphic Mycobacterium Isolated from Mouse Brains. I. Cultural and Morphologic Characteristics. Am. Rev. Tuberc. 71:88.

Ward, D. M., and Redmond, W. B., 1962. Spotting Method of Phage Typing Mycobacteria. Bacterial Clearing Due to Nonspecific Action. Am. Rev. Resp. Dis. 85:883-889.

Wayne, L. G., Juarez, W. J., and Nochols, E. G., 1958. Arylsulfatose Activity of Aerobic Actinomycetales. J. Bact. 75:367-368.

Wayne, L. G., 1961. Recognition of Mycobacterium fortuitium by Means of a Three-Day Phenolphthalein Sulfatase Test. Am. J. Clin. Path. 36:185-187.

Wayne, L. G., 1962. Differentiation of Mycobacteria by Their Effect on Tween 80. Am. Rev. Resp. Dis. 86:579-581.

Whitehead, J. E. M., Wildy, P., and Engbaeck, H. C., 1953. Arylsulfatase Activity of Mycobacteria. J. Path. Bact. 65:451-460.

Woolf, C. M. Syllabus for an Introduction to Biometry. University of Utah, Salt Lake City, Utah.

Zinnaka, Y., Takeya, K., Nakayama, H., and Toda, T., 1962. Further Studies of Purified Tuberculin Protein of Unclassified Mycobacteria: A Modification of Purification Method. Kyushu J. Med. Science. 13:151-158.