IDENTIFICATION OF THE UNCLASSIFIED MYCOBACTERIA IN THE CLINICAL LABORATORY

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ABSTRACT

IDENTIFICATION OF THE UNCLASSIFIED MYCOBACTERIA IN THE CLINICAL LABORATORY

An evaluation of methods for identifying the unclassified mycobacteria has been conducted. Mycobacteria cultures totalling 101 strains were collected and studied for microscopic and cultural characteristics, growth in thioglycollate broth, niacin production, hydrolysis of Tween 80, arylsulfatase production, tolerance to oleic acid, and drug resistance. These properties are described and discussed in separate chapters of the thesis, and are followed by a discussion of the problem and the results of this project.

A procedure for identifying mycobacteria according to Runyon's classification is presented. The scheme is based on temperature requirements, rate of growth in thioglycollate broth, and pigment production. The results indicate that the niacin test, the Tween 80 test, and the arylsulfatase test may be used for the identification of M. tuberculosis, M. kansasii and M. fortuitum, respectively.

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INTRODUCTION

Unclassified mycobacteria may be defined as acid-fast bacilli isolated from human material that differ significantly from the recognized species of the genus Mycobacterium. Mycobacteria are described as aerobic, nonmotile, nonspore-forming, rod shaped cells that are usually acid-fast, and that do not branch under ordinary cultural conditions (Breed, Murray and Smith, 1957). The genus is divided into two groups, the pathogenic species and the saprophytic species, on the basis of the ability of the organisms to produce disease in warm-blooded animals with the qualification that some organisms included in the saprophytic group are potential pathogens.

Wilson and Miles (1964) divide the mycobacteria into four groups:

1. The tubercle bacilli that produce progressive disease in mammals and birds, e.g., M. tuberculosis, M. bovis and M. avium.

2. The intermediate group, M. ulcerans, M. balnei and M. fortuitum, that are intermediate in virulence between tubercle and saprophytic acid-fast bacilli.

3. The anonymous (unclassified) group of mycobacteria that may be as virulent to man as tubercle bacilli or may be innocuous.

4. The saprophytic acid-fast bacilli that are unable to set up a progressive infection in mammals or birds, e.g., M. phlei, M. smegmatis and M. butyricum.

The unclassified mycobacteria exhibit some of the properties of the saprophytic species (lack of virulence for the guinea pig, and marked pigmentation), and some of the characteristics of the pathogens (slow growth). Prior to the last decade these organisms were probably confused with the saprophytes or the pathogens, and patients were treated accordingly.

Various authors have described tentative schemes to aid in the identification and classification of the unclassified mycobacteria. Marks and Richards (1962) have proposed a scheme based on morphology, pigment production, optimum temperature, and drug sensitivity, which divides these organisms into seven groups. A simpler classification, which is commonly used in North America, was devised by Runyon in 1959. The main criteria for this scheme are pigment production, growth rates, and animal pathogenicity. The names used in Runyon's classification are as follows:

Group I	Photochromogens	M. kansasii
Group II	Scotochromogens	
Group III	Nonphotochromogens	
Group IV	Rapid growers	

Runyon (1960) states that as much as 10% of the disease diagnosed as tuberculosis in some areas of the United States may be due to the unclassified mycobacteria. The pulmonary form of the disease has been given many names - mycobacterial pseudo-tuberculosis (Marks and Trollope, 1960a); atypical pulmonary tuberculosis (Phillips and Larkin, 1964); pulmonary mycobacteriosis (Elston, Farrillo,

Meiterger and Kleitsch, 1964). The pulmonary disease closely resembles tuberculosis in clinical, radiological and histological findings, but is usually a more benign and mild form (Runyon, 1959). Some fatal cases have been reported (Corpe, Smith and Stergus, 1961). At least three different organisms have been implicated as the etiological agent of pulmonary disease - M. kansasii (Kleton et al., 1964), Group III nonphotochromogens, Battey type, (Smith, Kovacs and Harris, 1964), and M. fortuitum (Dross, Abbatiello, Jenney and Cohen, 1964).

Unclassified mycobacteria have been known to cause cervical adenitis. Black and Chapman (1964) have reported cervical adenitis in children due to M. kansasii, Group II scotochromogens, and Group III nonphotochromogens, while M. fortuitum was isolated from the cervical lymph nodes of an adult by Wells, Aguis and Smith (1955). A case of fatal disseminated osteomyelitis due to Group III nonphotochromogens, Battey type, in a child was described by Yakovac, Baker, Sweigert and Hope (1961), and destructive polyarthritis due to M. kansasii was reported by Klinenberg, Grimley and Sugmiller (1965).

The unclassified mycobacteria have also been implicated as the etiological agent of cat-scratch fever on the basis of the isolation of photochromogenic mycobacteria (M. kansasii) and scotochromogens from eight cases of cat-scratch fever (Boyd and Craig, 1961). The organisms were not isolated from the claws nor the saliva of cats, but attempts to isolate acid-fast bacilli from

the fur of the animals were not made. Unclassified mycobacteria have been isolated repeatedly from soil samples (Kubica, Beam and Palmer, 1963).

A single isolation of an unclassified mycobacterium may be of no clinical significance. Some organisms are more frequently proven to be pathogenic than others, e.g., M. kansasii and Group III nonphotochromogens, Battey type, are more often associated with pulmonary disease than the Group II scotochromogens or the Group IV rapid growers which are usually isolated only once (Runyon, 1959).

This difference in clinical significance among the species emphasizes the need for a method to identify mycobacteria correctly. The purpose of this study was to evaluate various means of identifying mycobacteria, and in particular, the unclassified mycobacteria encountered in a bacteriology department of a general hospital.

Many tests (cultural, biochemical and animal virulence) have been reported to be useful in identifying mycobacteria. After reviewing the literature it was decided to evaluate the following tests:

1. Morphological and cultural properties
2. Growth in thioglycollate broth
3. Niacin test
4. Tween 80 test
5. Arylsulfatase test
6. Effect of oleic acid on growth
7. Drug sensitivity

Wayne, in 1962, stated that a successful laboratory test should require a minimum of handling of large masses of bacilli, employ a simple technical procedure, produce rapid results and allow a minimum of ambiguity in results. These criteria were considered while evaluating the tests used in this study.

To enable a more critical evaluation of the individual tests, this thesis is divided into chapters with each test being presented separately. The chapters are divided into the following five sections:

1. Review of the Literature
2. Materials and Methods
3. Results
4. Discussion
5. Conclusions

A discussion of the study as a whole is presented in Chapter X along with a scheme for identification of the unclassified mycobacteria arising from the results obtained from the various tests.

The term "unclassified mycobacteria" is used rather than "atypical mycobacteria" or "anonymous mycobacteria" as recommended by the Subcommittee on Unclassified Mycobacteria of the American Thoracic Society (Corpe, Runyon and Lester, 1963). Unclassified mycobacteria identified by other laboratories were classified according to Runyon's classification, and are designated accordingly throughout the text.

CHAPTER II

GENERAL MATERIALS AND METHODS

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Cultures Examined

Cultures of mycobacteria were obtained from various laboratories. *M. tuberculosis* H37Rv, *M. bovis* #4228-2 and *M. avium* Sheard were obtained from Dr. W. Steenken, Jr., Trudeau Laboratories, Saranac Lake, New York. *M. bovis* Copenhagen was obtained from the Ontario Health Department, *M. kansasii* from the American Type Culture Collection, and *M. phlei* from the Bacteriology and Immunology Department, University of Manitoba.

Forty-eight cultures of *M. tuberculosis* were obtained from the Bacteriology Department of the Winnipeg General Hospital where they were identified by the guinea pig virulence test.

Unclassified mycobacteria were requested from laboratories throughout Winnipeg. Unidentified cultures of mycobacteria were obtained from the following laboratories:

1. Bacteriology Department, Winnipeg General Hospital - 25 cultures.

2. Central Tuberculosis Clinic - 9 cultures.

3. Manitoba Provincial Laboratories - 10 cultures.

4. Bacteriology Department, Deer Lodge Hospital - 3 cultures.

Sixteen cultures were sent to the Hamilton Health Association where they were identified according to Runyon's classification. One culture was an avirulent strain of *M. tuberculosis*; the others were

M. kansasii (1), Group II scotochromogens (4), Group III non-chromogens "Battey" type (4) and M. fortuitum (6). The collection of mycobacteria thus consisted of 101 cultures, 32 of which were not identified outside this laboratory (Table I).

In 1964 the Bacteriology Department of the Winnipeg General Hospital isolated 14 strains of unclassified mycobacteria from specimens and 29 strains of M. tuberculosis. The percentage of mycobacteria belonging to the unclassified group is 32.6% of all acid-fast bacilli isolated. Information about the specimens and patients from whom these organisms were obtained was supplied by Dr. Shirley Parker and is shown in Table Ia.

M. kansasii was isolated repeatedly from the patient noted in Table Ia. M. fortuitum was isolated twice (once from sputum, and later, in 1965, from gastric washings) from the patient suffering from purpura. Group III nonchromogens obtained from the Manitoba Provincial Laboratories were isolated twice from the same patient, but no clinical information was obtained. The only patient with evidence of an infection due to an unclassified organism is the patient harbouring M. kansasii. No conclusions can be drawn from the information available on the other patients.

Medium

The organisms were propagated on Löwenstein-Jensen medium prepared as follows:

Löwenstein-Jensen Medium (Cruickshank, 1960).

A. Asparagin Mineral Salt Solution:

KH_2PO_4 (AR) 0.4%

TABLE I

CULTURES OF MYCOBACTERIA

Organism	No. of Strains
M. tuberculosis	50
M. bovis	2
M. avium	1
M. phlei	1
M. kansasii	2
Group II scotochromogens	4
Group III nonchromogens "Battey" type	4
M. fortuitum	6
Unidentified cultures	31
Total	101

TABLE Ia

ISOLATION OF UNCLASSIFIED MYCOBACTERIA FROM FOURTEEN PATIENTS

Organism	Specimen	Patient's Complaint
M. kansasii	Sputum	Chronic respiratory ailment with cavitation in right apex of lung - resistant to antituberculosis treatment.
Group II scotochromogens	Urine	? Tuberculosis of epididymis.
	Stool	Renal disease.
Group III "a"	Sputum	Acute respiratory ailment.
III "b"	Br. sec'ns.	Respiratory - ? sarcoidosis.
M. fortuitum	Sputum	Purpura.
	Br. wash.	Chronic asthma and bronchitis.
	Br. sec'ns.	Barbiturate poisoning.
	Asp. fluid	Atresia of oesophagus.
	Lung biopsy	Respiratory ailment with history of tuberculosis.
	Sinus swab	Draining sinus on chest.
	Urine	Renal and respiratory ailment.
	Urine	Abscess on hip.
Group IV rapid grower	Sputum	Myocardial infarction.

Br. sec'ns. and Br. wash. = bronchial secretions.
Asp. fluid = aspirated fluid.

$MgSO_4 \cdot 7H_2O$ (AR)	0.04%
$Na_3(C_6H_5O_7)_2 \cdot H_2O$ (AR)	0.1%
Asparagin	0.6%
Glycerol	2.0%
Distilled water	to 100 ml.

1. The asparagin mineral salt solution (A) was boiled over low heat for 15 minutes and cooled overnight.

2. Six gm. potato starch was added to 150 ml. of A and heated until a smooth mixture was obtained.

3. The mixture was sterilised in the autoclave at 10 lbs. pressure for 20 minutes.

4. Five eggs were broken, under aseptic conditions, into a sterile flask, shaken well, and filtered through sterile gauze.

5. The eggs were mixed with the cooled asparagin-potato starch mixture and 5 ml. of 2% malachite green solution were added.

6. The medium was distributed in 12 to 15 ml. amounts, under aseptic conditions, to Universal containers and sterilised in a slanted position by inspissation at 75° to 80°C. for one hour on two consecutive days.

The organisms were subcultured every three months and the stock cultures were stored at 4°C. after incubation at 37°C. to obtain heavy growth.

Acid-Fast Stain

Smears were stained by Ziehl-Neelsen method as follows:

Ziehl-Neelsen Staining Technique (Cruickshank, 1960).

Carbol Fuchsin (strong):

Basic fuchsin	5.0 gm.
Phenol (AR)	25.0 gm.
Alcohol (95%)	50.0 ml.
Distilled water	500.0 ml.

The dye and the phenol were dissolved over boiling water. The alcohol and water were added to the solution and filtered before use.

Acid Alcohol (3%):

Alcohol (95%)	485 ml.
HCl (conc.)	15 ml.

Malachite Green (0.5%):

Malachite green	2.5 gm.
Distilled water	500 ml.

1. A film of the organisms was prepared, dried and fixed in the usual manner.

2. The slide was flooded with carbol fuchsin and heated to steaming for 10 minutes.

3. It was washed with water.

4. The film was decolorized with 3% acid-alcohol until no more fuchsin was washed off the film.

5. It was washed with water.

6. The film was counterstained for 15 seconds with 0.5% malachite green.

CHAPTER III

CELLULAR AND COLONIAL MORPHOLOGY

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CELLULAR AND COLONIAL MORPHOLOGY

I. REVIEW OF THE LITERATURE

Attention was first drawn to unclassified mycobacteria by their distinctively different cultural characteristics as compared to M. tuberculosis. Chromogenic acid-fast bacilli isolated from human material were reported by Pinner in 1935, and rapid growing acid-fast bacilli were described by Beaven and Bayne-Jones (1931), and Cummings and Williams (1933). Feldman, Davies, Moses and Andberg (1943) reported frequent isolations of nonchromogenic, smooth, slow growing, acid-fast cultures from a case diagnosed as bilateral pulmonary silico-tuberculosis.

It remained for Timpe and Runyon (1954) to attempt to classify the atypical mycobacteria. Their classification consisted of three groups based on colony morphology and pigment production. Further studies by Runyon (1955) and other workers (Wood, Buhler and Follak, 1956; Bogul, Keller and Cabelli, 1956) revealed some discrepancies in the classification and suggested that a revision was required.

Following an extensive survey of mycobacteria isolated from clinical specimens, Runyon (1959) published a classification containing four major groups, based on pigmentation of colonies, rate of growth, temperature relations, and lack of virulence for guinea pigs. The salient features of the groups are shown in Table II and are

A COMPARISON OF RUNYON'S CLASSIFICATION WITH MARKS AND RICHARDS' SCHEME

Runyon	Chromo-genicity	Cells	Growth Rate	Temperature Range	Semi-solid Medium	Drug Sensitivity	Name
Group I	Photo-chromogenic	Long	Slow			Variable	M. kansasii
II	Scoto-chromogenic	Variable	Slow			Resistant	Scotochromogens
III	None	Short	Slow			Resistant	Nonphotochromogens "Battey" type
IV	None		Rapid			Resistant	Rapid growers M. fortuitum
Marks & Richards Group I	Photo-chromogenic	Long		25° - 37°C.	Super-ficial	Sensitive except INH	M. kansasii
II	Scoto-chromogenic	Short		25° - 37°C.	Deep	Variable	Scrofula scoto-chromogens
III	Weak	Long		37° - 42°C.	Deep	Sensitive	Isoniazid-sensitive thermophiles
IV	None	Short		37° - 42°C.	Deep	Resistant	Isoniazid-resistant thermophiles
V	None	Short		37°C.	Deep	Resistant	Dysgonic mesophilic nonchromogens
VI	None	Variable		25° - 37°C.	Super-ficial	Resistant	Eugonic mesophilic nonchromogens
VII	None	Long		25° - 37°C.	Super-ficial	Resistant	Psychrophilic nonchromogens

as follows:

Group I Photochromogens

Pigmentation - no pigment produced if grown in the dark, but yellow to orange pigment develops if continuously exposed to light.

Growth rate - slow growing at 37°C. and 25°C.

Cells - long, banded and beaded.

Animal pathogenicity - no disease produced in guinea pigs, but the organisms are usually pathogenic to mice.

Group II Scotochromogens (from Greek "skotos" - darkness)

Pigmentation - yellow to orange pigment in the dark or in the light.

Growth rate - slow growing at 37°C. and 25°C.

Cells - variable in size.

Animal pathogenicity - not pathogenic to animals.

Group III Nonphotochromogens

Pigmentation - weak or no pigment production in the light or in the dark.

Growth rate - slow growing at 37°C. and 25°C.

Cells - pleomorphic, but often very short.

Animal pathogenicity - no disease produced in guinea pigs, but disease produced in mice with some strains.

Group IV Rapid growers

Pigmentation - no pigment produced in the light or in the dark.

Growth rate - rapid growing, requiring 48 hours at 37°C. and 25°C.

Animal pathogenicity - no disease produced in guinea pigs, but disease produced in mice with some strains.

Runyon's classification has been widely used in North America for the past five years. The Group I photochromogens have been accepted as a homogeneous group constituting a single species. The name Mycobacterium kansasii Maudurey has been adopted. "Mycobacterium scrofulaceum" was suggested by Prissick and Hanson (1956) as the name for scotochromogens (Group II), but this has not been accepted yet as there is some doubt that the group contains only one species. Differences in virulence for man among the Group II scotochromogens has led to the idea that two or more species may exist (Wayne, Doubek and Russel, 1964a).

Group III is known to be a heterogeneous group. Marks and Trollope (1960b) suggested the term "nonchromogen" for Group III rather than Runyon's term "nonphotochromogen" which could be interpreted to include the scotochromogens. (In the remainder of this text "nonchromogen" will be used to designate the Group III organisms). Several strains of Group III nonchromogen were isolated at the Battey State Hospital, Georgia, so have been called the "Battey bacillus". These strains closely resemble M. avium. The only distinguishing feature is that they are not pathogenic for fowl (Youmans, 1963). Wayne includes two other types of

organisms in Group III - the "radish" bacillus as described by Richmond and Cummings (1950) and a "J" subgroup of nonpigmented strains resembling cultures of low catalase activity of M. kansasii.

The Group IV rapid growers as described by Runyon include M. fortuitum and possibly some species of Nocardia. Wayne, and many others, include the saprophytic species M. smegmatis, M. phlei and M. rhodochrous in this group.

Marks and Richards (1962) criticized Runyon's classification because they found the pigment production was difficult to evaluate and the rate of growth was not a very distinctive character. They offered an alternative scheme designed to classify the pathogenic strains of unclassified mycobacteria, based on morphology, pigment production, temperature range, drug sensitivity and growth in semi-solid medium. A comparison of Runyon's classification and Marks and Richards' scheme is given in Table II.

Criticism of a classification based on pigment production is supported by the findings of Lambe and Rubica (1964) who studied the factors affecting pigmentation in mycobacteria. Interpretation of pigment production was considered difficult for the following reasons:

1. Light of certain intensity bleaches the medium.
2. Some strains produce more luxuriant growth in the dark than in the light.
3. Time at which results are observed is critical.
4. Colonies at the top of the Lowenstein-Jensen medium are

more pigmented than those at the bottom.

Wayne and Doubek (1964b) reported that the exclusion of air from cultures of M. kansasii prevented the production of pigment when exposed to light.

II. MATERIALS AND METHODS

Cultures Examined

The cultures examined for pigmentation, rate of growth and temperature requirements included the following:

Tubercle bacilli	-	35 human strains
		2 bovine strains
		1 avian strain
Unclassified mycobacteria	-	2 M. kansasii
		4 Group II scotochromogens
		4 Group III nonchromogens
		6 M. fortuitum
Saprophytes	-	1 M. phlei
Clinical isolates	-	31 unidentified cultures

Inoculum

A small portion of a colony of the acid-fast bacilli was used to streak the surface of three slants of Löwenstein-Jensen medium.

Method

Eighty-six cultures of mycobacteria were transferred to

three tubes of Löwenstein-Jensen medium. One tube was incubated in a slanted position 25 cm. from a 24 watt electric light bulb, and the second tube of medium was wrapped in black paper to exclude any light from the culture. Both tubes were incubated at 37°C. and the third tube was left at room temperature (22°C.) and exposed to the light of the room. The plants were examined daily for 48 days and the following were noted:

1. First appearance of colonies (number of days).
2. Texture of colonies (dry and rough, or smooth and moist).
3. Pigmentation of the colonies.

All unclassified mycobacteria and unidentified cultures and M. tuberculosis H37Rv were smeared and stained by the Ziehl-Neelsen technique and examined microscopically at a power of 1000x. The following characteristics were noted:

1. Size and shape of the cells.
2. Degree of acid-fast staining.
3. Degree of beading.

III. Results

Eight different growth patterns were observed: (Table III).

Pattern 1. Tubercle bacilli

The human and bovine strains of tubercle bacilli grew slowly at 37°C. (average of 8.4 days for colonies to appear), and failed to grow at room temperature. The colonies were raised and thick with a rough, wrinkled

surface, and cream to buff in color in the dark and in the light. A Ziehl-Neelsen stained smear of H37Rv exhibited the typical microscopic morphology of tubercle bacilli - long, thin rods in threads, staining evenly with beads appearing throughout the cells (Plate I.)

Pattern 2. Photochromogens

M. kansasii strains and three clinical isolates grew moderately slowly at 37°C. (average of 5.8 days) and more slowly at room temperature (average of 7.6 days). The colonies were usually dry, rough and heaped, but some were smooth and moist. Deep yellow pigment was produced when incubated continuously under light, but not in the dark (Plate II). The bacilli were long and thin, strongly acid-fast and heavily beaded.

Pattern 3. Scotochromogens

Runyon's Group II scotochromogens and nine clinical isolates grew slowly at 37°C. (7.2 day average) and at room temperature (9.9 day average). The majority of the strains developed smooth, moist, dome-shaped colonies, but a few developed the dry, rough variety. A deep yellow-orange pigment developed in both the light and the dark (Plate III). The cells were pleomorphic with short and long forms, and were moderately acid-fast with many beads.

Pattern 4. Nonchromogens "a".

The Group III nonchromogens, "Battey" type, M. avium, and three clinical isolates grew slowly at 37°C. (average of 12.3 days) and at room temperature (average of 20.9 days). No distinct colonies were formed, but a thin smooth film grew over the surface of the slant, with the exception of the avian strain which exhibited both rough and smooth types of colonies. Pigment production was lacking. Microscopically, the cells appeared to be mostly short, wide bacilli or coccobacilli. They were strongly acid-fast and showed only occasional beading.

Pattern 5. Nonchromogens "b".

Four unidentified cultures exhibited growth intermediate between the nonchromogens and the rapid growers. At 37°C. growth was fairly rapid (average of 3.6 days) but much slower at room temperature (average of 6.3 days). The growth was smooth, flat and moist, and a light yellow pigment was produced by one culture and a light pink pigment by the other three cultures (Plate IV). The presence of light had no effect on the pigment production. Microscopically, the cells resembled those of the nonchromogens in that they were very short bacilli, strongly acid-fast with a few beads.

Pattern 6. Rapid growers "a".

M. fortuitum strains and eleven clinical isolates grew

rapidly at 37°C. and room temperature (averages of 3.2 and 3.5 days, respectively). The colonies were smooth, moist and mucoid with no pigment production (Plate V). The bacilli were long and thin, slightly acid-fast with a few beads.

Pattern 7. Rapid growers "b".

One unidentified culture exhibited a growth pattern sufficiently different from the others to be described separately. Rapid growth at 37°C. and room temperature (2 days and 5 days, respectively) was characterized by dry, rough, heaped colonies which were cream in color but became light orange in time (Plate V). Light had no effect on the pigment production. The bacilli were long and thin, and slightly acid-fast.

Pattern 8. Saprophytes

Only one culture, M. phlei, exhibited this growth pattern. Growth was rapid (2 days) at 37°C. and at room temperature, and the dry and rough, or smooth and moist colonies were lemon yellow under all conditions (Plate V). Long, thin bacilli, slightly acid-fast, with a few beads, predominated in the Ziehl-Neelsen smear.

To determine if incubation under light affects the rate of growth of the mycobacteria, the average number of days required for the appearance of growth in light and in darkness was calculated for each type of growth (Table IV). The student's t test was

TABLE XXI

CHARACTERISTICS OF MYCOBACTERIA CULTURES

Growth Pattern	1	2	3	4
Cultures:	35 M. tuberculosis 2 M. bovis	2 M. kansasii 3 unidentified cultures	4 Group II scotochromogens 9 unidentified cultures	1 M. avium 4 Group III nonchromogens 3 unidentified cultures
Rate of growth in days: 37°C. Average 22°C. Average	3 - 21 8.4 none	3 - 25 5.8 4 - 10 7.6	2 - 17 7.2 3 - 14 9.9	3 - 32 12.3 3 - 47 20.9
Colony texture:	Raised, dry and rough	Raised, dry and rough, or smooth and moist.	Smooth and moist or dry and rough	Thin, smooth film
Pigmentation: Light Dark	Cream to buff Cream to buff	Deep yellow Cream to buff	Yellow-orange Yellow-orange	Cream to buff Cream to buff
Ziehl-Neelsen stain: Size Acid-fast Beading		Long, thin 3 - 4* 4*	Short or long 2* 4*	Coccobacilli 3 - 4* 1*
Classification:	Tubercle bacilli	Photo-chromogens	Scoto-chromogens	Nonchromogens "a"

TABLE XII (continued)

Growth Pattern	5	6	7	8
Cultures:	4 unidentified cultures	6 M. fortuitum 11 unidentified cultures	1 unidentified culture	1 M. phlei
Rate of growth in days:				
37°C.	2 - 7	1 - 7	2	2
Average	3.6	3.2	2	2
22°C.	3 - 11	1 - 6	5	2
Average	8.3	3.5	5	2
Colony texture:	Smooth, flat and moist	Smooth, moist and mucoid	Rained, dry and rough	Smooth, moist and mucoid, or dry and rough
Pigmentation:				
Light	Yellow or pink	Cream to buff	Cream to buff	Lemon-yellow
Dark	Yellow or pink	Cream to buff	Cream to buff	Lemon-yellow
Ziehl-Neelsen stain:				
Size	Coccobacilli	Long, thin	Long, thin	Long, thin
Acid-fast	4*	1*	1*	1*
Beading	1*	1*	1*	1*
Classification:	Nonchromogens "b"	Rapid growers "a"	Rapid growers "b"	Saprophytes

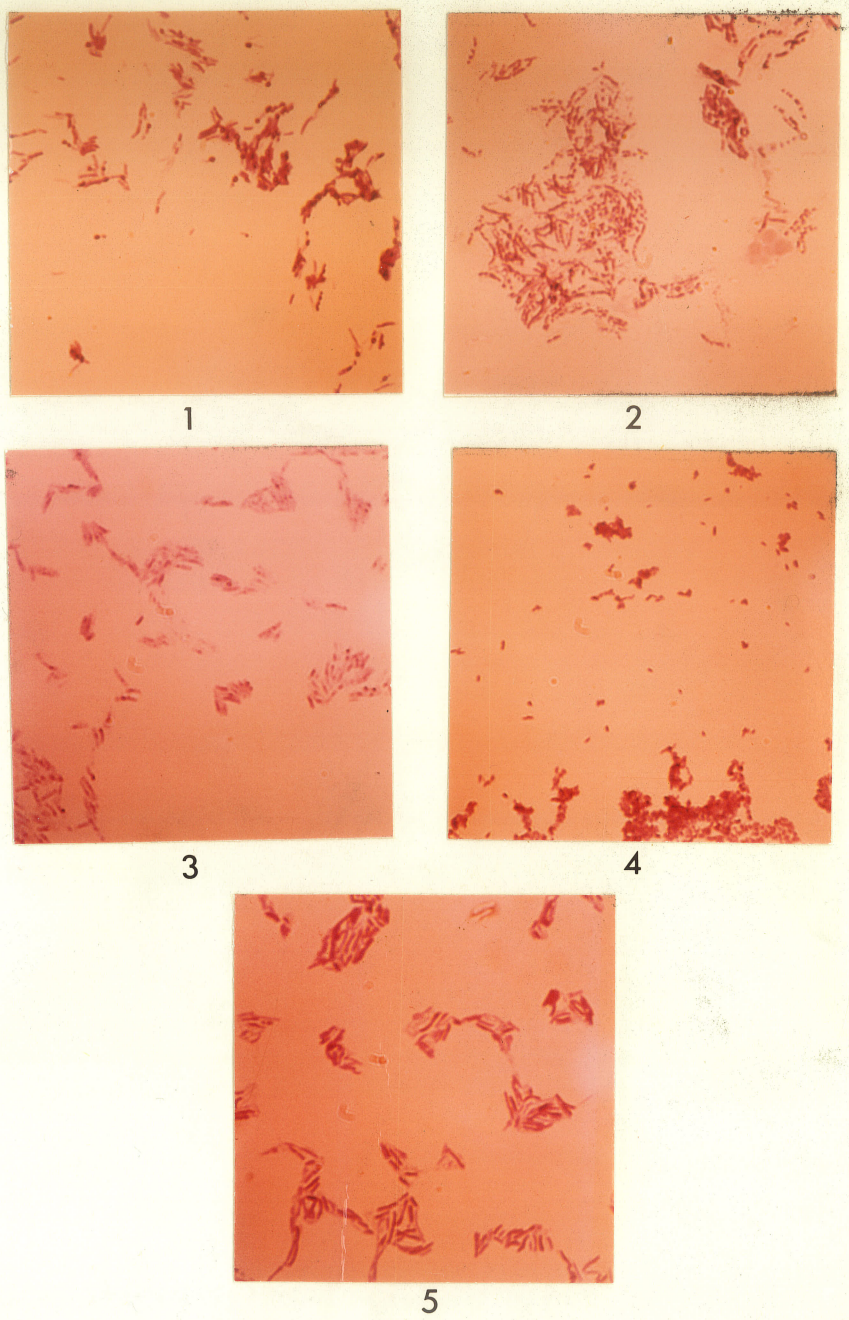

Plate I. Microscopic morphology of mycobacteria; 1. M. tuberculosis, 2. M. kansasii, 3. Group II scotochromogen, 4. Group III non-chromogen, 5. M. fortuitum.

Plate II. Group I photochromogens.
Left: incubated in the dark.
Right: incubated in the light.

Plate III. Group II scotochromogens.
Left: incubated in the dark.
Right: incubated in the light.

Plate IV. Group XXI nonchromogens.
Left: "Batty" type.
Center: nonchromogen "b" yellow pigment.
Right: nonchromogen "b" pink pigment.

Plate V. Group IV rapid growers.
Left: M. fortuitum.
Center: rapid grower "b".
Right: M. phlei.

TABLE IV

A COMPARISON OF GROWTH RATES IN THE LIGHT AND IN THE DARK

Growth Pattern	Number of Cultures	Average No. of Days in Light	Average No. of Days in Dark	t
I	37	8.75	8.1	0.76
II	5	4.60	7.0	1.15
III	13	7.69	6.9	0.59
IV	8	8.75	15.75	2.2
V	4	4.25	3.0	1.31
VI	17	2.59	3.88	2.7
VII	1	2	2	-
VIII	1	2	2	-

t = student's t test.

calculated, but the only significant difference existed between the rate of growth of the rapid growing organisms which grew more quickly in the light than in the dark.

IV. DISCUSSION

Identification of mycobacterial cultures has always been hampered by the variability in cultural characteristics within the different species. The tubercle bacilli develop rough or smooth colonies, the human type may develop yellow or faint red pigment on prolonged incubation, and the rate of growth, described as being slow, may range from a few days to a few weeks for the appearance of colonies. The saprophytic species are no more constant in their characteristics than the pathogens. The saprophytes exhibit smooth and rough colony variants, and develop pigments ranging from creamy white to deep orange. The problem is compounded by the appreciation of the role of the group of mycobacteria now designated as "unclassified", some of which closely resemble the pathogens, and others the saprophytes. Stringent control over the conditions of incubation, such as temperature and exposure to light, are required to separate one group from another.

Pigment production in the presence of light has been given an important role in the separation of the recently recognized species of the genus Mycobacterium, - Mycobacterium kansasii. This photochromogenic property was exhibited quite clearly by the five cultures described in growth pattern 2., but by none of the other

cultures studied. It was noted that old cultures of M. kansasii that had been grown in the dark and then stored at 4°C. failed to become yellow when exposed to light for 24 hours. This supports Runyon's findings that young cultures of M. kansasii exhibit photochromogenicity but in mature colonies the reaction is slow or non-existent. There was little difference in pigment production in the light as compared to the dark with any of the other types. The scotochromogens were deep yellow-orange as soon as growth became apparent, and the others were cream colored with the exceptions of the cultures described in patterns 5 and 7 which exhibited a small amount of pigment production which was not light dependent.

Runyon placed his cultures 45 cm. from a 30 watt lamp, while Marks and Richards used a 25 watt lamp 30 to 60 cm. from the cultures. A 25 watt lamp 25 cm. from the cultures was used in this study. No bleaching of the medium was observed. The cultures were placed almost horizontally so that all portions of the slant were approximately the same distance from the source of light. The intensity of pigment produced by the colonies at the top of the medium was observed to be the same as the colonies at the bottom. The light did not inhibit the growth of the mycobacteria (Table IV). There was no appreciable difference noted in the amount of growth on the slants incubated in the light as compared to the dark. These findings indicate that the difficulties in interpreting pigment production encountered by Lambe and Rubica (1964) were not observed with the method followed in this study.

The rate of growth was found to be extremely variable and not a reliable criterion for differentiating various groups. Considering just the averages presented in Table II, the cultures may be divided into two groups.

1. The slow growing organisms (Patterns 1 - 5) which require at least one week for growth at 37°C. or room temperature.
2. The rapid growing organisms (Patterns 6 - 8) which form visible colonies in less than one week at both 37°C. and room temperature.

This division is artificial and of little value when considering individual cultures as shown by the fact that at least one strain from each of the eight growth patterns exhibit growth in only three days at 37°C.

Growth at room temperature was useful in separating human and bovine strains of tubercle bacilli from the other mycobacteria. The one strain of M. avium tested produced slight growth at 22°C. within 21 days. All other cultures (Patterns 2 - 8) grew readily at room temperature. The rate of growth at 22°C. was slower than at 37°C. for Patterns 2 - 5 but was the same for Patterns 6 - 8.

Colony texture was variable and offered little assistance in identifying the organisms. The same applies for the appearance of the Ziehl-Neelsen stained smear. The descriptions given in the results and Table II represent the majority of the strains in each section but the variation was so great as to render the properties of colony texture and microscopic morphology unreliable for use in identification.

In order to conform with the present mode of classifying the unclassified mycobacteria, attempts were made to fit the eight types of mycobacteria into the classifications presented by previous authors. As growth at temperatures greater than 37° C., catalase activity, growth in semi-solid medium, and antibiotic resistance were unknown it was impossible to place the cultures into Marks and Richards' scheme. Runyon's classification, being less stringent, offered more agreement, and throughout the remainder of the text it will be used as follows:

Runyon's Classification	Growth Pattern No.
Tubercle bacilli	1
Group I Photochromogens	2
Group II Scotochromogens	3
Group III Nonchromogens	4 and 5
Group IV Rapid Growers	6 and 7
Saprophytes	8

When only "Group III nonchromogens" is used, it will include nonchromogens "a" and nonchromogens "b", and "Group IV rapid growers" will include M. fortuitum, M. fortuitum-like strains, and the rapid grower "b".

V. CONCLUSIONS

Eighty-six cultures of mycobacteria were studied for pigmentation, rate of growth, temperature requirements, and microscopic morphology. Microscopic morphology and colony texture

varied greatly among strains of the same species or group, rendering these characteristics unreliable for use in identification. The most reliable properties were found to be:

1. Pigment production - photochromogenicity or scoto-chromogenicity.

2. Temperature requirements - the failure of human and bovine tubercle bacilli to multiply at 22° C.

The rate of growth also varied greatly within the same species or group. It was difficult to determine if a strain was a slow grower or a rapid grower. Detection of additional characteristics are required to aid in a conclusive identification of the mycobacteria.

CHAPTER IV

GROWTH IN THIOGLYCOLATE BROTH

CHAPTER IV

GROWTH IN THIOGLYCOLLATE BROTH

I. REVIEW OF THE LITERATURE

Brewer's thioglycollate broth has been used for many years in bacteriology laboratories for anaerobic cultures, but it has been seldom considered as a medium for mycobacteria. Tarshis and Frisch (1952) compared the growth of tubercle bacilli, chromogenic acid-fast bacilli and saprophytic mycobacteria on many different media including Brewer's thioglycollate medium. They noted that the thioglycollate broth supported growth of nine chromogenic mycobacteria within 6 to 35 days; none of the tubercle bacilli were able to multiply, while the saprophytic strains grew rapidly within 24 to 48 hours forming a thick, wrinkled pellicle on the surface.

Gabelli, Elliott and McElroy (1954) studied the oxygen requirements of chromogenic acid-fast bacilli and noted the following:

1. Growth of chromogenic acid-fast bacilli in 18 to 24 hours at the interphase of the aerobic and anaerobic portions of the thioglycollate broth.
2. Removal of thioglycollic acid or agar rendered the medium unsuitable for growth of the chromogens.
3. Successive transfers of the acid-fast bacilli on egg media resulted in poor growth in thioglycollate broth.

The majority of Group I photochromogens and Group II scotochromogens grew slowly, requiring from 11 to 39 days for growth to first appear in thioglycollate broth, while a few strains failed to show any growth (Koch, Griffin and Agostini, 1958; Tarshis, 1959; Marks and Trollope, 1960a). Group III nonchromogens and M. avium strains also grew slowly while the saprophytic species and Group IV rapid growers (M. fortuitum) grew quickly forming a surface pellicle within one to two days (Tarshis, 1963a; Rodda and Singer, 1963).

Prolonged incubation of thioglycollate broth results in the formation of flocculent or crystalline precipitates due to concentration, and may simulate growth (Tarshis, 1959). To overcome the difficulties in reading growth in fluid thioglycollate medium, Smith and Steenken (1961) prepared agar slants of thioglycollate. They also used two types of thioglycollate media - one without an indicator and one with methylene blue added. They tested the growth of tubercle bacilli, unclassified mycobacteria and saprophytic mycobacteria on four different thioglycollate preparations:

1. Fluid thioglycollate without indicator.
2. Fluid thioglycollate with methylene blue.
3. Agar (1.5%) slants of thioglycollate without indicator.
4. Agar (1.5%) slants of thioglycollate with methylene blue.

Human and bovine strains of tubercle bacilli were inhibited by all four media, while M. avium produced growth in the fluid medium only. The Group IV organisms, which these authors called the saprophytic mycobacteria, produced rapid, heavy and opaque

growth in all four media in three days. The unclassified mycobacteria (Group I photochromogens, Group II scotochromogens and Group III nonchromogens) developed a slow dysgonic type of growth. The methylene blue exerted an inhibitory effect which was more marked in the fluid medium than the agar slants. Agar inhibited the growth of the Group III nonchromogens.

Thioglycollate medium exerts selective activity against human and bovine tubercle bacilli and separates the Group IV rapid growers and saprophytic species (M. fortuitum, M. phlei and M. smegmatis) from the unclassified slow growing mycobacteria. The growth patterns in this medium suggests that the unclassified slow growing mycobacteria are related to M. avium.

II. MATERIALS AND METHODS

Cultures Examined

The cultures examined for growth in thioglycollate medium included the following:

Tubercle bacilli	-	23 human strains
		2 bovine strains
		1 avian strain
Unclassified mycobacteria	-	5 Group I photochromogens (3)*
		12 Group II scotochromogens (8)*
		11 Group III nonchromogens (7)*
		18 Group IV rapid growers (12)*
Saprophytes	-	1 M. phlei

* Strains not identified outside this laboratory.

Medium

A. Thioglycollate medium without indicator, Brewer modified, (B.B.L.) was used as the base for the following preparations:

1. Fluid thioglycollate without indicator prepared according to manufacturer's instructions.

2. Semisolid thioglycollate - fluid thioglycollate plus 0.5% agar (B.B.L.)

3. Agar slants of thioglycollate - fluid thioglycollate plus 1.5% agar.

4. Fluid thioglycollate plus neutral red indicator - fluid thioglycollate plus 0.01 mgm./ml. neutral red.

All four preparations were dispensed in 10 ml. amounts in 16 x 150 mm. test tubes with metal caps, and sterilized at 15 lbs. pressure for 15 minutes. The semisolid medium was allowed to cool in the upright position while the agar slants were cooled at a 30° angle.

B. Fluid thioglycollate without indicator for the second part of the experiments was dispensed in 10 ml. amounts to screw-capped test tubes measuring 18 x 95 mm.

Method

A. Twenty-one cultures of mycobacteria were tested for growth in fluid thioglycollate without indicator, and with neutral red, semisolid thioglycollate, and solid slants of thioglycollate. A suspension of the test organism was prepared

by adding a 3 mm. loopful of solid culture to 10 ml. of sterile saline (0.65%) in a Universal container with twelve 5 mm. glass beads. The container was placed in a Vortex test tube mixer (model K-500-2) and shaken at high speed for five minutes. A small aliquot (0.1 ml.) of the suspension was added to one tube of each of the four types of media. The liquid media was rotated to disperse the inoculum, the semisolid medium was inoculated several times with a straight inoculating needle and the agar slant was streaked with a loop to insure the inoculum was spread over the entire slant. All tubes were incubated at 37°C. and were examined daily for 48 days to observe the growth pattern. Each organism was tested in duplicate.

B. Seventy-two cultures of mycobacteria were tested in duplicate for growth in fluid thioglycollate without indicator for 28 days at 37°C. A suspension of the test organism was prepared by adding a small portion of a colony from Löwenstein-Jenson medium slants to 0.5 ml. sterile water in a one-quarter ounce screw-cap bottle with two 5 mm. glass beads. The bottle was shaken manually for at least one minute. One loopful of the suspension was used as inoculum for each tube of media. The tubes were examined daily for growth.

XII. RESULTS

A. The rate of growth of the twenty-one mycobacteria strains in the four types of media is shown in Table V. The figures represent

TABLE V

GROWTH OF MYCOBACTERIA IN THIOGLYCOLLATE MEDIUM

Organism	Strain	Number of Days for Growth to Appear in Fluid - No Indicator	Semi-Solid	Agar Slant	Fluid with Neutral red
M. tuberculosis	H37Rv	-	-	-	-
	#226	-	-	-	-
	#7036	-	-	-	-
M. bovis	#4228-2	-	-	-	-
	Copenhagen	-	-	-	-
M. avium	Sheard	9	8	14	10
M. kansasii		24	13	-	26
Group II	#8021	-	-	-	-
scotochromogens	CTC #4	25	21	24	12
	CTC #5	25	12	20	20
	PL35699	27	22	26	47
Group III	#609	8	8	-	10
nonchromogens	#643	8	8	38	8
"Battey" type	#766	7	8	-	6
	#2382	5	8	-	5
Group IV	#172	2	2	2	2
M. fortuitum	#23848	2	2	2	2
	#81	2	3	3	2
	PL32810	2	2	2	2
	#3213	2	2	3	2
	#29095	2	3	3	3

the average number of days for the first appearance of growth. There is no appreciable difference in rate of growth with the fluid medium and the medium containing agar. The semisolid medium supported growth only at the surface (top 1 cm. of medium) while the agar slants showed only a few colonies of M. avium, Group II scotochromogens, and one of the Group III nonchromogens. Group IV M. fortuitum strains produced a thick, luxuriant growth on all four media in a short time.

Growth in the liquid media was of two types: (Plate VI)

1. Light flocculent growth which required up to two or more weeks to be apparent as in the case of the slow growing unclassified mycobacteria and M. avium.

2. Heavy growth at the surface of the liquid resulting in the formation of a dry, wrinkled pellicle which adhered to the glass surface as in the case of the M. fortuitum strains.

The presence of 0.01 mgm./ml. neutral red dye in the fluid thioglycollate had no effect on the growth rate of any of the organisms, nor the pattern of growth. The M. fortuitum strains took up the neutral red dye with the result that the thick surface pellicle was a brilliant red color. In time the liquid medium beneath the pellicle turned a bright yellow color indicating an alkaline pH which was confirmed by the use of phydrion paper (Micro Essential Laboratory).

B. The rate of growth in fluid thioglycollate without indicator by 72 strains of mycobacteria is given in Table VI.

Plate VI. Growth of unclassified mycobacteria in thioglycollate broth.
From left to right: Group II scotochromogen; Group III nonchromogen with slight flocculent growth; M. fortuitum, rapid grower "b"; and M. phlei with surface pellicle.

TABLE VI

MYCOBACTERIA IN FLUID THIOGLYCOLLATE MEDIUM

Organism	No. of Strains + for Growth	No. of Days Range	No. of Days Average	Type of Growth
Tubercle bacilli:				
M. tuberculosis	0/22	-	-	-
M. bovis	0/2	-	-	-
M. avium	1/1	28	28	flocculent
Unclassified mycobacteria:				
Group I photochromogens	4/5	10-28	17	flocculent
Group II scotochromogens	8/12	10-21	13.7	flocculent
Group III nonchromogens	9/11	12-25	15.3	flocculent
Group IV rapid growers	18/18	2-7	3.9	heavy pellicle
Saprophytes:				
M. phlei	1/1	4	4	heavy pellicle

None of the human nor bovine strains of tubercle bacilli produced growth within the 28 day incubation period while M. avium produced slight flocculent growth visible on the 26th day. Among the slow growing unclassified mycobacteria (Group I photochromogens, Group II scotochromogens and Group III nonchromogens) seven cultures failed to grow within the 28 days and 21 other cultures developed light flocculent growth after an average incubation period of two weeks. The shortest incubation period required for visible growth with these organisms was 10 days, and 28 days was the longest.

The Group IV rapid growers, including M. fortuitum strains, and the one saprophytic species, M. phlei, grew rapidly in thioglycollate broth producing a surface pellicle in less than one week. Seven days incubation was the longest period required for heavy growth on the surface of the medium.

IV. DISCUSSION

Thioglycollate medium, liquid or solid form, failed to support the growth of human and bovine tubercle bacilli, but did support sparse growth of slow growing mycobacteria such as M. avium, M. kansasii, Group II scotochromogens and Group III nonchromogens. Not all of the slow growing unclassified mycobacteria were capable of multiplying in the fluid medium, e.g., one photochromogen, four scotochromogens and two nonchromogens failed to grow in 28 days, but all of the rapid growing mycobacteria flourished in the thioglycollate medium.

Addition of 0.5% agar did not appear to effect the growth of the organisms except to limit it to the top one centimeter of the medium, while the addition of 1.5% agar seemed to inhibit the growth of M. kansasii, and the Group III nonchromogens. Neutral red dye was not inhibitory to growth, and was taken up by the rapid growing mycobacteria to give a red pellicle on the surface. Growth of the other mycobacteria was sparse so that it was not possible to determine if the neutral red dye had also been adsorbed by these organisms. The preparation of thioglycollate medium plus agar or neutral red dye was time consuming, and these preparations did not offer any advantages over the fluid thioglycollate without indicator.

The seventy-two strains of mycobacteria cultured in thioglycollate broth could be divided into three groups based on the growth pattern in the liquid medium:

1. Organisms which failed to grow in 28 days - 22 M. tuberculosis, 2 M. bovis, 1 photochromogen, 4 scotochromogens and 2 nonchromogens.

2. Organisms which grew slowly (2 to 4 weeks) to produce a slight flocculation - 1 M. avium, 4 photochromogens, 7 scotochromogens and 9 nonchromogens.

3. Organisms which grew rapidly (2 to 7 days) to produce a pellicle over the surface of the medium - 6 M. fortuitum, 12 rapid growers and 1 M. phlei.

The rate of growth on solid medium (Löwenstein-Jensen) of the Group IV rapid growers may in some cases be as slow as that of

some of the Group III nonchromogens (Chapter III). In such cases the growth pattern in thioglycollate broth is useful in placing the organisms into the category of a "rapid grower" or a "slow grower".

V. CONCLUSIONS

Seventy-two cultures of mycobacteria were tested for growth in fluid thioglycollate. Twenty-one cultures were tested for growth in fluid thioglycollate without indicator, and with neutral red, semisolid thioglycollate, and solid slants of thioglycollate. Fluid thioglycollate provided a simple method of separating Group IV rapid growers and saprophytic mycobacteria from other mycobacteria. Some strains of the slow growing unclassified mycobacteria may be separated from human and bovine tubercle bacilli by the development of a light flocculent growth after two to four weeks incubation. The addition of agar or neutral red dye to the thioglycollate medium offers no advantages to the test.

CHAPTER V

MAGAN RIVER

CHAPTER V

NIACIN TEST

I. REVIEW OF THE LITERATURE

The production of nicotinic acid (niacin) by M. tuberculosis has been used by many investigators as a method of differentiating tubercle bacilli of human origin from other mycobacteria. Pope and Smith (1946) noted that mycobacteria are capable of synthesizing the B group of vitamins, of which niacin is a member, from a basic medium. They used culture filtrates to study the synthetic ability of human and bovine strains of mycobacteria in Proskauer-Beck medium. The human strain, H37Rv, produced almost 50 times as much niacin as compared to the bovine strain, Ravenal. The production of niacin exceeded any of the other B vitamins. Bird (1947) showed that whole cultures of human tubercle bacilli, H37Rv, gave higher values of nicotinic acid than culture filtrates (61 mcgm./ml. as compared to 37 mcgm./ml.).

Niacin may be detected chemically according to the two following reactions:

1. Koenig's reaction - the development of a yellow color when cyanogen bromide is added to a pyridine derivative, niacin, in the presence of an amine.
2. Pelnice reaction - the development of a yellow color when niacin is mixed with chloramine T and a cyanide salt.

Konno (1956) used 4% alcoholic aniline as the amine and 10% cyanogen bromide to test various mycobacteria by the Koenig reaction. Other amines that have been used are 1.5% o-tolidine (Gutiérrez-Vásquez, 1960) to give a coral-red color, and 3% alcoholic benzidine (Medveczky, 1960) to give a violet-pink color if niacin is present. Medveczky claimed the benzidine reagent to be superior to aniline in the following respects:

1. The pink color contrasts well with most fluid and solid media.

2. The color of chromogenic acid-fast bacilli does not interfere with the reading of the test.

3. Benzidine is four times as sensitive as aniline.

Tarshis (1961) compared the three amines by performing niacin tests after 7, 10, 14 and 21 days incubation, and concluded that the sensitivity of aniline and benzidine was slightly better than o-tolidine when used on cultures from 7 to 10 days old, but on older cultures the sensitivity of the three methods was the same.

The Koenig reaction has the disadvantage of requiring the toxic cyanogen bromide which has an unpleasant pungent odour. A fume chamber is a necessity when working with this reagent. The Feknicz reaction offers a technique which is less hazardous to the worker. Runyon, Selin and Harris (1959) indicated that preliminary studies with these reagents proved the method to be less sensitive than the cyanogen bromide method. This was substantiated by

Karlson, Martin and Harrington (1964) who compared the Pelnice reaction with the Koenig reaction by testing liquid cultures of mycobacteria after 7, 14 and 21 days incubation. After 7 days, 52 of 65 cultures of M. tuberculosis were positive by the Koenig reaction but only 36 of 65 by the Pelnice reaction. After 21 days incubation, there was no difference between the two methods except that the Koenig reaction yielded a deeper yellow color.

Masses of solid culture were used for niacin tests by Konno (1956), Runyon et al. (1959), Koch and Kroll (1960), and Medvecsky (1960). The disadvantages to this method are as follows:

1. It requires large bacterial masses. Insufficient growth results in false negative or doubtful reactions (Runyon et al., (1959).

2. The transfer of several loopfuls of organism to a test tube constitutes a hazard to the worker.

3. The yellow color of a positive reaction in some cases is faint and is seen only close to the bacilli which renders interpretation of the test difficult with yellow pigmented organisms such as M. kansasii, Group II scotochromogens and M. phlei.

Konno, Kurmann, Bird and Sbarra (1958) advocated the use of liquid medium, and used the culture supernatant for the test, but lengthy periods of incubation were required. A Dubos liquid Tween albumin which yielded sufficient growth of human tubercle bacilli after 10 days incubation was found to be more practical (Konno and Sbarra, 1959).

An aqueous extract of the mycobacteria, rather than the culture itself, was used by Runyon et al. (1959) to detect the presence of niacin. The mycobacteria were grown on a solid medium until the colonies were mature and well developed, the niacin was extracted from the colonies and surrounding media by the addition of sterile distilled water which covered the surface of the slant. Two drops of the aqueous extract were mixed with two drops of each reagent in the well of a porcelain spot plate. The advantages of using an extract rather than a whole culture were cited by Runyon following a comparison of the methods:

1. False negative results are eliminated.
2. The color obtained is distinct and easily read.
3. Subculture to a liquid medium is unnecessary.
4. No difficulty is encountered from malachite green.
5. The transfer by pipette of the aqueous extract is easier and more rapid than the transfer of bacterial masses.
6. The culture is preserved intact.
7. The yellow pigmentation of colonies does not interfere with interpretation of the test.

Table VII shows the results obtained by various workers with the niacin test on human tubercle bacilli strains. The results are separated into those tests performed on cultures, solid or liquid, and those performed on aqueous extracts. From the group performed on cultures, 11.95% of the strains gave a negative or doubtful reaction as compared to 2.47% negative or doubtful reactions using an extract.

TABLE VII

RESULTS OF NIACIN TESTS OF *M. TUBERCULOSIS* BY VARIOUS WORKERS

Authors	Cultures				Extracts			
	Positive	Doubtful	Negative	Total	Positive	Doubtful	Negative	Total
Konno, et al.	584	85	0	669				
Runyon, et al.	113	21	80	214	207	2	2	211
Koch, et al.	14	0	6	20	97	0	0	97
Cilani, et al.	13	0	0	13				
Selkon, et al.	2	0	0	2				
Medvecsky	342	0	10	352				
Karlson, et al.	520	0	0	520				
Marks, et al.					144	6	3	153
Turnbis					9	0	0	9
Seamon, et al.					1	0	0	1
Gutiérrez-Vázquez					54	0	0	54
Total	1588	106	96	1690	512	8	5	525

Runyon used Löwenstein-Jensen, American Trudeau Society medium, Stoenken and Smith medium, 7H9 agar base and Tarshis blood agar to propagate the mycobacteria. The Tarshis blood agar imparted a pink color to the aqueous extracts, while the other media proved to be satisfactory. Konno, who used Löwenstein-Jensen medium, noticed a green discoloration of the fluid in tests performed on uninoculated media. Runyon did not experience this interference when he used an extract rather than the solid culture.

Production of niacin by mycobacteria other than M. tuberculosis is too minute to be detected by the methods described. Slight, or doubtful reactions have been reported with M. bovis, M. kansasii and Group II scotochromogens (Tarshis, 1960; Marks and Trollope, 1960a), but no positive reactions have been encountered with these organisms. Koch and Kroll (1960) and Virtanen (1963) reported positive and doubtful niacin results with Group III nonchromogens. Koch, Daigneault and Gehrke (1961) later reported that with Runyon's method of niacin detection they obtained results that verified that only human tubercle bacilli produce niacin in detectable quantities. This was at variance with the results they obtained with Konno's method.

Virtanen tested 57 strains of "Battey" type nonchromogens with a number of tests including the niacin test. Aqueous extracts of 33 of the nonchromogens gave positive niacin reactions. With the exception of this report, the niacin test is regarded as a reliable means of identifying M. tuberculosis.

II. MATERIALS AND METHODS

Cultures Examined

The cultures examined for niacin production included:

Tubercle bacilli	-	41 human strains
		2 bovine strains
		1 avian strain
Unclassified mycobacteria	-	4 Group I photochromogens (2)*
		11 Group II scotochromogens (7)*
		11 Group III nonchromogens (7)*
		9 Group IV rapid growers (3)*
Saprophytes	-	1 *M. phlei*

Reagents

1. Aniline - cyanogen bromide test:
   4% v/v aniline in 95% alcohol was prepared from aniline (AR) and 10% w/v aqueous cyanogen bromide was prepared in a fume chamber and was stored in a brown glass bottle with a tightly fitting screw-cap.
2. Benzidine - cyanogen bromide test:
   3% w/v benzidine in absolute alcohol was prepared from benzidine (AR). The cyanogen bromide solution was the same used in (1).

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* Strains not identified outside this laboratory.

3. Peknine reagents:

1% w/v aqueous potassium cyanide and 5% w/v aqueous chloramine T were prepared.

All reagents were prepared every 3 to 4 weeks in small quantities (25 ml.) and were stored at 4°C. when not in use.

Test

Niacin was extracted by adding 1 ml. sterile normal saline (0.85%) to each culture tube. The cultures were slanted so that the fluid was layered over the mycobacterial growth. After 10 to 20 minutes in this position, the fluid was removed from the cultures by sterile Pasteur pipettes. The saline extract was placed in 10 x 100 mm. serological test tubes which were then stoppered with cotton wool. Equal quantities (2 drops) of the extract and the other two reagents (aniline or benzidine and cyanogen bromide or chloramine T and potassium cyanide) were placed in the depressions in white glazed porcelain spot plates by means of Pasteur pipettes. Plastic disposable white spot plates replaced the porcelain spot plates for many of the experiments. The contents of the spot plates were mixed thoroughly by means of sliding the plate back and forth in a rotary motion. Any color change in the fluids was noted. The procedure was performed under a fume hood at all times.

Method

Using a small loopful of solid bacterial mass, 34 strains of mycobacteria were subcultured to six tubes each of Löwenstein-Jensen

medium. The tubes were incubated at 37°C. and after 2 weeks incubation one tube was tested for niacin production, then returned for further incubation. Seven days later the same tube plus one more were removed, tested for niacin, and returned for further incubation. This continued until 49 days of incubation had elapsed at which time all six tubes were tested for niacin production and discarded. The saline extracts obtained after 2, 3, 4, 5, 6 and 7 weeks were tested by three different methods:

1. aniline - cyanogen bromide
2. benzidine - cyanogen bromide
3. pelnice reaction

Color changes were noted following the addition of the reagents to the saline extracts, and were graded as positive (+), doubtful (D) or negative (-) as follows:

1. Aniline - cyanogen bromide method:

distinct yellow color	+
slight yellow color	D
no color change or green-blue color	-

2. Benzidine - cyanogen bromide method:

distinct pink precipitate	+
slight pink precipitate	D
white precipitate	-

3. Pelalice reaction:

distinct yellow color	+
slight yellow color	D
no color change	-

Niacin tests were performed on 46 strains of mycobacteria that had been studied for cultural characteristics (Chapter III). When one of the three slants exhibited moderate growth, the culture was tested for niacin production, and once a week thereafter, until heavy growth occurred on the slant for two successive weeks. The aniline - cyanogen bromide and benzidine - cyanogen bromide methods were used to test the saline extracts prepared from these slants. Each strain was tested until a positive result was obtained by one of the methods, or a negative result was obtained two weeks in succession from a slant exhibiting heavy growth of the organisms.

The reagents were checked for interfering color reactions by performing the test described above but omitting the saline extract. Similarily, the medium (Löwenstein-Jensen) was tested by replacing the test fluid with the saline extract from an uninoculated tube of medium. Following the completion of the test 4% sodium hydroxide was added to each depression in the spot plate to detoxify the cyanogen bromide. The plates were then immersed in a Sudol solution prior to sterilization.

An attempt was made to grade the amount of bacterial growth from which the saline extract was prepared. The grading was based on the amount of visible growth as follows:

± occasional colonies (1 - 10)

1+ growth covering 1/4 of the slant

2+ growth covering 1/2 of the slant

3+ growth covering all of the slant

4+ heavy growth

VII. RESULTS

Color production did not occur with the reagents alone. Aniline plus cyanogen bromide yielded a colorless solution, as did chloramine T plus potassium cyanide, while benzidine plus cyanogen bromide yielded a white precipitate. Saline extracts from uninoculated medium developed a blue-green flocculent precipitate upon the addition of aniline and cyanogen bromide. This precipitation was reduced considerably by discarding any fluid present in the culture tube prior to the addition of saline, but the blue-green color developed with many of the test culture extracts resulting in a green solution in the presence of niacin instead of the distinct yellow color required for a positive result (Plate VII). This interference was not noted with the other two methods used for detecting niacin.

White plastic disposable spot plates were found to be superior to the porcelain spot plates in two respects:

1. The white background of the plastic plate provided a better contrast for the positive tests as compared to the off-white color of the porcelain plates. The yellow solutions

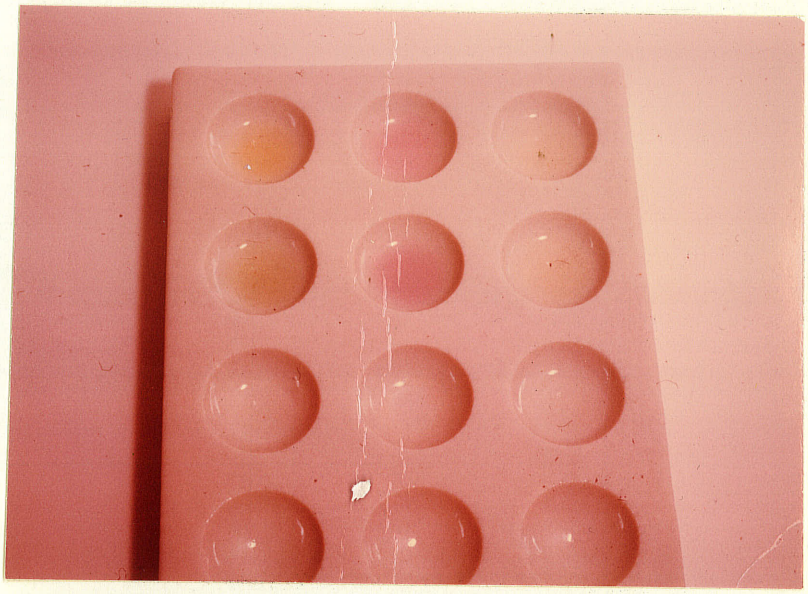

Plate VII. Positive niacin reactions.
Left: Aniline-cyanogen bromide method.
Center: Benzidine-cyanogen bromide method.
Right: Pelnics reaction.

were especially difficult to interpret in the porcelain spot plates.

2. The plastic spot plates were easy to handle and were discarded following sterilization.

Of the 34 strains of mycobacteria tested for niacin production over a 2 to 7 week period, only cultures of human tubercle bacilli yielded a positive niacin test by any method. The unclassified mycobacteria, bovine and avian strains of tubercle bacilli gave consistently negative results (Table VIII). Table IX shows the percentage of positive tests obtained by each of the methods with cultures of the 16 strains of M. tuberculosis and Table X shows the percentage of positive tests obtained with cultures of 15 strains of M. tuberculosis graded according to the amount of visible bacterial growth.

Of the 46 acid-fast cultures tested for niacin production following a study of cultural characteristics, 25 gave a positive niacin reaction with either or both of the aniline and benzidine methods (Table XI). All 25 of these niacin positive cultures were proven to be M. tuberculosis by the guinea pig virulence test or cultural studies. The 21 niacin negative cultures were those that resembled the unclassified mycobacteria or saprophytic mycobacteria on culture (Chapter III). Table XII shows the number of the 25 cultures of M. tuberculosis which gave a positive niacin reaction at various time intervals. Table XIII compares the aniline and benzidine methods on cultures of varying degrees of growth.

TABLE VIII

NIACIN TESTS ON 34 STRAINS OF MYCOBACTERIA

Organism	No. of Strains Tested	No. of Strains Niacin Positive after Incubation for Weeks at 37°C. 2	3	4	5	6	7 weeks
M. tuberculosis	16	9	12	14	15	16	16
M. bovis	2	0	0	0	0	0	0
M. avium	1	0	0	0	0	0	0
M. kansasii	1	0	0	0	0	0	0
Group II scotochromogens	4	0	0	0	0	0	0
Group III nonchromogens	3	0	0	0	0	0	0
Group IV rapid growers	7	0	0	0	0	0	0

TABLE IX

EFFECT OF INCUBATION TIME ON NIACIN REACTION

Incubation Time in Weeks	No. of Tubes Tested	Percent Positive By		
		Aniline	Benzidine	Peknice
2	16	38 %	56 %	0 %
3	32	44	50	13
4	46	44	52	35
5	60	48	48	18
6	76	40	55	22
7	91	51	62	15

TABLE X

EFFECT OF AMOUNT OF BACTERIAL GROWTH ON NIACIN REACTION

Amount of Bacterial Growth	No. of Tubes Tested	Percent Positive By Aniline	Benzidine	Pelnlue
1+ (1/4 of slant)	4	0 %	0 %	0 %
2+ (1/2 of slant)	76	0	1	0
3+ (all of slant)	88	17	34	3
4+ (heavy growth)	133	90	99	42

TABLE XI

NIACIN TESTS USING ANILINE AND BENZIDINE

Organism	No. of Strains	Positive Test By Aniline	Positive Test By Benzidine
M. tuberculosis	25	12	25
Group I photochromogens	3	0	0
Group II scotochromogens	7	0	0
Group III nonchromogens	7	0	0
Group IV rapid growers	3	0	0
M. phlei	1	0	0

TABLE XII

NIACIN TESTS ON 25 STRAINS OF *M. TUBERCULOSIS* AFTER VARIOUS INCUBATION PERIODS

Incubation Time in Weeks	No. of Tubes Tested	Niacin Positive By Aniline Method	Benzidine Method	One Method Only	Neither Method
1	7	0	0	0	7
2	17	4	8	4	9
3	8	4	6	2	2
4	7	2	6	4	1
5	2	0	2	2	0
6	3	0	1	1	2
8	1	0	0	0	1
9	1	1	1	0	0
10	2	1	1	0	1
Total	48	12	25	13	23

TABLE XIII

NIACIN TESTS ON 25 STRAINS OF M. TUBERCULOSIS WITH VARYING AMOUNTS OF BACTERIAL GROWTH

Amount of Growth	No. of Tubes Tested	Positive By Aniline Method	Positive By Benzidine Method	Positive By One Method Only	Neither Method
1+ (1/4 of slant)	1	0	0	0	1
2+ (1/2 of slant)	9	0	0	0	9
3+ (all of slant)	27	7	17	10	10
4+ (heavy growth)	11	5	8	3	3
Total	48	12	25	13	23

IV. DISCUSSION

The purpose of this study was firstly to investigate the claims of other workers that the niacin test is specific for M. tuberculosis, and secondly, to determine the simplest and most dependable method of detecting niacin, which can be used in the routine of a hospital laboratory.

The results shown in Tables VIII to XI indicate that of the 80 strains of mycobacteria studied, only the human tubercle bacilli strains exhibited a positive niacin reaction. Other mycobacteria gave consistently negative results even after prolonged incubation of slants with heavy bacterial growth. In contrast to the results obtained by Virtanen in his study, all the Group III nonchromogens were niacin negative.

All 41 strains of M. tuberculosis eventually produced sufficient niacin to give a positive reaction by at least one of the methods under study, but negative and doubtful (only slight color production) reactions were encountered with some of the slants. Age of the cultures had little effect on the percentage of positive reactions after an initial incubation period of two weeks. Some slants gave a clear positive reaction after 14 days incubation while one slant was negative after 10 weeks incubation. The determining factor was the amount of bacterial growth on the slant at the time the saline extract was prepared. Thus, a strain of tubercle bacilli which yielded heavy growth (4+) on subculture after 2 weeks incubation was

positive, while a poorly growing organism which produced only moderate growth (3+) after lengthy incubation (10 weeks) was niacin negative. The sharp increase in the percentage of positive reactions for slants with heavy growth (4+) as compared to those with only moderate growth (3+) indicates the importance of the amount of bacterial culture tested.

The Bacteriology Department of the Winnipeg General Hospital, at the present time, identifies human tubercle bacilli by proving pathogenicity for guinea pigs. A portion of the original specimen, treated with sodium hydroxide, is inoculated on to two Löwenstein-Jensen slants, and 0.5 to 0.75 ml. is injected intraperitoneally into a guinea pig. A second guinea pig is injected with culture washings if acid-fast bacilli are isolated on culture. Six weeks after injection the animals are killed and examined for evidence of tuberculosis, which is confirmed by histopathological examination by the Department of Pathology of the Winnipeg General Hospital. In some cases the first guinea pig does not show conclusive evidence of disease, and a final report must be withheld until the second animal is examined. Death of the guinea pigs from extraneous causes, e.g., pneumonia and peritonitis is another factor in delaying identification of the tubercle bacilli.

The niacin test offers an alternate method of identification of M. tuberculosis. Time is of importance when dealing with communicable diseases such as tuberculosis; for this reason, the average length of time required for identification of human tubercle

bacilli by the guinea pig and by the niacin test were calculated. From the records of the Bacteriology Department of the Winnipeg General Hospital were obtained the date of isolation of acid-fast bacilli on culture and the date of identification by guinea pig for 19 strains of M. tuberculosis. The average length of time between isolation on culture and identification by the guinea pig was found to be 25.6 days as compared to an average of 23.7 days for the niacin test. A difference of 1.9 days indicates that the niacin test is equally as rapid as the present method of identification. For those laboratories that inject guinea pigs only when acid-fast bacilli have been isolated on culture, the difference in time is significant (6 weeks as compared to 23.7 days).

Other factors favouring the use of the niacin test are:

1. The low cost as compared to the expense of maintaining live animals.

2. The niacin test is positive for virulent and attenuated strains of M. tuberculosis, as well as drug-resistant strains. One of the cultures which was niacin positive was avirulent for guinea pigs, but was identified by the Hamilton Health Association as an "attenuated" M. tuberculosis.

Of the three methods of niacin detection used in this study, the Pelnice reaction was the least sensitive. The yellow color was faint and often difficult to interpret. Only 42% of the slants with heavy growth of M. tuberculosis produced sufficient niacin to be detected with chloramine T and potassium cyanide. Although the

procedure does not require a fume chamber, the method cannot be recommended for use because of the high percentage of false negative results.

The benzidine reagent produced a pink color that was easily detected as compared to the yellow color of the aniline - cyanogen bromide which at times was faint and difficult to discern from the white porcelain plate. The interfering green color from the malachite green in the medium occurred only with the aniline reagent (Plate VII). The sensitivity of the benzidine method thus appears to be greater than the aniline method.

V. CONCLUSIONS

Eighty strains of mycobacteria were tested for niacin production by the aniline - cyanogen bromide and the benzidine - cyanogen bromide methods. The Pakmice reaction was used on 34 strains of mycobacteria. The niacin test was specific for M. tuberculosis. It is as reliable or more so than the present day method of identification of human tubercle bacilli by proving virulence for guinea pigs. Acid-fast bacilli isolated in a laboratory may be identified as human tubercle bacilli by the niacin test within three weeks (on the average) after subculture. It is essential that the saline extract be prepared from a slant exhibiting heavy growth of the organism, in order to avoid false negative results. Alcoholic benzidine and cyanogen bromide proved to be the most reliable reagents, while the use of plastic disposable spot plates was more convenient than the conventional porcelain spot plates.

CHAPTER VI

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CHAPTER VI

TWEEN 80 TEST

I. REVIEW OF THE LITERATURE

A polyoxyethylene derivative of sorbitan monoleate (Tween 80) has been used in media for mycobacteria since 1946 when Dubos and Davis added Tween 80 to their liquid medium and obtained dispersed growth of tubercle bacilli due to depression of surface tension. The degradation of Tween 80 to form oleic acid by the lipase enzymes of mycobacteria was recognized by the same workers in 1948. They found that serum albumin was essential for the initiation of growth by small inocula of tubercle bacilli in liquid medium containining Tween 80. The serum albumin, acting as a protective factor rather than a nutritive factor, served to bind the bacteriostatic unesterified fatty acids which inhibited the growth of small inocula. They also noted that filtrates of M. phlei contained a lipase which released free fatty acid from Tween 80.

Andrejew, Cernez-Rieux and Tacquet (1960) studied the lipase activities of human, bovine and avian tubercle bacilli as well as unclassified mycobacteria with Tweens 20, 40, 60 and 80 by measuring the quantity of carbon dioxide released from the substrates. There was no significant difference between the values for different tweens, but the activity of the different organisms varied greatly. The tubercle bacilli showed low activity as did some of the Group III

nonchromogens, while M. kansasii and M. phlei gave much higher values than the other mycobacteria. Group II scotochromogens and Group III nonchromogens appeared as heterogeneous groups with some exhibiting high activity and others very low.

The Tween 80 test devised by Wayne (1962) is a semi-quantitative assay of the rate of hydrolysis of Tween 80, and is based on the difference in lipase activity of the photochromogen M. kansasii from the other mycobacteria. Wayne added a loopful of bacteria from an actively growing solid culture to a substrate of M/15 phosphate buffer, pH 7.0, 0.5% Tween 80, and neutral red (20 mgm./ml.) and incubated the tubes at 37°C. The neutral red dye, in the neutral phosphate buffer, produced a red solution with an absorption peak at 462μ and a shoulder at 523μ, but the addition of 0.5% Tween 80 reduced the absorption at 523μ resulting in an amber colored solution. Hydrolysis of the Tween 80 to produce oleic acid restored the red color to the substrate.

Wayne found that after 48 hours incubation at 37°C. M. kansasii had hydrolysed Tween 80 to produce a salmon pink to red color in the supernatant solution which he called a positive Tween 80 reaction. The results of his tests with various mycobacteria are shown in Table XIV. As indicated by Andrejew's work some of the organisms in Group II scotochromogens and Group III nonchromogens also hydrolysed Tween 80 to give a pink color in 48 hours. The Group III nonchromogens that produced a positive reaction were all members of a group of organisms isolated from the washings

TABLE XIV

WAYNE'S RESULTS OF TWEEN 80 TEST AFTER 48 HOURS

Organism	Number Tested	Percent Positive
M. tuberculosis	14	0 %
M. bovis	4	0
M. avium	11	0
Group I M. kansasii	45	98
Group II scotochromogens	47	21
Group III "Battey" organisms	29	0
Swine organisms	12	0
Radish organisms	10	100

of radishes (Richmond and Cummings, 1950). None of the Group III nonchromogens isolated from humans produced a positive Tween 80 reaction.

On further studies, Wayne, Doubek and Runnell (1964) noted a color change was produced within 48 hours with as little as 0.15 mg. (moist weight) of M. kansasii cells per ml., but inocula as large as 2.5 mg. of "battey" organisms failed to produce a color change. Continued incubation of the substrate with various mycobacteria resulted in color changes occurring after incubation at 37° C. for periods of 3 to 20 days. Turbidity in the substrate was noted after 10 days with M. kansasii due to an excess of oleic acid which was no longer soluble in the Tween 80 solution. Wayne concluded that the Tween 80 test, in conjunction with other tests, is useful in the classification of mycobacteria.

II. MATERIALS AND METHODS

Cultures Examined

The cultures tested for hydrolysis of Tween 80 included the followings:

Tubercle bacilli	-	20 human strains
		2 bovine strains
		1 avian strain

Unclassified mycobacteria	-	5 Group I photochromogens (3)*
		11 Group II scotochromogens (7)*
		11 Group III nonchromogens (7)*
		10 Group IV rapid growers (4)*
Saprophytes	-	1 M. phlei

Substrate

The substrate solution was prepared as follows:

M/15 phosphate buffer, pH 7.0	-	100 ml.
Tween 80	-	0.5 ml.
Neutral red stock solution (1 mgm./ml. in water)	-	2.0 ml.

The mixture was heated gently to dissolve the Tween 80, and was dispensed in 4 ml. amounts to screw-capped one-quarter ounce bottles. Sterilization was accomplished by autoclaving at 15 lbs. pressure for 15 minutes. The substrate was stored at 4° C. prior to use.

Method

Sixty-one strains of mycobacteria were tested for the ability to hydrolyze Tween 80. The test consisted of inoculating a tube of substrate with a 3 mm. loopful of bacteria from a solid culture, and incubating at 37° C. The color of the substrate was compared with the color of an uninoculated tube after 4, 16, 24 and 48 hours, and 1 week of incubation. With each series of organisms tested, one tube

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* Strains not identified outside this laboratory.

of substrate was inoculated with the stock culture of M. kansasii.

The production of a salmon-pink to red color in the supernatant solution was considered to be a positive reaction (Plate VIII). Each strain of mycobacteria was tested twice, and any organism producing a positive reaction was tested four more times.

III. RESULTS

The stock culture of M. kansasii produced a positive Tween 80 test in 48 hours every time it was tested (14/14). M. kansasii was used as a standard to insure that each batch of substrate was adequate, and to compare the reactions of the other organisms. The uninoculated tubes of substrate maintained the amber color consistently.

Hydrolysis of Tween 80 to produce a red color in the substrate solution occurred within 48 hours with all five of the photochromogenic strains. The reaction also occurred with a few of the other mycobacteria (Table XV). The strains of mycobacteria which hydrolysed Tween 80 in 48 hours were tested repeatedly, and these results are shown in Table XVI.

IV. DISCUSSION

The hydrolysis of Tween 80 to form oleic acid is not characteristic of only one group or species of mycobacteria. Group I photochromogens yielded more positive Tween 80 reactions after 48 hours incubation than any other group or species, but positive reactions were also obtained from a strain of M. tuberculosis,

Plate VIII. Tween 80 reaction.
Left: uninoculated substrate.
Right: positive reaction.

TABLE XV

EFFECT OF INCUBATION TIME ON TWEEN 80 REACTIONS

Organism	No. of Strains Tested	Number Positive After Hours 4	16	24	48	1 week
M. tuberculosis	20	0	0	1	1	3
M. bovis	2	0	0	0	0	0
M. avium	1	0	0	0	0	0
Group I photochromogens	5	0	2	3	5	5
Group II scotochromogens	11	0	1	1	3	7
Group III nonchromogen "a"	7	0	0	0	0	1
nonchromogen "b"	4	1	2	2	2	2
Group IV rapid growers	10	0	0	0	1	2
Saprophytes M. phlei	1	0	0	0	0	1

TABLE XVI

RESULTS OF REPEATED TESTING FOR HYDROLYSIS OF TWEEN 80

Organisms and No. of Strain	No. of Times Tested	Number Positive After Hours 4	16	24	48	1 week
M. tuberculosis #28372	6	0	0	1	3	5
Group I photochromogens						
M. kansasii	14	1	10	12	14	14
CTC #2	6	1	3	5	6	6
CTC #2	6	0	1	1	4	6
#23425	6	0	1	1	5	5
CTC #3	6	0	0	3	6	6
Group II scotochromogens						
CTC #4	6	0	0	0	1	3
PL35699	6	0	0	0	2	5
PL38206	6	0	2	2	3	6
Group III nonchromogen "b"						
DL1181	6	1	3	4	4	6
#26338	6	0	3	3	6	6
Group IV M. fortuitum PL32810	6	0	0	0	2	5

Group II scotochromogens, Group III nonchromogens and M. fortuitum.

Incubation for 48 hours proved to be the optimum time for separating Group I M. kansasii from the other mycobacteria. Some strains of M. kansasii showed no color change prior to the 48 hour reading, while more positive reactions from the other groups were observed after one week.

Wayne stipulated the use of actively growing cultures for the test, but the age of the culture appeared to have little significance in this study. The stock cultures of M. kansasii were stored at 4° C. for up to four months prior to use in this test. No difference was observed in the rate of hydrolysis between these old cultures and new subcultures. Similarily, the strain of M. tuberculosis which yielded a positive reaction in one of the two tubes used for routine testing was stored at 4° C. for six months and then tested for hydrolysis of Tween 80 four more times. Again 50% of the tubes showed a positive reaction after 48 hours while the others were negative.

The Group III nonchromogens that hydrolysed Tween 80 within 48 hours belonged to the "b" subdivision of the group, i.e., they exhibited slight pigment production in time. None of the Group III nonchromogens that resembled "Battey" organisms produced a positive reaction until one week.

V. CONCLUSIONS

Sixty-one strains of mycobacteria were tested for hydrolysis

of Tween 80. The Group I photochromogenic strains and a few strains belonging to Group II scotochromogens, Group III nonchromogens, and one strain of M. fortuitum and one of M. tuberculosis produced a positive Tween 80 reaction within 48 hours. The Tween 80 test is rapid, easy to perform and easy to read, but it lacks specificity and thus is of limited usefulness. As suggested by Wayne (1962) it may be used to rule out the Group III nonchromogen "Battey" type. It may also be used to confirm the identification of a photochromogen as M. kansasii.

CHAPTER VII

ARYLSULFATASE TEST

I. REVIEW OF THE LITERATURE

The property of hydrolyzing the salt of an arylsulfuric acid to yield a phenolic compound has been found to be useful in the identification of mycobacteria. The enzyme responsible for hydrolysis of the substrate is known as "arylsulfatase", or may be named according to the substrate, i.e., "phenolphthalein sulfatase" if tripotassium phenolphthalein disulfate is hydrolysed to release free phenolphthalein. The presence of free phenolphthalein may be detected by adding an alkaline sodium solution to form the red colored disodium salt of phenolphthalein.

Whitehead, Wildy and Engback (1953) detected arylsulfatase activity in mycobacteria by cultivating the organisms in a Tween-albumin liquid medium to which had been added potassium phenolphthalein disulfate to a 0.001 M concentration. Following incubation for 14 days 2N NaOH was added drop by drop to detect phenolphthalein. Whitehead tested 154 strains of mycobacteria, and found the majority of the saprophytic strains showed arylsulfatase activity, as did the avian and murine strains, while the human and bovine tubercle bacilli exhibited very little activity.

Using Whitehead's technique, Nelkon and Hitchison (1959) and Marks and Trollope (1960a) noted that the tubercle bacilli

exhibited little or no arylsulfatase activity while the majority of the unclassified mycobacteria showed moderate to considerable activity. Marks and Trollope replaced sodium hydroxide with a 2N sodium carbonate solution to eliminate fading of the red color due to the formation of the colorless trisodium salt of phenolphthalein by strong alkalis.

Kubica and Vestal (1961a) conducted an extensive study on Whitehead's technique for arylsulfatase activity of acid-fast bacilli. They used substrate concentrations ranging from 0.00025 M to 0.003 M, and incubation periods of from one day to three weeks. From their results they concluded the following:

1. Mammalian and avian tubercle bacilli may be separated from other mycobacteria by using a substrate concentration of 0.001 M and a two week incubation period.
2. Avian tubercle bacilli may be distinguished from Group III nonchromogens after two weeks incubation with 0.003 M substrate.
3. M. fortuitum may be distinguished from rapid growing saprophytes and other Group IV rapid growers after two to three days incubation with 0.001 M substrate.

Verification of Kubica's conclusions has been provided by the work of Tarshis (1963b). A point worth noting in Tarshis's report is the activity of M. balnei, which is a slow growing organism isolated from skin ulcerations. M. balnei has an optimum temperature of 32°C. and fails to grow at 37°C. It exhibited slight

arylsulfatase activity after three days incubation with the 0.001 - 0.002 M concentrations, but none with 0.0005 M substrate.

Similarly, Wayne (1961) reported that M. fortuitum may be recognised by its enzymic activity after three days incubation. His method involved inoculation of a small amount of solid test culture on to the surface of a solid medium composed of Dubos oleic agar base with additives of 1% glycerol and 0.001 M phenolphthalein sulfate. After testing 106 cultures of rapid growing mycobacteria, Wayne found that only M. fortuitum produced a positive arylsulfatase reaction and he concluded that the test was sufficiently specific, simple to perform and easy to interpret to use as a routine technique for recognizing M. fortuitum.

II. MATERIALS AND METHODS

Cultures Examined

The cultures tested for arylsulfatase activity included the following:

Tubercle bacilli	-	6 human strains
		2 bovine strains
		1 avian strain
Unclassified mycobacteria	-	5 Group I photochromogens (3)*
		11 Group II scotochromogens (7)*
		10 Group III nonchromogens (7)*
		13 Group IV rapid growers (7)* (including 6 M. fortuitum)

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* Strains not identified outside this laboratory.

Saprophytes - 1 M. phlei.

Substrate Medium and Reagents

The substrate medium was prepared as follows:

Dubos oleic acid agar base (Difco) - 4.0 gm.

Distilled water - 180 ml.

Boil, and to 155 ml. of base add:

Glycerol - 1.5 ml.

Phenolphthalein disulfate (tri-potassium) - 100 mgm. (0.001 M) or 50 mgm. (0.0005 M).

The medium was dispensed in 3.0 ml. amounts to one-quarter ounce screw-capped bottles, and sterilized at 15 lbs. pressure for 15 minutes. The medium was allowed to cool in a slanted position (0.0005 M), or an upright position (0.001 M).

A solution of 1 N sodium carbonate was prepared by dissolving 10.6 gm. of Na_2CO_3 in 100 ml. of water.

Method

Preliminary experiments using 0.001 M concentration of substrate, as recommended by Wayne (1961), were conducted with fifteen strains of mycobacteria. A small portion of a colony from Löwenstein-Jensen medium was used to inoculate the top of two bottles of medium which were incubated at 37° C. for 72 hours. Free phenolphthalein was detected by adding 0.5 ml. of 1 N Na_2CO_3 solution to the test medium. After 30 minutes the color of the

medium, colonies, and fluid was noted and graded as follows:

Negative	(-)	- no color production
Doubtful	(±)	- faint tinge of pink
Slight	(1+)	- pale pink
Moderate	(2+)	- pink
Moderately strong	(3+)	- light red
Strong	(4+)	- red
Very strong	(5+)	- deep red

Forty-nine strains of mycobacteria were tested in duplicate for arylsulfatase activity after 24, 48, 72 hours, 1 and 2 weeks incubation. Ten bottles of medium containing 0.0005 M concentration of substrate were inoculated with solid culture. Following incubation at 37° C. the alkaline solution was added and the bottles were placed in a slanted position to enable the alkali to cover the surface of the slope. The color produced following the addition of the alkali was compared to an uninoculated tube of medium to which Na_2CO_3 had also been added (Plate IX). With each series of organisms tested, a stock strain of M. fortuitum was inoculated to five tubes of medium.

XII. RESULTS

In the preliminary experiment with 0.001 M concentration of phenolphthalein disulfate, no color production occurred with the tubercle bacilli, faint pink color at the interface of the solid medium and the alkali solution with the Group I photochromogens,

Plate IX. Arylsulfatase reaction.
Left: positive reaction (5+).
Right: uninoculated substrate medium.

CHAPTER VII

ARYLSULPATASE TEST

Group II scotochromogens and Group III nonchromogen strains, and pink to red color production with the M. fortuitum strains (Table XVII).

The lower substrate concentration (0.0005 M) yielded better separation of M. fortuitum species from the other mycobacteria after 72 hours incubation (Table XVIII). Some strains of M. fortuitum showed no arylsulfatase activity with only 24 or 48 hours incubation, but were positive after 72 hours, while none of the other organisms tested showed any activity until after one week of incubation. Six clinical isolates that exhibited a growth pattern similar to the M. fortuitum strains also exhibited arylsulfatase activity after 72 hours. At no time did the uninoculated medium show evidence of free phenolphthalein.

IV. DISCUSSION

Mycobacterium fortuitum is a rapid growing acid-fast organism that is separated from M. phlei and M. smegmatis by comparing the ability to grow at 40°, 45° and 52° C., survival at 60°C. for 4 hours, and the utilization of various carbon sources (Gordon and Smith, 1955). Growth on MacConkey's agar was used by Jones and Kubica (1964) for identifying M. fortuitum. Both methods require several days for a final identification. Wayne's phenolphthalein sulfatase test required only three days, and the author claimed the test provided a sharp distinction between M. fortuitum and other mycobacterial species.

TABLE XVII

ARYLSULFATASE REACTION AFTER 72 HOURS WITH 0.001 M SUBSTRATE MEDIUM

Test Organism	Number of Strains	Color Reaction
M. tuberculosis H37Rv	1	-
M. bovis	1	-
M. avium	1	-
M. kansasii	1	±
Group II scotochromogens	4	- to ±
Group III nonchromogens	1	- to ±
M. fortuitum	6	2+ to 4+

TABLE XVIII

EFFECT OF INCUBATION TIME ON ARYLSULFATASE REACTION WITH 0.0005 M SUBSTRATE MEDIUM

Test Organism	Number of Strains	Color Reaction Incubation Time in Hours 24	48	72	Weeks 1	2
M. tuberculosis	6	-	-	-	-	-
M. bovis	2	-	-	-	-	-
M. avium	1	-	-	-	-	±
Group I photochromogens	5	-	-	-	- to 1+	± to 2+
Group II scotochromogens	11	-	-	-	- to ±	- to 2+
Group III nonchromogens	10	-	-	-	- to ±	- to 1+
Group IV rapid growers:						
M. fortuitum	6	- to 3+	- to 5+	1+ to 5+	3+ to 5+	5+
rapid grower "a"	6	- to 3+	- to 4+	1+ to 5+	3+ to 5+	3+ to 5+
rapid grower "b"	1	-	-	-	-	-
M. phlei	1	-	-	-	-	-

Wayne tested 106 cultures of rapid growing mycobacteria, but he did not report testing slow growing mycobacteria. The method outlined by Wayne was used in the preliminary experiment in this study, and it was found that weak arylsulfatase activity could be demonstrated with slow growing organisms belonging to Runyon's Groups I, II and III. This weak activity exhibited at the 0.001 M substrate concentration was interpreted as false positive reactions assuming the test is specific for M. fortuitum.

Tarshis also noted weak arylsulfatase activity after three days at the 0.001 M concentration with Group III nonchromogens, "Battey" type, but no activity at the 0.0005 M concentration. A reduction of the substrate concentration to 0.0005 M in this study resulted in clear separation of M. fortuitum strains from the other mycobacteria. The length of incubation proved to be crucial, with 72 hours being the optimum time for identification of M. fortuitum.

The procedure followed in this study is simple and requires little time. Tarshis used seven-day old liquid cultures in one-drop aliquots from a graduated serologic pipette for the inoculum. A small portion of a colony from solid medium is more practical for use in a routine laboratory and the size of the inoculum did not alter the results of the test. Wayne permitted the medium to harden in an upright position and then inoculated the top of the agar. It was found that slants of the medium were easier to inoculate and they offered more surface area for the growth of the organisms and activity of the enzyme.

V. CONCLUSIONS

Fifteen strains of mycobacteria were tested for arylsulfatase activity by Wayne's method. Forty-nine strains were tested with a modified medium containing only 0.0005 M substrate as compared to 0.001 M. The test conducted after 72 hours incubation with the 0.0005 M substrate concentration clearly separated M. fortuitum from the other mycobacteria. The test is simple to perform, requires little time and is easily interpreted. These attributes make it a desirable test for use in a hospital laboratory.

CHAPTER VIII

EFFECT OF OLEIC ACID ON GROWTH

CHAPTER VIII

EFFECT OF OLEIC ACID ON GROWTH

I. REVIEW OF THE LITERATURE

Oleic acid exerts an inhibitory effect on the growth of mammalian tubercle bacilli (Dubos and Middlebrook, 1947) but stimulates the growth of Group III nonchromogens and *M. avium* (Hawkins and Steenken, 1963). Oleic acid was added to media for tubercle bacilli by Dubos and Middlebrook (1947) who recognized that the soap of oleic acid had a marked bacteriostatic and bactericidal action, but serum albumin protected the bacilli enabling the fatty acid to serve as a nutrient. The oleic acid-albumin complex used was in the ratio of one part of oleic acid to 50 to 100 parts of albumin by weight. Various strains of tubercle bacilli were grown in the medium, and the avian strains utilized the fatty acid to a greater extent than the mammalian strains. The authors stated that the avian strains were resistant to the oleic acid, whereas the human and bovine strains of tubercle bacilli were susceptible.

Hawkins and Steenken (1963) studied the lipase activity of mycobacteria and noted that while the oleic acid was an inhibitor to growth of human tubercle bacilli, Group III nonchromogens were resistant to 50 mcgm./ml. and their growth was actually stimulated by it. Similar results were obtained with *M. avium*.

While investigating tests employing Tween 80 as the substrate, Wayne, Doubek and Russell (1964a) studied the tolerance of oleic acid of various species and subgroups of mycobacteria. Sodium oleate (0.025%) was incorporated into Dubos oleic agar which was dispensed in 3.5 ml. amounts to form slants. The conventional Dubos oleic agar, with the oleic-albumin complex (0.005% oleic acid), was used for comparison. The media was inoculated with 0.1 ml. of a barely turbid aqueous suspension of the test organism, and incubated for six weeks at 37°C. Human, and bovine tubercle bacilli, M. kansasii, some of the Group II scotochromogens, and some of the rapid growing mycobacteria were inhibited by 0.025% oleic acid. Some Group II scotochromogens, most of the Group III nonchromogens, M. avium, and the rapid growers M. fortuitum, M. smegmatis, M. phlei, and M. rhodochrous were both tolerant to and stimulated by the additional oleic acid. The authors concluded that oleic acid exerts a differential inhibitory effect on mycobacteria.

II. MATERIALS AND METHODS

Cultures Examined

The cultures examined for tolerance to oleic acid included the following:

Tubercle bacilli	-	16 human strains
		2 bovine strains
		1 avian strain
Unclassified mycobacteria	-	5 Group I photochromogens (3)*

12 Group II scotochromogens (8)*

10 Group III nonchromogens (7)*

12 Group IV rapid growers (6)*

Saprophytes - 1 M. phlei

Test Medium

Three types of medium were prepared from Dubos oleic agar base (Difco) with different concentrations of oleic acid and albumin. The oleic acid solution was prepared by adding 0.6 ml. oleic acid (U.S.P.) to 10 ml. N/20 sodium hydroxide and mixing thoroughly to obtain a uniform emulsion. A 0.25% solution of oleic acid was obtained by adding 5 ml. of the emulsion to 95 ml. normal saline (0.85%). Oleic acid-albumin complex (0.05% oleic acid and 5% albumin) was obtained commercially.

(1) 0.005% oleic acid and 0.5% albumin:

Dubos oleic agar base	4 gm.
Distilled water	180 ml.
Dubos oleic-albumin complex	20 ml.

(2) 0.025% oleic acid:

Dubos oleic agar base	4 gm.
Distilled water	180 ml.
Oleic acid solution	20 ml.

(3) 0.03% oleic acid and 0.5% albumin:

Dubos oleic agar base	3.6 gm.

* Strains not identified outside this laboratory.

Distilled water	160 ml.
Dubos oleic-albumin complex	20 ml.
Oleic acid solution	20 ml.

The oleic acid solution was added to the medium prior to sterilization at 15 lbs. pressure for 15 minutes while the Dubos oleic-albumin complex was added aseptically after sterilization. The medium was dispensed in 4 ml. amounts to one-quarter ounce screw-capped bottles and allowed to harden in the slanted position.

Method

Fifty-nine strains of mycobacteria were tested in triplicate for growth on the three types of media. A suspension of the test organism was prepared by adding a small portion of a colony from Löwenstein-Jensen medium slants to 0.5 ml. water in a one-quarter ounce screw-cap bottle with two 5 mm. glass beads. The bottle was shaken manually for at least one minute. One loopful of the suspension was used as an inoculum for each tube of media. The tubes were incubated for 14 days at 37°C. after which time the slants were examined for evidence of growth. The bacterial growth was most obvious when the tube was held close to a light source so that the light passed through the translucent medium. The conventional Dubos oleic agar (0.005% oleic acid plus 0.5% albumin) was used as the standard, and the amount of growth in the other two tubes was compared to this. Less than twenty colonies on the agar slant was considered to be evidence of inhibition of growth.

III. RESULTS

All the organisms developed good growth on the conventional Dubos oleic agar medium, but the tolerance to higher concentrations of oleic acid varied greatly. Of the 59 organisms tested, only 26 gave consistent results. The others developed growth in one or two of the bottles with 0.03% oleic acid, but showed no growth in the third. The results are presented as the percentage of tubes showing growth (Table XIX).

IV. DISCUSSION

Oleic acid exerted an inhibitory effect on the growth of all the test organisms except for Group IV rapid growers. The addition of 0.5% albumin neutralised this inhibitory effect, and permitted growth of the majority of the unclassified mycobacteria on medium containing 0.03% oleic acid. Up to 50% of the tubercle bacilli also developed good growth on the medium containing both oleic acid and albumin. There was little difference observed between the Group III nonchromogens and the other mycobacteria on the medium containing oleic acid and albumin.

These results do not agree with the results obtained by Wayne et al. (1964a). They stated that tubercle bacilli and M. kansasii were inhibited by 0.02% oleic acid, but the composition of the medium they used was not clearly stated. Sodium oleate was incorporated into Dubos oleic agar to a 0.02% level, but no mention was made of the albumin concentration, nor is there any mention of

TABLE XIX

GROWTH IN THE PRESENCE OF OLEIC ACID

Organism	No. of Tubes	Percentage Showing Growth in Medium Containing 0.025% Oleic Acid	Percentage Showing Growth in Medium Containing 0.03% Oleic Acid Plus 0.5% Albumin
Tubercle bacilli:			
M. tuberculosis	48	2 %	52 %
M. bovis	6	0	33
M. avium	3	0	100
Unclassified mycobacteria:			
Group I photochromogens	15	0	80
Group II scotochromogens	36	14	86
Group III nonchromogens	30	10	97
Group IV rapid growers	36	94	100
Saprophytes:			
M. phlei	3	0	67

the number of times the organisms were tested for tolerance to oleic acid.

In the present study, the results were not reproducible as shown by the fact that less than half (26/59) of the test organisms yielded consistent results when tested three times.

V. CONCLUSIONS

Fifty-nine strains of mycobacteria were tested for tolerance to oleic acid. Oleic acid at a 0.025% concentration in the absence of albumin inhibited the growth of most of the tubercle bacilli, and unclassified mycobacteria except for the rapid growing Group IV organisms. Growth of most mycobacteria occurred on medium containing 0.03% oleic acid with 0.5% albumin. Tolerance to oleic acid was variable in this study, and the results were not reproducible. This test cannot be recommended for use in identifying mycobacteria.

CHAPTER IX

DRUG RESISTANCE

CHAPTER IX

DRUG RESISTANCE

I. REVIEW OF THE LITERATURE

The resistance of the unclassified mycobacteria to antimicrobial agents has been of interest to many investigators ever since these organisms were recognised. Runyon (1959) and Marks and Richards (1962) included resistance to antituberculosis drugs in their description of unclassified mycobacteria. Resistance to isoniazid and thiosemicarbazone was an important criterion in Marks and Richards' classification, and served to differentiate their third group, the isoniazid sensitive thermophiles, from the fourth group, the isoniazid resistant thermophiles.

Tarshis, Wood, Parker and Dunham (1955) investigated the *in vitro* response of chromogenic acid-fast bacilli to antimicrobials including isoniazid, streptomycin and para-aminosalicylic acid. They found that their chromogenic strains were resistant to 100 mcg./ml. of isoniazid, but some were sensitive to 10 mcg./ml. of streptomycin or P.A.S. Rogul *et al.* (1957) noted that the photochromogens were more susceptible to drugs than the scotochromogens and nonchromogens.

Wolinsky, Smith and Steenken (1958) conducted a study using 39 strains of mycobacteria and 22 antimicrobial agents. The unclassified mycobacteria were generally more resistant than *M. tuberculosis* to the antituberculosis drugs, while none of the

other antimicrobials nor antifungal agents were active. The authors also noted that the photochromogens were more susceptible than other members of the unclassified mycobacteria.

The use of thiosemicarabasone in identifying mycobacteria was reported by Marks and Trollope (1960b). They noted that the unclassified mycobacteria, with the exception of the photochromogens, were resistant to 10 mcg./ml. of thiosemicarbasone while tubercle bacilli were sensitive. They cautioned that a heavy inoculum of tubercle bacilli would produce colonies on the medium containing thiosemicarbasone and that the occasional strain of tubercle bacilli is resistant to this drug.

The methods of testing drug sensitivity have been reviewed by Canetti et al. (1963), who recognize three main sensitivity tests:

1. the absolute-concentration method
2. the resistance ratio method
3. the proportion method

The absolute-concentration method has been used by the United States Veterans Administration, and involves the inoculation of a carefully standardized inoculum to drug-containing medium. Resistance is determined by the number of colonies appearing on the medium with known drug concentrations.

The resistance ratio method is used by the Medical Research Council of Great Britain and endorsed by the Association of Clinical Pathologists. It involves a comparison of the minimal concentration of drug inhibiting growth of the test strain with the minimal

concentration inhibiting growth of the standard sensitive strain M. tuberculosis, H37Rv. An organism is considered to be sensitive if the resistance ratio is 2 or less, and resistant if the ratio is 8 or more. The exact concentration of the drug is not as important in this method because the standard strain, H37Rv, is always tested against the same drug concentration as the test organisms.

The proportion method involves diluting the inoculum to determine the total number of viable bacteria and the number of resistant bacteria in the inoculum. If 1% or more of the viable bacteria in the inoculum are capable of growing on the medium containing known quantities of the drug, the strain is considered to be resistant.

II. MATERIALS AND METHODS

Cultures Examined

The cultures tested for drug sensitivity included the following:

Tubercle bacilli	-	8 human strains
		2 bovine strains
		1 avian strain
Unclassified mycobacteria	-	3 Group I photochromogens (2)*
		3 Group II scotochromogens (2)*
		3 Group III nonchromogens
		3 Group IV rapid growers(1)*

* Strains not identified outside this laboratory.

Saprophytes - 1 M. phlei

Test Medium

Löwenstein-Jensen medium was prepared as described in Chapter II. Drug dilutions of streptomycin (sulfate), isoniazid, and para-amino-salicylic acid (P.A.S.) were prepared asoptically in sterile distilled water and were added to the Löwenstein-Jensen medium in the ratio of one part drug dilution to nine parts medium. The final drug concentrations were:

Streptomycin: 0.4, 0.8, 1.6, 3.2, 6.4 and 10 mcg./ml. of medium.

Isoniazid: 0.012, 0.025, 0.05, 0.1, 0.2 and 1.0 mcg./ml. of medium.

P.A.S.: 0.1, 0.2, 0.4, 0.8, 1.6 and 3.2 mcg./ml. of medium.

Control: no drugs added.

The medium was dispensed in 2 ml. amounts to one-quarter ounce screw-capped bottles and inspissated at 75° - 80°C. for one hour with the bottles in a slanted position for two consecutive days. Before use the medium was stored at 4°C.

Commercially prepared drug sensitivity medium was obtained from the Oxoid Division of Oxo Limited, London, England. The medium was transported by jet and kept under refrigeration to prevent deterioration of the drugs. The drug concentrations were:

Streptomycin: 0, 1, 2, 4, 8, 16 and 32 mcg./ml. of medium.

Isoniazid: 0, 0.03, 0.06, 0.12, 0.25, 0.5 and 1.0 mcg./ml. of medium.

P.A.S.: 0, 0.25, 0.5, 1, 2, 4 and 8 mcg./ml. of medium.

Method

Twenty-four strains of mycobacteria, including M. tuberculosis H37Rv, were tested for resistance to the major antituberculosis drugs, streptomycin, isoniazid, and para-aminosalicylic acid. The inoculum consisted of a 3 mm. loopful of growth from stock cultures on Löwenstein-Jensen medium mixed in one-quarter ounce bottles containing 0.5 ml. sterile distilled water and two 5 mm. glass beads. The bottle was shaken manually for one minute to obtain an even suspension. The slants were inoculated with one loopful of the suspension streaked on the surface of the slant. The tubes were incubated at 37°C. for 28 days.

Following incubation, the slants were observed and the number of colonies were counted. Less than twenty colonies on the slant was considered to indicate inhibition of growth. The resistance ratio was determined by comparing the minimum inhibitory concentration of the test organism to that of M. tuberculosis H37Rv.

III. RESULTS

The resistance ratio for the various strains of mycobacteria on commercially and locally prepared medium are recorded in Table XI. All strains developed heavy growth on the Löwenstein-Jensen medium without drugs. The minimum inhibitory concentration for H37Rv was 6.4 mcg./ml. streptomycin, 0.1 mcg./ml. isoniazid, 0.2 mcg./ml. P.A.S. with the medium prepared locally. Thus, the

TABLE IX

RESISTANCE RATIOS OF MYCOBACTERIA

Organism	Strain	Local Medium Strept.	Local Medium INA	Local Medium PAS	Commercial Medium Strept.	Commercial Medium INA	Commercial Medium PAS
M. tuberculosis	#13810	0.5	0.5	1	1	1	0.25
	POST	0.5	0.5	1	1	1	0.25
	#24770	1	0.5	1	4	2	4
	#24899	1	0.5	2	2	1	0.5
	#24740	1.56	0.5	1	2	1	1
	#226	1.56	0.5	2	2	1	0.5
	#16927	1	1	2	1	1	0.25
M. bovis	Copenhagen	1	0.5	0	0.5	1	0.25
	#4228-2	1	10	>16	0.5	4	0.25
M. avium	Sheard	>1.56	2	16	8	>16	>8
Group I	M. kansasii	>1.56	>10	>16	>8	>16	>8
photochromogens	#23425	>1.56	>10	>16	>8	>16	>8
	CTC #3	>1.56	10	16	4	>16	>8
Group II	#8021	0.25	10	>16	1	>16	>8
scotochromogens	CTC #7	>1.56	>10	>16	>8	>16	>8
	CTC #8	>1.56	>10	>16	>8	>16	>8
Group III	#609	>1.56	>10	>16	8	>16	>8
nonchromogens	#643	>1.56	>10	>16	>8	>16	>8
	#766	>1.56	>10	>16	8	>16	>8
Group IV	M. fortuitum	>1.56	>10	>16	>8	>16	>8
rapid growers	#30184	>1.56	>10	>16	>8	>16	>8
	#3213	>1.56	>10	>16	>8	>16	>8
Saprophytes	M. phlei	0.5	10	16	2	>16	>8

> 8 indicates greater than 8, etc.

maximum resistance ration that could be obtained with the dilutions used are 1.56 for streptomycin, 10 for isoniazid, and 16 for P.A.S. If growth occurred with the highest concentration of drug, the resistance ratio is indicated as being greater than the maximum that can be calculated.

With the commercially prepared medium, the minimum inhibitory concentration for H37Rv was 4 mcgm./ml. streptomycin, 0.06 mcgm./ml. isoniazid, and 1 mcgm./ml. P.A.S., and the maximum resistance ratios that could be obtained were 8, 16.6 and 8 respectively.

IV. DISCUSSION

The majority of the unclassified mycobacteria and M. avium exhibited resistance to all three major antituberculosis drugs while the mammalian tubercle bacilli were sensitive. One Group I photochromogen and one Group II scotochromogen exhibited sensitivity to streptomycin, but were highly resistant to isoniazid and P.A.S.

The resistance ratios obtained with the commercially prepared medium and with the locally prepared medium do not agree in all cases, but the interpretation of the resistance ratios (a ratio of 4 indicates probably resistance, and a ratio of 8 indicates definite resistance) is the same with the exceptions of M. bovis #4228-2 with P.A.S., M. avium Sheard with isoniazid, and one strain of human tubercle bacilli. M. tuberculosis #24770 exhibited slight resistance to the antibiotics with the commercial

medium but not with the other medium. The resistance ratios of the human tubercle bacilli were higher on the Oxoid medium with streptomycin and isoniazid, and lower with P.A.S. Since sensitivity testing is a quantitative procedure requiring a high degree of accuracy, the use of medium prepared by a manufacturer with considerable experience in the field is desirable.

The testing for resistance to antibiotics was not found to be useful in the identification of unclassified mycobacteria. The procedure requires 28 days incubation during which time the organism can be identified by other methods previously described. On the other hand, the testing of drug resistance cannot be disregarded because it offers useful information to a clinician contemplating treatment of a patient harbouring acid-fast bacilli.

V. CONCLUSIONS

Twenty-four strains of mycobacteria were tested for resistance to streptomycin, isoniazid and para-aminosalicylic acid using commercially prepared medium (Oxoid Limited) and locally prepared medium. Resistance ratios were calculated by comparing the minimum inhibitory concentration of the test organism with the minimum inhibitory concentration of M. tuberculosis H37Rv. Drug resistance was not found to be useful in the identification of unclassified mycobacteria but it does offer useful information to the clinician.

CHAPTER X

DISCUSSION

CHAPTER V

DISCUSSION

The work described in this thesis was an investigation of a number of tests and morphological characteristics of mycobacteria for the purpose of identifying them in the clinical laboratory.

The results described have indicated that the first step in identification of the unclassified mycobacteria is to show that they are capable of multiplying at room temperature (approximately 22°C.) which distinguishes them from M. tuberculosis and M. bovis. In this study 35 M. tuberculosis and 2 M. bovis strains failed to grow at 22°C. whereas 49 strains of unclassified mycobacteria and M. avium multiplied at this temperature. This confirms the work of Runyon (1959) who showed that all the unclassified mycobacteria in his study grew at room temperature whereas it has been long established that M. tuberculosis and M. bovis are not able to grow at 25°C. (Wilson and Miles, 1964). M. ulcerans and M. balnei may also grow at room temperature although the optimum for their growth is 32°C., and they are unlikely to be confused with the unclassified mycobacteria as they are isolated from superficial skin lesions while the other mycobacteria from human sources are almost always isolated from lymph nodes or sputa.

The 41 strains of M. tuberculosis tested for niacin production all yielded a positive reaction provided care was taken to ensure that only cultures with heavy growth were tested, while none of the

other mycobacteria gave a positive reaction with this test. This is in agreement with the findings of numerous workers; thus, the test served to identify M. tuberculosis and separate it from other mycobacteria such as M. bovis and M. avium as well as the unclassified organisms that do not produce sufficient niacin to be detected. The use of an amine and cyanogen bromide as test reagents was found to be more reliable than the Feknicz reagents which confirms the work of Runyon, Selin and Harris (1959), and Karlson, Martin and Harrington (1964). Benzidine was found to be slightly better than aniline as the amine reagent although the difference was not as great as indicated by Hodvecsky (1960).

Having thus separated the unclassified - M. avium group of mycobacteria from M. tuberculosis and M. bovis, we found the rate of growth in thioglycollate broth distinguished the "rapid growers" of Runyon's Group IV and saprophytic species from the "slow growers", Groups I, II, III, and M. avium. All "rapid growers" grew in less than one week to develop visible growth. Similar results were obtained by Tarshis (1959) who tested 25 human tubercle bacilli, 10 photochromogens, 15 scotochromogens and 5 saprophytic species for growth in thioglycollate. Only the saprophytic species grew rapidly, the photochromogens and scotochromogens grew slowly or not at all, and the human tubercle bacilli failed to grow.

A consideration of the results of the above tests will leave Groups I, II, and III to be separated, and it appears from our work and consideration of the literature that this can be satisfactorily

achieved by the demonstration of pigment production in the presence or absence of light. The first group consisted of five strains that were photochromogenic. These cultures readily produced a yellow pigment if they were incubated 25 cm. from a 25 watt electric light bulb, but failed to develop pigment if the tubes were wrapped in black paper to exclude any light.

The scotochromogens, of which there were 13 different strains, produced a deep yellow-orange pigment in the light and in the dark. These descriptions agree with Runyon's Groups I and II, and with Marks and Richards' Groups I and II.

The remaining group of mycobacteria consisted of the non-chromogens and M. avium. The latter is indistinguishable from the "Battey" type of nonchromogen unless pathogenicity tests were conducted in chickens. M. avium strains produce a progressive disease in fowl while the "Battey" bacillus is avirulent for fowl (Yousuns, 1963). Four members of the nonchromogenic group produced a slight amount of pigment, yellow or pink, after prolonged incubation. Group III is known to be a heterogeneous group (Runyon, 1959) and Marks and Richards (1962) described organisms which exhibited weak yellow pigmentation but they did not grow at 25°C. and were sensitive to isoniazid.

The above procedure will readily separate and group the unclassified mycobacteria according to Runyon's classification. However, it may be important to distinguish M. fortuitum from other members of Group IV and saprophytic species because M. fortuitum has

been shown, occasionally, to be the cause of disease in man. In our study the arylsulfatase test conducted after 72 hours with a 0.0005 M substrate concentration clearly separated M. fortuitum from other mycobacteria. This verifies the results of Wayne (1962), Tarshis (1963b), and Kubica et al. (1961).

The Tween 80 test was found to be useful in confirming the identification of a photochromogen as M. kansasii but the reaction was not specific for this organism. Similar results were obtained by Wayne, Doubek and Russell (1964).

Characteristics that were not found to be useful in the identification of unclassified mycobacteria were microscopic morphology, rate of growth on solid medium, colony texture, tolerance to oleic acid, and drug resistance. The Ziehl-Neelsen stained smears exhibited bacillary forms and arrangements that did not resemble the typical morphology of tubercle bacilli but this was not a reliable criterion. The rate of growth and colony texture of acid-fast bacilli on solid medium were found to be extremely variable features and could not be depended upon for the identification of species or groups.

Oleic acid (0.025%) in the absence of serum albumin inhibited the growth of most mycobacteria with the exception of the species belonging to Runyon's Group IV rapid growers, while 0.03% oleic acid in the presence of albumin was non-toxic to the majority of the unclassified mycobacteria and some of the tubercle bacilli.

Drug resistance, although used by Marks and Richards, does

not assist in separating unclassified mycobacteria into Runyon's groups. Although M. tuberculosis encountered in a general hospital laboratory are sensitive to antituberculosis drugs, there are occasional strains which are not only resistant but also are not pathogenic to guinea pigs. We encountered one such strain which was satisfactorily separated by the above methods and identified by the niacin test.

Although drug resistance does not aid in the identification of unclassified mycobacteria, the results of the test provide useful information for the treatment of the patient harbouring acid-fast bacilli, and on this basis can be recommended as a routine procedure for laboratories dealing with many cultures of mycobacteria.

One culture obtained from the Manitoba Provincial Laboratories was an isolate of acid-fast bacilli from a sapphire mink. The organism was studied as described and it exhibited the characteristics of a Group III nonchromogen "Battey" type and is so included in the results presented. The staff at the Provincial Laboratory injected this organism into guinea pigs, mice, rabbits, and roosters. The roosters developed signs of tuberculosis but the other animals did not. This would indicate that the organism is a strain of M. avium. Marks and Richards noted that some of the cultures they included in their study of unclassified mycobacteria may have been attenuated or even virulent strains of M. avium. In 1963 Marks and Kim reported that 10 of 17 cultures believed to be Group III non-chromogens were proven to be M. avium strains by pathogenicity for

fowl. It is possible that other cultures described here as resembling Group III nonchromogens could in fact be M. avium.

Thus, to conclude, our work indicates that the following steps will satisfactorily separate and identify the mycobacteria likely to be isolated from patients with systemic disease in a general hospital laboratory.*

1. Growth at room temperature and at 37°C. will separate the unclassified, the saprophytic, and M. avium strains from M. tuberculosis and M. bovis. M. tuberculosis is identified by the niacin test.

2. Having excluded M. tuberculosis and M. bovis, the rate of growth in thioglycollate broth separates the Group IV rapid growers and saprophytes from the other mycobacteria. M. fortuitum is separated from other rapid growers by the arylsulfatase test.

3. A study of the pigment production in the light and in the dark will separate the remaining organisms into three groups, Group I photochromogens, Group II scotochromogens and Group III nonchromogens.

4. Finally, M. avium is separated from other Group III nonchromogens by the pathogenicity test for fowl.

Other tests found to be useful but not absolutely necessary are the Tween 80 test for identifying M. kansasii, and drug resistance tests.

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* See Appendix for detailed test procedure.

CHAPTER XI

SUMMARY

CHAPTER XI

SUMMARY

In an attempt to determine a method of identifying mycobacteria in the clinical laboratory, 50 strains of *M. tuberculosis*, 2 *M. bovis*, 1 *M. avium*, 1 *M. phlei*, and 47 strains of unclassified mycobacteria representing Runyon's four groups were studied. The organisms were examined microscopically, studied for pigment production, growth rates, colony texture, temperature requirements, and growth in thioglycollate broth, and tested for niacin production, hydrolysis of Tween 80, production of arylsulfatase, tolerance to oleic acid, and drug resistance.

Runyon's classification with subdivision of Group III into the "Battey" bacillus and the slight pigment producing organisms, and subdivision of Group IV into *M. fortuitum* and other rapid growers is an adequate guide for naming unclassified mycobacteria.

The following steps will satisfactorily separate and identify the mycobacteria likely to be isolated from patients with systemic disease in a general hospital laboratory.*

1. Growth at room temperature and at 37°C. will separate the unclassified, the saprophytic, and *M. avium* strains from *M. tuberculosis* and *M. bovis*. *M. tuberculosis* is identified by the niacin test.

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* See Appendix for detailed test procedure.

2. Having excluded M. tuberculosis and M. bovis, the rate of growth in thioglycollate broth separates the Group IV rapid growers and saprophytes from the other mycobacteria. M. fortuitum is separated from other rapid growers by the arylsulfatase test.

3. A study of the pigment production in the light and in the dark will separate the remaining organisms into three groups, Group I photochromogens, Group II scotochromogens, and Group III nonchromogens.

4. Finally, M. avium is separated from other Group III nonchromogens by the pathogenicity test for fowl.

Other tests found to be useful but not absolutely necessary are the Tween 80 test for identifying M. kansasii, and drug resistance tests.

CHAPTER XII

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CHAPTER XIII

APPENDIX

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PROCEDURE FOR IDENTIFYING MYCOBACTERIA

1. Subculture the acid-fast organism to three Lowenstein-Jensen slants.
   a) Incubate one tube at 37°C. and exposed to light.
   b) Wrap another tube in black paper and incubate at 37°C.
   c) Incubate the third tube at room temperature (22°C.)
2. Subculture to thioglycollate broth.
3. Perform the niacin test on organisms that grow at 37°C. but not at room temperature nor in thioglycollate broth.
4. Perform the Tween 80 test on photochromogens.
5. Perform the arylsulfatase test on organisms that grow rapidly (less than one week) in thioglycollate broth.

With the results of these tests consult the flow chart below.

I. Growth at 37°C., no growth at room temperature nor in thioglycollate broth.
   A. Niacin positive - M. tuberculosis
   B. Niacin negative - M. bovis

II. Growth at 37°C. and at room temperature.
   A. Slow growth (2 to 3 weeks) or no growth in thioglycollate broth.
      1. Photochromogenic.
         a. Tween 80 positive - M. kansasii

2. Scotochromogenic - M. scrofulaceum

3. Nonchromogenic.

a. Pathogenic to fowl - M. avium

b. Nonpathogenic to fowl -

i. Cream colored - Battey bacillus

ii. Slight pigmentation - Group III "b"

B. Rapid growth (less than 1 week) in thioglycollate broth with formation of a pellicle on the surface.

1. Arylsulfatase positive - M. fortuitum

2. Arylsulfatase negative -

a. Yellow pigmentation - M. phlei

b. Nonpigmented - Group IV rapid grower