STUDIES ON CHROMOGENIC MYCOBACTERIA

by


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1. INTRODUCTION AND PURPOSE
INTRODUCTION AND PURPOSE

The term "chromogenic mycobacteria" is but one of several commonly used to describe acid-fast bacilli which differ from \textit{M. tuberculosis}, and which for the past ten years have been isolated from clinical specimens with steadily increasing frequency. Additional terms which have been used in recent scientific literature and which are usually, but not necessarily synonymous with "chromogenic mycobacteria", include: "atypical", "anonymous","paratubercle", "unclassified", "nontuberculous acid-fast bacilli","unidentified mycobacteria", and "MOTT bacilli" (mycobacteria-other-than-tubercle-bacilli). The terms "yellow bacilli", "orange bacilli", and "Battey" refer to specific varieties within the general group. The current trend in terminology favors "unclassified" as suggested by the American Thoracic Society (1961), but the American Review of Respiratory Diseases in recent months (1964) has indicated its intention to use only the term "atypical". In this thesis "chromogenic mycobacteria" may be considered synonymous with "unclassified", "atypical" and "anonymous".

Of primary importance is the fact that certain members of this poorly defined group can cause disease in man which is clinically, radiologically and pathologically indistinguishable from tuberculosis. A positive diagnosis of such infections, however, is most difficult because indistinguishable strains are often isolated from healthy, symptomless persons.

The types of clinical specimens from which chromogenic mycobacteria have been isolated include: sputum, urine, faeces, exudates from various types of lesions, skin, tonsils, ear and nasal secretions, mucous membranes, lymph nodes,
synovial fluid, bone, spleen, cerebrospinal fluid and blood. Obviously, significance is attached to isolations from cerebrospinal fluid, blood, or closed lesions and abscesses; however, by far the most commonly encountered isolations are from pulmonary infections where, in the absence of other recognizable pathogenic organisms, the presence of chromogenic mycobacteria in sputum cannot be relied upon as positive diagnosis. The same situation exists when members of the unclassified mycobacteria are isolated from urine specimens in suspected cases of renal tuberculosis.

Considerable interest has been aroused in these bacteria, especially with regard to their exact relation to true human tubercle bacilli, their pathogenicity, and their sensitivity to antituberculous therapeutic agents.

The candidate first became interested in this particular group of mycobacteria during 1957 when a chromogenic strain was isolated from a sputum specimen of a patient with pulmonary disease. On only three previous occasions, all during 1953, had pigmented mycobacteria been isolated from clinical specimens in the Province of Newfoundland. A careful search of the literature in 1957 revealed that relatively little was known about slow-growing chromogenic mycobacteria. Furthermore, no agreement existed among bacteriologists concerning the clinical significance and the classification of these organisms. It was apparent that most workers ignored such isolations, especially when made from sputum and urine specimens, on the basis of their being "something other than M. tuberculosis". In instances where microscopic examination of cultural growth revealed the presence of acid-fast bacilli, a few workers had carried out animal pathogenicity studies. At this level, the conscientious few were frequently misled, since these mycobacteria are nonpathogenic for the guinea
pig. The old concept that the guinea pig parallels man in susceptibility to pathogenic mycobacteria is still difficult for many to reject despite the fact that chromogenic mycobacteria are now known to cause tuberculosis-like disease in man.

In Newfoundland, following the finding of chromogenic mycobacteria in a clinical specimen, the routine procedure for reading TB cultures was modified to facilitate a thorough search for these organisms. Almost immediately additional strains were found. During the years 1957 - 1960 unclassified mycobacteria were isolated from 36 of 35,555 clinical specimens cultured for *M. tuberculosis* in the Provincial Public Health Laboratories. The majority of isolations came from patients suspected of having tuberculosis and from whom repeated attempts to isolate typical tubercle bacilli had failed.

With increasing experience it became apparent that these organisms could be easily recognized. The difficulty then arose concerning their classification and clinical significance. Although some workers suggested that certain specific varieties of chromogenic mycobacteria possessed greater clinical significance, there were no reliable methods available to permit strain recognition.

A scheme for the grouping of unclassified mycobacteria was suggested by Runyon in 1959. It was based upon the growth rate and the ability (or inability) of strains to produce pigment under specified conditions. Runyon introduced the terms *photochromogen*, *scotochromogen*, *nonphotochromogen*, and rapid growers for his Groups I, II, III and IV respectively.

At the time this research was started the author had already isolated over 50 strains of chromogenic mycobacteria from patients suspected of having tuberculosis.
Cultural studies had been undertaken in an effort to group these isolates but it was frequently experienced that repeat, or duplicate cultures, under apparently identical conditions, displayed such marked differences as to affect their grouping within Runyon's scheme. Identical experiences had been reported by others and it was personally felt that the classification suggested was ineffectual, and even potentially misleading.

In the absence of specific biochemical, immunological or pathogenicity tests to differentiate members of the chromogenic mycobacteria, this investigation was undertaken to determine the cause, or causes responsible for the variation so frequently encountered in the pigmentation of these bacteria. To facilitate diagnosis it is naturally essential that bacteriologists be able to establish the identity of strains isolated from clinical specimens and also to be able to determine whether or not repeat isolations from an individual patient are the same strain. It was also initially intended to extract and characterize the major pigments of these organisms.

Just prior to these studies the Subcommittee on Mycobacteria, American Society for Microbiology (1962), decreed that photochromogens (Runyon's Group I) formed a homogenous group regardless of their geographical occurrence. They designated the species' name _M. kansasii_ for all photochromogens previously referred to as Group I, "Yellow Bacilli", and _M. luciflavum_. This species was usually associated with disease processes and appeared to be a distinct pathogen.

_M. kansasii_, because of its clinical significance, was chosen for most of the present studies. Fortunately, chromogenesis is best studied by employing photochromogens. Pigment formation occurs rapidly, following exposure of a mature culture
to light. The type species ATCC 12478 (Bostrom) has been used routinely, and in many instances was paralleled by representatives of photochromogens, scotochromogens and nonphotochromogens kindly supplied by Dr. Runyon.
II. HISTORICAL REVIEW
HISTORICAL REVIEW

1. SIGNIFICANCE, GEOGRAPHICAL DISTRIBUTION, INCIDENCE AND EPIDEMIOLOGY OF CHROMOGENIC Mycobacteria.

A. Chromogenic mycobacteria prior to 1950

In 1891, less than ten years following the isolation of \textit{M. tuberculosis} by Koch, Straus and Gamaleya reported on the chromogenic nature of certain tubercle bacilli (cited from Xalabarder, 1961). A review of the literature indicates that prior to 1950 at least 66 investigators reported the isolation of one or more mycobacteria, pathogenic for man but not for the guinea pig (Xalabarder, 1961).

B. Clinical significance during the past decade

The year 1952 marked the beginning of a keen and growing interest in chromogenic mycobacteria. In that year Tarshis and Frisch (1952 a, b, c) reported cultural, pathological and hypersensitivity studies on twenty-six strains of chromogenic mycobacteria isolated from patients with, or suspected of having tuberculosis. The patients from whom these organisms had been isolated were scattered throughout the United States in Minnesota, Washington, Oregon, California and New York. Also in the same year Prissick and Masson, in Montreal, released a report on fourteen strains of pigmented mycobacteria, ten of which had been obtained in pure culture from pus aspirated from suppurating facial, submaxillary or cervical lymph nodes in children (1952; also 1956 and 1957). In all their cases the clinical picture suggested a tuberculous infection. Because the organisms were in pure culture from closed tuberculous-like lesions they were considered to be the
actual cause of the lymphadenitis. Since then others have demonstrated unclassified mycobacteria as the causative agents in cervical adenitis (Weed et al., 1956; Runyon, 1959a; Davis and Comstock, 1961; Marsden and Hyde, 1962; Hsu, 1962; Chapman and Guy, 1958, 1959; Nouflard et al., 1961; Wolinsky, 1963; Gerszten et al., 1964). The role of unclassified mycobacteria in pulmonary tuberculosis-like disease has likewise been established since the organisms have frequently been isolated from resected lung tissue following the isolation of identical strains over long periods of time prior to surgery (Buhler and Pollak, 1953; Timpe and Runyon, 1954; Nassau and Hamilton, 1957; Huppert et al., 1957; Miyamoto et al., 1959; Corpe and Laing, 1960; Lester et al., 1962; and Law et al., 1963).

The increased interest in mycobacteria other than tubercle bacilli has also revealed that the previously recognized and classified M. fortuitum, considered to be a saprophytic mycobacterium, can also cause infection in man (Wells et al., 1955; Gordon and Smith, 1955; Prather, 1960; Corpe et al., 1961; Wayne, 1961; Hartwig et al., 1962; and Dross et al., 1964).

Three separate reports support the view that unclassified mycobacteria may be etiological agents in cases of sarcoidosis (Chapman, 1961; James, 1961; Mankiewicz, 1963). The atypical acid-fast bacilli have also been implicated as the causative agent in cat scratch fever (Boyd and Craig, 1961; Reikes and Washington, 1962).

With present knowledge, infection due to unclassified mycobacteria cannot be differentiated from tuberculosis by any means other than bacteriological studies.

C. Geographical distribution and incidence

From reports to date, a world wide distribution of these organisms is indica-
Significant numbers of cases of human disease have been identified in the United States, in Wales, the Netherlands, Spain, Romania, Malta, Australia and Jamaica. Isolated cases of pulmonary disease caused by these organisms have been reported also from Sweden, Finland, France, Switzerland, Peru and Canada (Lewis et al., 1960). Although there may well be areas of high or low incidence, sufficient valid information is not available upon which to base an opinion.

Experienced workers, specifically looking for these organisms, have reported the isolation of atypical mycobacteria from the sputum of approximately 2 per cent of admissions to tuberculosis hospitals in Florida (Lewis et al., 1960). Approximately 1 per cent of 15,180 patients admitted to tuberculosis hospitals in Georgia during the ten year period 1951 - 1961 had lesions associated with these organisms (Crow et al., 1961). Chaves in New York City (1960) reported that 8.9 per cent of the specimens positive for acid-fast bacilli were atypical, while Keltz et al. in Illinois (1958) reviewed 649 culture-positive consecutive cases and found 164 positive for atypical mycobacteria. This represented an incidence of slightly more than 25 per cent. Dunbar et al., (1963) released figures from the Florida State Board of Health stating that in one year 28 per cent of the cultures isolated at the Central Laboratories proved to be mycobacteria other than M. tuberculosis. Gerszten et al., (1963) after a four year study said that the finding of unclassified mycobacteria in Virginia (5%) was midway between that found in the South (10%) and in the North (2%). Mercks et al., at the Mayo Clinic (1963) also claimed that 5 per cent of their cases were caused by unclassified mycobacteria. Youmans (1963) in quoting Lester et al., (1958) cited an interesting epidemiological study of 49 cases due to M. kansasii.
These workers found that the incidence of infection with *M. kansasii* in the Chicago suburbs of Cicero, Berwyn, and Oak Park, with a total population of 184,129 was 10.7 per cent, whereas the incidence of infection in the remainder of Cook County with a population of 940,000 was only 1.4 per cent. From Texas, LeMaistre (1963) reported that 9 per cent of all newly discovered mycobacterial disease in Dallas was due to *M. kansasii*.

Foreman (1962) of the Central Tuberculosis Laboratory in Wales felt that, compared with pulmonary tuberculosis due to the tubercle bacillus, disease due to anonymous mycobacteria was a rarity. In Wales, in the ten years from 1950 - 1960 only 59 cases could be traced. This figure is fewer than 6 cases per year in a population of 2 million. Besta (1959) cited the incidence in Italy as 0.5 per cent.

Apart from the author's work, figures concerning the incidence of unclassified mycobacteria in Canada are limited to one report. Mankiewicz (1958) reported that over a three year period approximately 4 per cent of the cultures positive for acid-fast microorganisms were chromogenic. Her study was performed at the Royal Edward Laurentian Hospital, Montreal. The author (Butler and Josephson, 1963), culturing 35,555 specimens during the four year period 1957 - 1960, found 2.1 per cent of the positive cultures examined at the Newfoundland Provincial Public Health Laboratory to be of the unclassified varieties. Figures for the years 1961 - 1964 are included in the body of this thesis.

D. Epidemiological aspects

Epidemiological studies carried out by several workers have failed to show any evidence of contagiousness of infections by the unclassified mycobacteria (Lewis et al., 1959; Nassau and Hamilton, 1957; Runyon, 1959a; Kubica et al., 1961;
Crow et al., 1961). A single instance of conjugal disease has been reported (Beek et al., 1963).

What is known concerning the distribution of these organisms is presented in the following paragraphs.

a) **Soil, water, milk and house dust**

Kubica et al., (1961) undertook a study of 1200 samples of soil and water. Preliminary results released on 452 samples in Georgia revealed that more than 45 per cent of the samples yielded acid-fast bacilli. They identified photochromogens, scotochromogens, and "Battey" strains, as well as members of Group IV rapid growers. Later (1963) they reported that the marked similarity of the unclassified acid-fast bacilli isolated from soil and animals strengthened the belief that these agents of disease in man and lower animals originated in soil. The ubiquity of the organisms in nature suggests that they are relatively nonaggressive. In Australia large numbers of atypical acid-fast bacilli were found in swimming-pools, beach sand, water tanks, and in mud from dams and creeks (Singer and Rodda, 1963; Singer, 1964).

Prather et al., (1961) have isolated unclassified mycobacteria from house dust, hospital dust, soil, vegetables and tap water. Others also reported isolations from tap water, house dust and soil (Pellman and Runyon, 1964; Jefferies, 1963; Rodda and Singer, 1963).

Chapman et al., (1965) in Texas found 261 of 270 samples of raw milk to contain mycobacteria. Representatives of Runyon's Groups II, III and IV were identified among the isolates. Biochemical, serological, and biological tests suggested that a small minority of the organisms shared certain characteristics with _M. kansasii_ but the
findings were inconsistent and inadequate for more precise identification. Also in Texas, Jones and Jenkins (1965) isolated 101 strains of mycobacteria from 77 of 92 samples of soil. They failed to isolate photochromogens but did find representatives of Groups II, III and IV.

b) Animals other than man

Anonymous mycobacteria have been isolated from dogs in Japan (Toda et al., 1960) and from swine in California (Froman et al., 1961). The strains isolated from swine were indistinguishable from the "Battey" type, and it was suggested that swine may serve as potential reservoirs for human infection. Scammon et al., (1963) also isolated Group III organisms from swine.

In a study of the distribution and significance of mycobacteria isolated from cattle and swine in the United States, 2,244 tissue specimens of cattle and 172 tissue specimens of swine were cultured. All were negative for Group I organisms, but mycobacteria of Groups II, III and IV, as well as M. avium and M. bovis were not uncommon isolates (Ellis and Yoder, 1964). Others had earlier isolated members of all four Runyon Groups from cattle, and had found some of the Group III organisms to be more virulent than those of human origin (Mallman et al., 1962). Rodda and Singer (1963) reported on anonymous mycobacteria isolated from cattle, sheep, pigs, rats, birds, a toad and a fish.

c) Man

It is possible that man himself may act as a symptomless healthy carrier of these organisms occasionally developing clinical disease when body resistance is lowered. In support of this theory are the reports on the isolation of unclassified mycobacteria from healthy individuals (Edwards and Palmer, 1959; Atwell and Pratt, 1960;
E. Origin of chromogenic mycobacteria

Several possibilities have been suggested concerning the origin of the chromogenic mycobacteria. Thus far epidemiological studies coupled with laboratory investigations have not yielded satisfactory answers. One must accept the fact that scores of recorded cases of human infection involving mycobacteria that differed from Koch's bacillus either in cultural morphology or animal virulence, occurred prior to 1950. Indeed some of the early isolates were so well described that one cannot consider them as differing from the chromogens encountered today. We lack, however, a satisfactory explanation for the apparent increase in the incidence of mycobacterial infections caused by these organisms during the past decade.

It has been suggested that the chromogenic mycobacteria are variants of previously existing forms, dissociation occurring either spontaneously, or due to the influence of drug therapy or bacteriophage. As yet there is inconclusive evidence to favour either of these as the sole answer, however, there is sufficient evidence to suggest each as possible.

Dissociation has been observed in mycobacteria by many bacteriologists. Petroff reported dissociation of both tubercle bacilli and the BCG strain in 1927 and 1929 (Petroff, 1927a, b; Petroff et al., 1929). Orange colonies were isolated from H37 human tubercle bacilli (Petroff and Steenken, 1930). (See also Miller, 1931; Pinner, 1935a, b.) Winn and Petroff (1933) studied dissociated avian bacilli which had been accidentally exposed to increased temperature. Colonies became chromogenic and were found to have lost their pathogenicity for chickens.
A theory upheld by some workers (Burrows and Barclay, 1959; Tarshis, 1958, 1960, 1962; and Sweany, 1961) is that antituberculous chemotherapy has increased the frequency of isolation of chromogenic mycobacteria. Tarshis has produced chromogenic mycobacteria from H37 Rv organisms by culturing them for prolonged periods in the presence of either streptomycin or isoniazid. The variants, on the basis of cultural, pathological, allergenic and antimicrobial susceptibility studies, appeared genetically related to the parent organisms. Other workers have reported similar observations (Tirunarayanan et al., 1959). In a survey, including tuberculous patients who had received, or were receiving, streptomycin therapy, Tarshis isolated chromogenic mycobacteria from 19 per cent of 405 patients compared to a control series of 203 nontuberculous patients of whom less than 1 per cent yielded chromogens. Runyon (1959b) does not accept the drug theory as a source of chromogens; however, he points out that because chromogens are drug resistant, they are favoured by chemotherapy which removes other species. This would be a situation similar to that of Candida and drug resistant staphylococci which emerge in large numbers following use of broad spectrum antimicrobial agents.

It has also been noted that drug-resistant strains of M. tuberculosis isolated from patients receiving para-aminosalicylic acid (PAS) and isoniazid (INH) exhibit either a partial or an absolute growth requirement for oleic acid. In this respect, Groups I, II and III of the unclassified mycobacteria resemble drug-resistant strains of tubercle bacilli more closely than normal drug-susceptible strains (Hedgecock, 1958; 1962).

Recent studies with mycobacteriophage have proven most interesting, and
speculation concerning the role played by phage is not without support. White and Knight (1958) reported that smooth colonies of mycobacteria could be isolated from previously rough strains after exposure to mycobacteriophages. Mankiewicz (1961a) showed that a phage immune variant of a strain of chromogenic mycobacteria differed from the parent strain in rate of growth, colonial morphology and pigmentation, drug sensitivity, Neutral red reaction, animal virulence and in ability to sensitize guinea pigs to Old Tuberculin. The same year (1961b) she was able to demonstrate the presence of mycobacteriophages in stool specimens of patients with tuberculous and nontuberculous conditions. Up to then mycobacteriophages active on acid-fast organisms had been isolated only from fertilized soil.

Examining 50 stool specimens from tuberculous patients Mankiewicz found 10 to contain mycobacteriophage. Most interesting was the finding that only 1 mycobacteriophage, isolated repeatedly from one patient was active on pathogenic mycobacteria. Sputum cultures tested in parallel revealed that the sputum of the patient excreting mycobacteriophage active against pathogenic mycobacteria contained atypical chromogenic mycobacteria, while the sputum of the 9 patients excreting mycobacteriophage active against saprophytic and anonymous mycobacteria contained *M. tuberculosis*.

More recent studies reported by Mankiewicz and van Walbeek (1962) revealed further interesting results. Human tubercle bacilli H37 Rv when infected with the mycobacteriophage found to be active against virulent tubercle bacilli, showed the emergence of phage-resistant bacteria which differed in colonial morphology (smooth, rounded colonies) and which reacted to the photochromogenicity test, i.e.
pigmentation increased when, after 30 minutes exposure to light, incubation was resumed. Upon repeated exposure to bacteriophage, bacteria from the smooth colonies underwent further changes involving growth rate, nutritional requirements, loss of niacin production, and loss of catalase activity. The lysogenic bacteria did not elicit tuberculin reaction in guinea pigs. Cytological changes occurred showing elongated and branching bacillary elements, only the intracellular granules of which were acid-fast by Ziehl-Neelsen staining.

White et al., (1962) also reported additional results which they felt were evidence suggesting that lysogeny with certain mycobacteriophages altered colony morphology.

It is also an interesting fact that chromogenic mycobacteria are frequently isolated from patients when they are in a clinically improved phase of their disease. At times both chromogenic and typical mycobacteria are found together, although it is more common to isolate chromogenic mycobacteria when \( M. \) \textit{tuberculosis} no longer can be demonstrated (Huppert et al., 1957; Nassau et al., 1958; Keltz et al., 1958; Runyon, 1959b; Tarshis, 1960; Mankiewicz, 1961a; Butler and Josephson, 1963).

2. 

CLASSIFICATION OF CHROMOGENIC MYCOBACTERIA

Since the beginning of bacteriology, the ability of certain organisms to produce pigment has been utilized as a criterion in the identification of several bacterial species. Although, in most instances, bacterial classification is today based on combinations of morphology, staining reactions, cultural characteristics, biochemical tests, animal pathogenicity and antigenic analysis, the ability of microorganisms to
produce pigment still remains important in the identification of certain species. This is particularly true for this group of slow growing chromogenic mycobacteria now incriminated as etiological agents of tuberculous-like disease in man. Indeed, it was through their chromogenicity that they were first recognized and subsequently named.

Following the increase in the rate of isolation of chromogenic mycobacteria from clinical specimens during the early fifties, Timpe and Runyon (1954) proposed a tentative grouping for strains isolated from 120 patients, all of whom were thought, or known, to have pulmonary disease. An extended scheme proposed later by Runyon (1959a) has served as the basis of classification until the present time. Runyon's groups are described as follows:

**GROUP I, PHOTOCHROMOGENS:**

("Yellow Bacilli" of Buhler and Pollak, 1953; *M. kansasii*, Hauduroy, Subcommittee on Mycobacteria, American Society for Microbiology, 1962; *M. luciflavum* of Middlebrook, 1956). Pigmentation: Little or none if grown in the dark; bright yellow to orange or brick red if grown in continuous light. Young actively growing non-pigmented colonies will become yellow in the dark incubator 6 to 12 hours after exposure for 1 hour, 45 cm. from a 30-Watt lamp. Photochromogenicity is a pronounced and
GROUP II, SCOTOCHROMOGENS: ("Orange Bacilli", of Buhler and Pollak, 1953). Pigmentation: Yellow or orange from the beginning of growth in the dark; more reddish if grown continuously in light.

Growth rate: About as for tubercle bacilli or a little more rapid at 37°C.

GROUP III, NONPHOTOCHROMOGENS: ("Battey" type of Crow et al., 1957).

Pigmentation: Usually weak or none; if present slowly developing and not as described for Groups I and II.

Growth rate: As described for Groups I and II.

GROUP IV, RAPID GROWERS: Growth rate: Growth within 48 hours at 20 - 25°C from invisible small inocula.

This Group includes described species of Mycobacterium, as M. fortuitum, M. phlei, M. smegmatis or Nocardia species.
The current classification for recognized members of the genus Mycobacterium is outlined in Table I. A description of \textit{M. kansasii}, designated in 1962 by the Subcommittee on Mycobacteria, American Society for Microbiology, as a type species for Group I photochromogens appears in Table II.
<table>
<thead>
<tr>
<th>GENUS MYCOBACTERIUM</th>
</tr>
</thead>
</table>

**TABLE I**

**SAPROPHYTES**

including potential parasites; grow rapidly on most media at 28°C.

1. *M. phlei*
2. *M. smegmatis*
3. *M. fortuitum*
4. *M. marinum*
5. *M. thamnopheos*
6. *M. platypoecilus*

**PARASITES**

on warm-blooded animals

A. **GROWTH ON ORDINARY OR SPECIAL MEDIA**

7. *M. ulcerans*
8. *M. tuberculosis*
9. *M. bovis*
10. *M. microti*
11. *M. avium*
12. *M. paratuberculosis*

B. **HAVE NOT BEEN GROWN ON NON-LIVING CULTURE MEDIA**

13. *M. leprae*
14. *M. lepraemurium*
<table>
<thead>
<tr>
<th>COMMON NAMES:</th>
<th>PHOTOCHROMOGEN; &quot;YELLOW BACILLUS&quot;.</th>
</tr>
</thead>
<tbody>
<tr>
<td>GLYCEROL EGG SLANTS:</td>
<td>AFTER TWO WEEKS IN DARK INCUBATOR, RAISED WITH IRREGULAR SURFACE AND MARGINS, IVORY, OR OFF-WHITE. IF GROWN IN LIGHTED INCUBATOR, LEMON-YELLOW BECOMING ORANGE OR EVEN RED-ORANGE WITH AGE.</td>
</tr>
<tr>
<td>PATHOGENICITY:</td>
<td>IN MAN IT PRODUCES PULMONARY AND EXTRAPULMONARY DISEASE VERY SIMILAR TO TUBERCULOSIS. VERY SLIGHT OR NO PATHOGENICITY FOR GUINEA PIGS, RABBITS, AND FOWL.</td>
</tr>
<tr>
<td>DISTINCTIVE CHARACTER:</td>
<td>DEFINITE YELLOW PIGMENTATION WITHIN 24 HOURS AFTER 1 HOUR EXPOSURE OF AN ACTIVELY GROWING CULTURE TO BRIGHT LIGHT.</td>
</tr>
<tr>
<td>TYPE CULTURE:</td>
<td>AMERICAN TYPE CULTURE COLLECTION STRAIN 12478 (Bostrom).</td>
</tr>
</tbody>
</table>
3. METHODS PROPOSED FOR THE DIFFERENTIATION OF CHROMOGENIC MYCOBACTERIA FROM M. TUBERCULOSIS, AND FOR THE DETECTION OF SPECIFIC VARIETIES AND SUB-GROUPS

A. Animal virulence studies

Very few of the many strains of acid-fast bacilli which occur in nature are capable of causing progressive disease in man or animals. Furthermore, virulent forms exhibit a well-marked degree of specificity in host range (Dubos, 1948). This characteristic is utilized in the current classification of human, bovine and avian strains (Bergey, 1957).

The most commonly employed animals for virulence testing of mycobacteria include guinea pigs, rabbits, chickens, hamsters and mice. Until the early nineteen fifties much reliance was placed on the close correlation in pathogenicity of mycobacteria for guinea pigs and man. This no longer can be maintained as many INH-resistant strains of tubercle bacilli, as well as numerous unclassified strains of mycobacteria have since been found to possess either very low or undetectable levels of virulence for the guinea pig.

Animal virulence studies, as related to the unclassified mycobacteria, have been of little value either in differentiating types within the group, or in evaluating the pathogenicity of such strains for man. Runyon (1959a), reported that unclassified mycobacteria, in general, failed to produce progressive disease in guinea pigs. Photochromogens usually cause disease in mice if 1/100 mg. is inoculated intravenously, or 3 mg. intraperitoneally. Scotochromogens are non-pathogenic for laboratory animals, and Group III nonphotochromogens ("Battey")
are variable in pathogenicity for animals, some strains resembling Group 1 by showing limited pathogenicity for mice, and others lacking pathogenicity for all laboratory animals.

Hardy et al., (1958) performed virulence studies on 73 atypical strains of human origin. Not one produced generalized or progressive disease in guinea pigs. They found all 4 photochromogens tested to be pathogenic for mice, as were 25 of 47 nonphotochromogens tested. None of the 5 scotochromogens studied infected mice. In contrast, others found scotochromogenic mycobacteria to be virulent for mice, possessing a special affinity for the kidney and forming epithelioid tubercles 8 weeks after inoculation (Matsumoto et al., 1963). Some workers feel that differences in results of animal virulence studies for unclassified mycobacteria are due to too short a period of observation. Characteristic lesions have been observed to develop in guinea pigs and mice that have been retained under observation for longer periods of time (Xalabarder, 1961; Kertay et al., 1962). Chromogenic mycobacteria exhibit limited pathogenicity for the rhesus monkey (Schmidt, 1957). It has recently been shown that dogs may become infected with photochromogenic mycobacteria (M. kansasii) but the infection is not serious or progressive (Leon et al., 1964).

B. Antigenic analysis

Cross-sensitization experiments indicated an immunological relationship between human tubercle bacilli and the chromogens (Tarshis and Frisch, 1952c; Nassau and Hamilton, 1957). It appears that they not only share antigenic properties but that they also possess specific components. Using a hemolytic modifi-
cation of the Middlebrook-Dubos hemagglutination test it was shown that sera from tuberculous patients reacted to antigens prepared from atypical "yellow" mycobacteria, and sera from patients with atypical mycobacterial disease reacted with human Old Tuberculin antigens (Nassau et al., 1958). Sera from guinea pigs infected with human strains usually react to Old Tuberculin antigen only, whereas sera of animals infected with strains of the yellow bacillus type cross react with the Old Tuberculin antigen. Absorption of these sera with heat-killed H37 Rv and "yellow" bacilli gives a typical absorption pattern. The H37 Rv removes antibodies leaving the "yellow" titer unchanged, but the "yellow" bacilli remove both human and "yellow" antibodies.

Mankiewicz (1958) using two serological techniques (agar diffusion precipitation and complement fixation) attempted to classify chromogenic acid-fast bacilli, and found both methods revealed evidence of overlapping of antigens between chromogens, and both typical tubercle bacilli and saprophytic mycobacteria. This was also the finding of Beck (1959) using culture filtrates of acid-fast bacilli. He showed cross reaction between the unclassified saprophytes and tubercle bacilli when performing skin sensitivity tests in guinea pigs. There was serological specificity to some degree on a quantitative basis in that animals infected with unclassified mycobacteria, or with tubercle bacilli, showed strongest reactions with homologous PPD extract (Beck, 1960). The difference in strength of the reactions with homologous and heterologous was not marked and consistent enough to distinguish between various mycobacteria (Beck, 1961; Affronti, 1959).
In a study using mammalian tuberculin (PPD-S) and nonphotochromogen "Battey" tuberculin (PPD-B) it was found that patients infected with tubercle bacilli had definite strong reactions to PPD-S and weaker or no reactions to PPD-B. Patients infected with "Battey" organisms had larger reactions to PPD-B than to PPD-S. Comparative testing provided a way to separate tuberculin sensitivity produced by the "Battey" organism from that produced by tubercle bacilli. Using PPD-Y (yellow bacilli) and PPD-S the sensitivity produced by the two organisms was too similar to be distinguished by comparative testing. An interesting epidemiological finding was made using PPD-S and PPD-B. It was observed that two-thirds of the men recruited for navy services from the states of Georgia and Florida were reactors to PPD antigen prepared from the "Battey" mycobacterium while only about 6 per cent were considered reactors to PPD-S (Edwards and Palmer, 1958).

The study of Magnusson et al. (1961) suggests antigenic differences among strains isolated in different areas of the world. "Sensitins" prepared from Indian orange-pigmented strains were similar to "sensitins" prepared from Danish orange-pigmented strains, but differed from those produced from orange-pigmented African strains.

It is proven that a significant number of non-specific tuberculin reactions results from infections with unclassified mycobacteria (Kendig, 1962, 1963; Flynn, 1962; Smith and Johnston, 1963).

The amount of protection or immunity conferred upon animals by exposure to unclassified mycobacteria is under active study. Guinea pigs and mice vaccinated with unclassified mycobacteria have been observed to possess some degree of immunity (Wenkle et al., 1948; Youmans et al., 1961; Klugh and Pratt, 1962; Larson and Wicht, 1963). By means of protection tests in mice, it has been shown that immuni-
zation with atypical mycobacteria representing Runyon's Groups I, II and III gave partial protection against infection with *M. tuberculosis*. A photochromogenic strain equalled or surpassed BCG in protective power. Vaccination of mice with BCG conferred a significant degree of protection against pathogenic photochromogenic strains (Siebenmann, 1964). In other studies the degree of immunity to *M. tuberculosis* H37 Rv induced by a photochromogen in guinea pigs and mice was as good as that produced by BCG (Satake, 1963).

Immunological cross reactions between mycobacterial organisms may eventually be eliminated with purer antigens. Kniker (1961) in a study of H37 Rv as well as representatives of all groups of unclassified mycobacteria, employed ion-exchange chromatography and was able to demonstrate that while some antigens were common to all or most organisms, others possessed specificity for individual organisms.

A large number of strains have been studied by agglutination and antibody absorption techniques. This approach may have greater application in epidemiological studies than in clinical diagnostic laboratories (Schaefer and Reggiardo, 1963; Saito et al., 1964). Using agar diffusion methods Chapman (1961) obtained a serological reaction between sera of patients with sarcoidosis and antigens from photochromogenic mycobacteria.

**C. Resistance to drugs and other agents**

Primary drug resistance is a characteristic of the unclassified mycobacteria. The level of resistance naturally varies with strains; however, broadly speaking the photochromogens, scotochromogens and nonphotochromogens show partial resistance to 1 mcg. per ml. streptomycin, 10 mcg. per ml. PAS and 1 mcg. per ml. INH (Runyon, 1959a). Much higher levels of resistance are frequently reported for these
organisms especially when they are isolated from treated cases. Prissick and Masson (1957) found some strains to be inhibited by PAS only in 1000 mcg. concentration. Tarshis et al., (1955) testing a series of chromogens, observed that INH failed to inhibit the growth in concentrations up to 100 mcg. per ml. Chromogens have also been found to grow in medium containing 750 mcg. per ml. of streptomycin (Butler and Josephson, 1963). Results of susceptibility tests performed on these organisms with the lesser used antituberculous drugs are too scarce and variable to provide useful information. Resistance to various agents at specific concentrations has been suggested for the separation of mycobacteria, but division on this basis has not been too practical (Gastambide-Odier and Smith, 1958; Collins, 1962; Eidus et al., 1959, 1960; Hedgecock and Faucher, 1961; Tsukamura, 1962; Jones and Kubica, 1963, 1965; Minsley, 1964).

D. Bacteriophage typing

The application of bacteriophages for the classification of mycobacteria was suggested in 1956. Hnatko (1956) phage typed 34 classified acid-fast microorganisms and compared them with 33 unclassified mycobacteria which had been isolated from tuberculous patients. Seventy-three per cent of the unclassified organisms were lysed by one or more of the 9 phages used.

Mankiewicz (1961b) also studied the phage susceptibility of chromogenic acid-fast bacteria isolated from patients with tuberculosis-like disease and found that 22 of 93 were affected by exposure to one or more of the six phages used. Other workers have since isolated mycobacteriophages from stool specimens of patients with pulmonary disease (Carter and Redmond, 1963).
It should be mentioned that non-specific clearing occurs when heavy concentrations of phage are employed in the typing of mycobacteria. This finding decreases the value of many results reported from earlier studies in which typing was performed while using heavy suspensions of phage. The routine test dilution method and its modifications give promise of more reliable results (Tokunaga et al., 1961; Tokunaga and Murohashi, 1961, 1963; Redmond, 1963a, b; Redmond et al., 1963). It would appear, however, that all the technical problems are not yet solved and more remains to be known before reliable methods are available to give reproducible results thus permitting the phage typing of mycobacteria on a basis similar to that employed with other groups of bacteria (White et al., 1963; Tokunaga et al., 1964; Manion and Bradley, 1964; Redmond, 1964).

E. Growth at various temperatures

Information of limited value can be obtained by preparing subcultures of mycobacterial isolates and incubating them at 45°C, 22°-25°C, and 37°C. Mammalian tubercle bacilli, both human and bovine, grow slowly and only at 37°C. The avian tubercle bacilli grow slowly at all three temperatures, while the described species of saprophytic mycobacteria (M. phlei, M. smegmatis) grow rapidly at all three temperatures. Most of the unclassified mycobacteria grow slowly at 37°C, and at room temperature, but variable results are obtained at 45°C (American Thoracic Society, 1961).

F. "Cord" factor and neutral red test

Both the ability to form "cords" in fluid medium and the ability to bind neutral red in alkaline buffer solution have been associated with virulence in tubercle bacilli (Middlebrook et al., 1947; Dubos and Middlebrook, 1948). Unclassified
mycobacteria give variable results in both tests (Huppert et al., 1957; Wayne et al., 1957; Hardy et al., 1958; Tarshis, 1960b; Xalabarber, 1961; Butler and Josephson, 1963; Karlson et al., 1964).

G. Lipid content

Specific lipids have been extracted from mycobacteria and it is possible that this may serve to differentiate species and strains. Each strain possesses several lipid fractions, and each fraction has an infrared spectrum sufficiently characteristic to differentiate it from other fractions. Using this principle H37 Rv and H37 Ra strains of *M. tuberculosis* can be distinguished from each other. Bovine and human strains have also been separated. Extending the method to the unclassified mycobacteria and using a combination of column chromatography and infrared spectroscopy it has been possible to extract a specific glycolipid from all 17 photochromogens examined. The fraction has been identified and has been found to be entirely independent of pigment formation and photoactivation (Randell et al., 1951, 1952; Smith et al., 1954, 1957, 1960a, b). In 1964 approximately 150 cultures of mycobacteria were examined for their lipid content. Basically the results indicated that among the 20 to 30 substances recognized in the lipids of a given culture, all but one or at most two were shared between that culture and most other cultures of mycobacteria regardless of species. The substance that is limited in distribution would appear to permit the recognition of the species or sub-group of mycobacteria to which the culture belongs (Randell and Smith, 1964). It has also been demonstrated that relative affinities of acid-fast bacilli for the fat solvents may have taxonomic importance. Partition affinity for a given strain of bacillus was found reproducible and characteristic (Wayne and Jaurez, 1955).
H. Tissue culture

In HeLa cells photochromogenic and "Battey" strains have been shown to grow intracellularly in characteristic patterns, the most prominent feature was a distinct beading which occupied the entire length of the organisms (Shepard, 1958). Branching filaments were also observed.

Morigi (1959) studied phagocytosis of atypical mycobacteria belonging to the chromogenic group. He found that the chromogens did not show invasive capacity comparable to that of virulent tubercle bacilli, and their multiplication in the HeLa cells did not produce cytopathological changes. Scotochromogens were phagocytosed at a faster rate than photochromogens. From his studies on scotochromogens and photochromogens it appeared evident that intracellular multiplication of the scotochromogen proceeded more rapidly and that they showed a more active invasion. This is contrary to clinical and experimental findings which indicate that scotochromogens are generally nonpathogenic. Others (Brosbe et al., 1962), also using HeLa cells, studied avian and "Battey" mycobacteria and found a difference in growth rate, but concluded that the difference observed did not permit differentiation of species. Branching filaments were observed with variable frequency in all of the strains studied, the branching occurred at right angles and was seen more often in "Battey" strains. Within three to five days, tissue cultures showed numerous intracellular organisms for all of 9 "Battey" and 4 avium strains tested.

1. Morphology and staining

The morphology of chromogenic mycobacteria studied by Tarshis and Frisch (1952a), varied considerably depending upon the medium used and the age of
the culture.

In general, most reports agree with Runyon (1959a) in which he described his Groups as follows:

**Photochromogens:** Average size larger than tubercle bacilli, often quite long, banded and beaded; strongly acid-fast.

**Scotochromogens:** Variable in size, strongly acid-fast.

**Nonphotochromogens:** Highly pleomorphic, but often very short, characteristically containing a single hyperchromic granule.

Kappler and Janowiec (1963) are of the opinion that photochromogens are not strongly acid-fast. This has also been the experience of the author (unpublished data). Others reported that photochromogens were easily differentiated from other mycobacteria when stained by the Ziehl-Neelsen method, stating that skilled observers would seldom miss them in direct smears or histological sections (Nassau and Hamilton, 1957).

Periodic acid-Schiff stain has been used to differentiate photochromogenic and scotochromogenic mycobacteria from other varieties (Csillag, 1960).

The validity of attempts to differentiate photochromogens on a morphological basis from other mycobacteria is weakened somewhat by the finding of Gale (1961) who showed a dramatic difference in the morphological appearance of these organisms before and after exposure to light. Prior to exposure to light these or-
organisms were typical of tubercle bacilli in appearance, but following exposure to light and subsequent pigment formation the organisms were twice as long, 4 - 6 times as wide, and contained more than three times as many granules. Similar observations are reported by the author in this thesis.

J. **Eh potential**

Early attempts to separate human, bovine and BCG strains by measuring their Eh potential in buffer solutions showed that there was no distinct difference in types (Aksianzew, 1933; Wilson et al., 1952). Likewise nothing conclusive was derived from studies involving methylene-blue reduction (Bloch, 1950a, b; Aksianzew, 1933; Desbordes and Fournier, 1950).

K. **Adansonian classification (electronic computer)**

Bojalil et al., (1962) using the electronic computer method of Sneath (1957a, b, 1958), studied 229 unclassified strains of mycobacteria and classified them into 12 different categories on the basis of physiological properties. Their study revealed the existence of intermediates between categories (or branches as they called them) which formed a continuous metabolic spectrum and made difficult the separation of related categories.

L. **Biochemical tests**

a) **Introduction**

For the classification of microorganisms, biochemical and metabolic differences are naturally preferred to those of cultural morphology and animal pathogenicity. The slow growth rate of mycobacteria, however, presents technical difficulties along these lines.
In several tests that have been suggested for the detection of biochemical and metabolic differences in members of the mycobacteria, the differences observed have been quantitative rather than qualitative in nature. Such tests have limited value in the recognition of specific types of organisms because results depend upon the metabolic state of the organism under test, as well as the degree of sensitivity and specificity of the test employed. Thus far few useful qualitative differences for strain recognition have been observed among the mycobacteria. Certain tests which distinguish only between rapidly growing saprophytes and \textit{M. tuberculosis} are not of much practical value.

Biochemical tests which have served to help in differentiating \textit{M. tuberculosis} from chromogenic mycobacteria, and to detect specific varieties or sub-groups of the unclassified organisms are listed below under appropriate headings.

\textbf{b) Niacin test}

One of the more useful tests available for the differentiation of \textit{M. tuberculosis} from other members of the mycobacteria is that of niacin testing, based on the principle that tubercle bacilli can synthesize nicotinic acid from certain amino acids (e.g. asparagine and glutamic acid) if they are the only source of nitrogen in the medium. Konno (1953) reported on a marked quantitative difference in the niacin production of human type tubercle bacilli and other mycobacteria when grown in a synthetic culture medium. These workers (Konno, 1956; Konno et al., 1957, 1958a,b) introduced a test designed to differentiate human from all other mycobacteria. Bovine avian, nonpathogenic and unclassified acid-fast bacilli were reported to be negative while the human type gave positive results irrespective of INH susceptibility, cata-
lase content, or pathogenicity for the guinea pig. Rare exceptions have been reported but it is generally accepted that only *M. tuberculosis* var. *hominis* produces enough niacin to give a positive reaction by the Konno test or its modifications. It has recently been reported that a culture of *M. fortuitum* was isolated which gave a positive reaction for niacin (Karlson et al., 1964). As this organism grows rapidly it is not likely to be confused with *M. tuberculosis*. The niacin test may be considered as reliable as any other test currently available for differentiating human tubercle bacilli from all other mycobacteria (Konno and Sbarra, 1959; Konno, 1960).

c) **Catalase activity**

Unclassified mycobacteria as a group show strong catalase activity (Runyon, 1959a). In this respect they differ from strains of INH resistant *M. tuberculosis* which generally show decreased catalase activity (Middlebrook, 1954). It is known that catalase of human and bovine strains, regardless of their virulence, is inactivated by suspending cultures in a phosphate buffer of pH 7 at 68°C for 20 minutes. Unclassified mycobacteria retain their catalase activity under these conditions (Kubica and Pool, 1960). A correlation appears to exist between catalase activity and animal virulence (Peizer et al., 1960). A study of 46 strains of tubercle bacilli from INH treated patients showed that the organisms could be grouped as follows:

1. Those of high catalase activity which were virulent for guinea pigs;

2. Those of moderate catalase activity which were or were not virulent for guinea pigs;
3. Those of low catalase activity which were usually non-virulent for guinea pigs.

That catalase activity is in some way related to virulence was further supported by the finding that the degree of catalase activity in photochromogens paralleled the clinical significance of the strains in the patients from whom they were isolated (Wayne, 1962).

d) Arylsulfatase activity

Early studies on arylsulfatase activity of mycobacteria revealed variable quantitative differences for most members including unclassified varieties (Whitehead et al., 1953; Engbaek, 1954; Wayne et al., 1958), but recent reports indicate that this test under controlled combination of substrate-concentration and bacterial inoculum may be employed in the differentiation of mammalian and avian tubercle bacilli from all other mycobacteria. The arylsulfatase test promises to provide a method of distinguishing M. avium from Group III nonphotochromogens ("Battey"). Avian and "Battey" mycobacteria have been indistinguishable except on the basis of pathogenicity of the former for the chicken (Kubica and Vestal, 1961; Kubica and Beam, 1961). Arylsulfatase testing has also been used to separate M. fortuitum from other rapidly growing saprophytes (Kubica and Rigdon, 1961; Wayne, 1961). M. fortuitum is the only "rapid grower" which is capable of causing infection in man.

e) Hydrolysis of Tween "80"

It was recently noted that certain mycobacteria when grown in media containing "Tween 80" produced a turbidity out of proportion to the actual number of viable cells present (Wayne, 1962). Some organisms could attack "Tween 80" and
form opalescent degradation products. Using this principle as the basis of a test, *M. tuberculosis* and *M. bovis* can be differentiated from Group I photochromogens (*M. kansasii*). Photochromogens have the ability to attack "Tween 80" whereas the tubercle bacilli and *M. bovis* as well as all Group III nonphotochromogens which produce disease in man, birds or other animals give negative results. Group II strains are variable. This principle has been used by Wayne et al., (1964) in a scheme for the classification and identification of mycobacteria.

f) **Growth in the presence of thioglycollate**

A useful observation made by Tarshis and Frisch (1952a) (also Tarshis, 1959, 1960b; Potuznik and Matejka, 1962) was that chromogens would grow slowly in fluid thioglycollate-containing medium. The saprophytes grew rapidly and luxuriantly within 24 - 48 hours forming thick wrinkled pellicles. *M. tuberculosis* was unable to multiply in such a medium. A modified solid thioglycollate medium containing methylene blue has been used to separate Group I and II organisms from those of Group III, the latter failing to grow on the modified medium. (Smith and Steenken, 1961.)

*M. Miscellaneous tests*

In attempts to find reliable criteria for the recognition and identification of slow growing mycobacteria a great variety of tests have been proposed. Conventional differential utilization of carbohydrates, although suggested from time to time, has not proven useful. Recently Sweeny and Jann (1961, 1962) developed a new technique which may be of possible value. They use a massive inoculum in various carbohydrates and polyhydric alcohols superimposed on a solid basal medium contain-
ing phenol red indicator. Without multiplication of the organisms specific metabolic activities can be detected. The authors have used the term "non-growth" testing for this procedure. The organism being tested must first be grown in a rich medium and harvested in its logarithmic phase of growth to provide cells of high metabolic activity.

Differentiation based on carbon utilization has been investigated by some workers (Karlson and Ulrich, 1962; Cerbon and Trujillo, 1963) without leading to practical results. Several workers have had partial success in differentiating groups, species and types of mycobacteria on the basis of amidase activity (Juhlin, 1960; Bonicke, 1960; Halpern and Grossowicz, 1957; Cerbon and Trujillo, 1963; Satake, 1963). On the basis of ribonucleic acid-desoxyribonucleic acid ratio, mycobacteria can be divided into two sub-groups (Tsukamura, 1960).

A test designed to differentiate M. tuberculosis from all other mycobacteria was recently described by Karlson et al. (1964). It is based upon the appearance of growth within a modified Proskauer and Beck liquid medium. Their test, in principle, is similar to a procedure developed by the candidate in the present research (Butler and Josephson, 1963).

An indirect approach has been used by McCuiston and Hudgins (1960). They compared the electrophoretic patterns of sera from patients with sarcoidosis, tuberculosis and disease due to unclassified mycobacteria. The sera from patients with sarcoidosis and infection caused by unclassified mycobacteria showed certain similarities whereas those from patients with tuberculosis gave dissimilar patterns. The authors studied the sera of seventy-four cases suffering from infections caused by
unclassified mycobacteria and in all instances the changes observed were more like those in sarcoidosis than tuberculosis. Electrophoresis showed a decrease in serum albumin and an increase in gamma globulin without an increase in alpha-2 globulin.

N. Characterization of pigments in mycobacteria

a) M. tuberculosis

Anderson and Newman (1933a, b) isolated a yellow crystalline pigment from saponified acetone-soluble fat of M. tuberculosis var. hominis. They identified the pigment as a naphthoquinone and named it phthiocol.

Cold acetone extracts of M. tuberculosis var. avium cultivated on synthetic media containing trace element supplements are often heavily pigmented (Patterson, 1960). The pigment involved was isolated and identified as coproporphyrin III.

M. tuberculosis BCG, sensitive to INH, produces pigment when exposed to the drug whereas a resistant strain of the organism does not (Youatt, 1962).

Pterin-like pigments were isolated from tubercle bacilli by Crow and Walker (1949).

b) Saprophytes (M. phlei, M. smegmatis, M. lacticola, etc.)

Chargaff (1930, 1933) isolated beta and gamma carotenes from M. phlei by separating fractions on columns of aluminum oxide. The same pigments were found in the acid-fast organisms, Bacillus lombardo pellegrini and Bacillus grassberger, however the latter also contained lycopene (Chargaff and Lederer, 1935).

From M. phlei Ingraham and Steenbock (1935) isolated alpha and beta carotenes, cryptoxanthin, esters of lutein, zeaxanthin and azafrin. They also reported
a fraction resembling the naphthoquinone phthiocol. The ratio of the various pigments and their absolute amounts varied with age and cultural conditions.

Leprotin, a carotenoid hydrocarbon similar to beta carotene was found by Grundmann and Takeda (1937) in an acid-fast organism isolated from a leprous lesion. This organism was most likely a saprophyte. Takeda and Ohta (1939, 1940, 1944) found leprotin in M. phlei. These workers questioned the findings of Chargaff, Ingraham and Steenbock and they felt that what had been reported as beta carotene was really leprotin.

M. lacticola (M. smegmatis) when grown on mineral oil media produced the acidic carotenoid astacin as well as carotene, but no xanthophylls (Haas and Bushnell, 1944). The same organism grown on nutrient agar produced carotenes, xanthophylls, but not astacin.

Turian (1950b) described a yellow pigment, acid in character, produced by M. phlei. It differed slightly from astacin and he proposed the name chrysoflein. Later (1951, 1953) Turian reported beta carotene, xanthophyll, leprotene, gamma carotene, a "rhodopin-like" lycopenoid, an oxycarotenoid, phytofluene and chrysoflein. The pigments differed quantitatively depending on whether growth occurred at 30°C or 37°C. Using diphenylamine to inhibit carotenoid biosynthesis in M. phlei, Turian and Haxo (1952) isolated two fractions not detectable in uninhibited normal bacteria. One fraction was a visible yellow and the second was colorless, but possessed a greenish-grey fluorescence.

Fisher et al., (1955) found leprotene and beta carotene as epiphasic frac-

ions when partitioning extracted pigments of M. phlei between petroleum ether and
90 per cent methanol. A hypophagic red pigment was also observed but not identified. Goodwin and Jamikorn (1956) investigated three strains of \textit{M. phlei} and found that they all produced the same carotenoids in the same relative amounts. Included were beta carotene, zeta carotene, leprorene, lycopene and two unidentified xanthophylls. The xanthophylls were pigments earlier considered to be cryptoxanthin and zeaxanthin. These workers were of the opinion that Turian's chryroflein was a mixture of these two unidentified fractions.

Also working with \textit{M. phlei} Schlegel (1959) found phytoene, phytofluene, beta carotene, zeta carotene, gamma carotene, neurosporene, lycopene and myxoxanthophyll. Quantitatively the oxycarotenoid myxoxanthophyll accounted for approximately 95 per cent of the total carotenoids.

A group of mycobacteria isolated from the soil and presumed to be saprophytes, was found to form a red product from PAS (Tsukamura, 1961). The colored matter was not identified. It could be produced if cells of mycobacteria were incubated at 37°C for 12 hours in a phosphate buffer solution containing 2 mg. of PAS per ml.

Rilling (1964) extracted and identified phytoene and phytofluene from \textit{M. lacticola}. Other fractions were obtained which were not identified but which were similar to zeta carotene, beta carotene and leprorene. A hypophagic fraction was observed but not identified.

c) \textbf{Unclassified slow growing pathogenic mycobacteria}

Pigments of a photochromogenic mycobacterium (Runyon P-8) and a scotochromogenic mycobacterium (Runyon P-6) were investigated by Ebina et al. (1962).
By chromatography on an alumina column these workers observed four colored fractions. Near the top of the column was a very narrow brown band. A very narrow red band was immediately below. These were not identified. A broad orange band occurred midway down the column, and just below it was a narrow yellow band. These were identified as beta and alpha carotene respectively. It was reported that exposure of nonpigmented photochromogenic mycobacteria to light resulted in an increase in the beta carotene content. The appearance of the chromatogram for P-6 (scotochromogen) was similar to that for the "light" culture of P-8. By ultraviolet illumination five fluorescent bands were observed but only one was investigated. It exhibited the absorption maxima at 348 and 368 μ in petroleum ether and was considered to be phytofluene. Since this band was broader in the "dark" culture of P-8 than in the "light" culture, the workers proposed it as a precursor for beta carotene.

Tsukamura (1962) studied two photochromogenic mycobacteria. From one strain he isolated beta carotene and lycopene. Only beta carotene was isolated from the second strain.

Costello et al., (1962) working with representatives of Runyon's Groups I, II and III isolated a fraction for which the absorption spectrum was a bell shaped curve with a single broad peak at 452 μ in petroleum ether and 462 μ in ethanol. This was similar to Turian's chrysoflein. A second fraction was considered to be leprotin. Costello (personal communication, 1963) felt that Group I and II organisms contained essentially similar pigments, however they only studied two strains of each Group. Studies on three Group III strains revealed that one developed pigments similar to those of Group I organisms, but two Group III strains were markedly different.
4. FORMATION OF PIGMENT IN MYCOBACTERIA

A. Introduction

Previous observations reported in the literature concerning pigment formation in mycobacteria relate to a variety of species and strains. Just how applicable earlier studies are, in terms of the pathogenic chromogens being encountered today, is unknown. It is unfortunate that practically all detailed chromogenicity and pigment studies on mycobacteria, including those involving pigment extraction and characterization, have been performed on saprophytes. Pathogens do not lend themselves to the extensive manipulations necessary for detailed analysis. Furthermore, the association of chromogenic mycobacteria with human disease has only recently been established.

The factors which have in the past been observed to influence pigment production in various acid-fast bacilli are reviewed below under appropriate headings.

B. Oxygen tension

Schwabacher in 1933, when studying saprophytic chromogenic acid-fast bacilli concluded that the most important factor influencing pigment production appeared to be the oxygen supply. In tightly sealed tubes little or no pigment was produced even after incubation for several weeks, while in lightly corked tubes pigment formation was often well marked within a few days. Petroff and Steenken (1935) two years later cultured M. phlei in two series of flasks, one series plugged with cotton and capped with perforated rubber nipples, and the second series tightly sealed with melted paraffin. Growth in the sealed tubes was much smaller and less chromogenic, and they concluded that chromogenicity
of *M. phlei* was an unstable characteristic influenced by several factors including that of available oxygen supply. The following year Reid (1936 – 1937) investigated seventy-six strains representing twenty-four genera of chromogenic bacteria. His collection included *M. phlei* and *M. butyricum*. No pigment was produced when bacteria were grown under low oxygen tension. Abundant growth occurred in freshly inoculated tubes sealed with special tube closures, but lack of oxygen was reported to have prevented pigmentation. Similarly Baker (1938), using controlled experiments, studied pigment formation in a mycobacterium pathogenic for small tropical killifish. He likewise found that pigment would not form in the absence of molecular oxygen. The same has recently been reported for the saprophyte *M. lacticola* (Rilling, 1962) and it has been postulated within recent months that a compound capable of inducing a carotenogenic enzyme is formed in the same organism as a result of exposure to light and oxygen (Rilling, 1964). At the same time, and following release of results in this present study (Butler, 1964), Wayne and Doubek (1964) reported on the role of air on the photochromogenic behavior of *M. kansasii*.

*M. tuberculosis* BCG has been found to produce pigment when exposed to isoniazid in the presence of oxygen. This may, however, be a straight chemical reaction since it was not determined whether the pigments were derived from the isoniazid, or whether they were a product of disordered cell metabolism. Indirect evidence favoured the view that the pigments were derived from the cells (Youatt, 1961).

C. Temperature of incubation

As in other bacterial genera, the effect of temperature on pigment formation has been examined in mycobacteria. Petroff and Steenken (1935) reported
M. phlei deepened in pigment content when left at room temperature. Turian (1953) has shown qualitative differences in pigment formation by M. phlei when grown in glycerol broth at 30°C and 37°C. Decrease in pigment production with increase in temperature was recorded by Reid (1936 - 1937) and also Darzins (1939), while Baker (1938) found the opposite to be true. He observed greater chromogenicity at 37°C, a temperature at which the strain studied did not grow. Still another peculiar finding was that of Winn and Petroff (1933). They accidentally subjected a culture of M. avium to a high temperature and obtained a dissociated form having all the properties of the original, except it became ochre in color and lost its pathogenicity. Buhler and Pollak (1953) when first describing their "Yellow Bacillus", stated that cultures grown at a temperature of 37°C could lose chromogenicity and be cream colored. Their "Orange Bacillus" retained its deep orange irrespective of the temperature of incubation.

D. pH of medium

There is little agreement in the observations recorded concerning the effect of pH on chromogenesis in mycobacterial species. One study indicated that carotenogenesis in a saprophyte, (M. lacticola) was favoured by an alkaline pH over a very broad maximum, a pH of 8 being considered satisfactory (Rilling, 1962). In another study, pigment production of M. tuberculosis BCG was diminished at pH 6 compared with that obtained at pH 7 (Youatt, 1961). Steenken (1935), in contrast, reported tubercle bacilli grew with chromogenicity at pH 6 but lacked color at pH 7.6. In a large survey involving 76 cultures of chromogenic bacteria, including representatives of mycobacteria, the reaction of several media was adjusted over a pH range from 6.0 - 8.4. It was found that below pH 6.6 or above pH 8.0 pigmentation be-
came less marked. Within this range differences in reaction had little effect upon the production of pigments although changes in shade or tint were detectable (Reid, 1936 - 1937). Bovine tubercle bacilli have been observed to produce definite pigment below pH 8.0, while a more alkaline reaction resulted in growth without pigment (Reed and Rice, 1929). Ingraham and Steenbock (1935), determined the optimum reaction for pigmentation of *M. phlei* to be between pH 6.0 and 7.0. When the pH was above 8.6 pigmentation was very poor in any medium.

E. Composition of medium including the presence of trace elements and drugs

Many sporadic reports have been made on the effect of various media, or specific chemical substances in a particular medium, on the pigmentation of mycobacteria. It is of no advantage to cite observations related to cultural systems in which several variable factors existed. Without controlled pH, temperature, and oxygen tension, it is obvious from the foregoing, that depending upon various combinations of variables, pigment may or may not be formed. This was the finding of Reid (1936 - 1937) when 96 media of different composition were investigated to determine which factors and substances favoured, and which inhibited pigment formation. In the study of 76 species of chromogenic bacteria, results led him to conclude that pigmentation was governed by a variety of factors, the presence of one or several of which may be adequate to produce sufficient pigment so that the absence of some other factor or factors would not be noticed. The study indicated, however, that the principal element essential for production of pigment in bacteria was nitrogen. Carbohydrates increased pigmentation probably by virtue of stimulating the amount of growth, but in the absence of nitrogen they were not able to support pigmentation.
Others studying *M. phlei* also demonstrated the importance of nitrogen, but further found that the type of compound which served as a source of nitrogen had little effect on pigmentation if the hydrogen ion concentration was maintained around neutrality (Ingraham and Steenbock, 1935). Striking changes were noted, however, when glycerol was substituted for glucose. The rate of growth was slower on glycerol than on glucose-containing medium, but cells grown on glycerol became yellow earlier in the growth period and pigment during the later stage was many times that obtained on glucose.

Strains of tubercle bacilli have been noted to produce green pigment in Sauton medium. Kolle (1932) stated that this was first observed by Lange and Piscatore at the time of the tragedy in Lubeck caused by contamination of BCG vaccine with the "Kiel" strain of tubercle bacilli. The strain could be readily recognized by its abundant pigment production after recovery from organs of children who had died from administration of the contaminated vaccine.

Cultures of *M. phlei*, *M. leprae* (?), and *M. smegmatis* were pigmented when grown on ordinary media but failed to yield pigments when grown on media containing n-decane, light mineral oil, heavy mineral oil, or paraffin wax. In contrast *M. lacticola* when grown in mineral oil media produced a bright orange pigment (Hass and Bushnell, 1944).

For certain mycobacteria the enhancement of pigment production in serum-containing medium is recognized. The human form of *M. tuberculosis* generally develops a creamy to yellow or faint red pigment especially on media-containing serum, while the bovine and murine forms are not pigmented (Bergey, 1957). Griffith (1916), almost 50 years ago, described the effect of bovine serum on pigment formation in
cultures of tubercle bacilli.

Reed and Rice (1929), demonstrated the influence of iron on the pigmentation of acid-fast bacilli. When grown in the absence of iron, human, bovine, avian, and saprophytic strains were colorless. When iron-containing medium was employed, all strains produced pigment. In contrast, pigmented non-acid-fast bacteria tested produced approximately equal amounts of pigment on both iron-free and iron-containing media. *M. tuberculosis* var. *avium* when grown on synthetic media containing trace-element supplements (cobalt, copper, zinc) formed pigments which could be extracted from the cells with cold acetone (Patterson, 1960).

Pinner (1935a, b), one of the first to study a significant number of chromogenic mycobacteria isolated from clinical material, found strains with lemon, yellow and dark orange pigments. The fact that some transplants failed to develop pigment, but that pigment usually returned on repeated transplants, led him to conclude that the changes were not caused by the medium.

Tubercle bacilli produced a yellow pigment when grown in Long or Sauton media with 1:1000 - 1:3000 p-aminobenzoic acid, or in medium containing 1:200 - 1:1000 procaine (Mayer, 1943, 1944a, b). The production of pigment by strains of *M. tuberculosis* occurs upon exposure to INH. *M. tuberculosis* BCG, which is sensitive to INH, produced pigment when exposed to the drug, whereas resistant strains of the organism did not (Youatt, 1961, 1962).

Chromogenic mycobacteria have been produced from the H37 Rv strain of *M. tuberculosis* by serial passage of the organism over a period of fourteen months in media containing increasing concentrations of streptomycin and INH (Tarshis, 1958,
1960, 1962). On continued incubation all of the colonies changed in color from yellow to yellow-orange and finally to orange.

An amithiozone-resistant mutant of *M. tuberculosis* H37 Rv was observed to produce a yellow pigment whereas the parent susceptible strain did not (Tirunarayanan et al., 1959). Several strains of human origin made resistant to amithiozone all gave the same pigment formation.

F. Exposure to light

Light is obviously an important factor influencing the production of pigment in the photochromogenic mycobacteria. However, very little is known concerning the type of light (wavelength), and duration of exposure, which best stimulates the formation of pigment in these bacteria. Baker (1938) studied the effect of sunlight, incandescent lamp, and ultraviolet rays on *M. platypoecilus*. An exposure for 1 minute to ultraviolet rays (Wood filter) resulted in full pigment development equal to that resulting from 30 minutes exposure to a 100-Watt incandescent lamp. Exposure to sunlight for 15 minutes also permitted full pigment development. Longer exposure to either sunlight (100 minutes) or ultraviolet light (20 minutes) produced no pigment. These exposures were apparently lethal since the organism failed to grow on subculture. This indicates the necessity of living cells for pigment formation.

The blue portion of the spectrum 400 - 500 mu has been reported most stimulatory for pigment formation in *M. lacticlea* (Rilling, 1962). Using monochromatic light, Gale (1961) found that 10 minutes exposure to wavelengths ranging from 400 - 580 mu was adequate for stimulating pigment development in a photochromogenic mycobacterium. It has recently been determined that scotochromogens are most resistant to ultraviolet irradiation, with nonphotochromogens showing intermediate resistance and photochro-
mogens possessing least resistance (Tsukamura, 1964).

G. Exposure to mycobacteriophage

It was observed by Mankiewicz that exposure to mycobacteriophage could influence pigment formation in mycobacteria (1961a). She isolated phage-immune variants upon exposure of chromogenic mycobacteria to mycobacteriophages. One of the strains thus obtained, in addition to phage immunity, showed marked differences in a variety of characteristics including that of pigmentation. The original strain produced smooth light-yellow colonies in 11 - 12 days, and pigmentation was unaffected by light. It was considered to belong to Runyon's Group III. In contrast, the phage-immune variant grew rapidly producing orange colored growth in 3 days and the pigmentation was affected by light. In light at room temperature (22°C) very little if any pigment was formed, while at 37°C in the dark, deep orange pigment developed. As reported, both temperature and light were varied at the same time. Whether the lack of pigmentation at 22°C was due to decrease in temperature or increase in light, or on factors depending on the simultaneous variation of both light and temperature, was not reported. The possibility of decrease in pigmentation resulting from exposure to light must be considered. Two such strains of acid-fast bacilli have been isolated from clinical specimens in Newfoundland (personal unpublished data). It was recorded in one other instance by Rogul et al., (1957). They proposed the name "photophobe" for organisms producing more pigment in the dark than in the presence of light.

Returning to phage-immune variants and pigmentation, a further report was made by Mankiewicz and van Walbeek in 1962. They obtained from virulent human tubercle bacilli, strain H37 Rv, phage-immune variants which differed in a
A wide variety of characteristics from those of the original strain. Among other changes, the phage-resistant bacteria responded to the photochromogenicity test, i.e., pigment developed when after 30 minutes exposure to light, incubation was resumed in the dark. This photoreaction is listed as a distinctive characteristic of the recently designated species *M. kansasii* (Subcommittee on Mycobacteria, American Society for Microbiology, 1962).

**H. Specific inhibitors of pigment synthesis**

Inhibition of pigment formation in microorganisms can be achieved by the use of certain chemicals. The most widely investigated is diphenylamine. Kharasch et al. (1936) found that all chromogenic organisms investigated, when grown in media containing diphenylamine, gave colorless growth. They concluded that the type of medium was of minor importance since various media, normally supporting growth of the organisms with pigment formation, all gave rise to colorless growth when diphenylamine was added in concentrations of 1:2000 - 1:6000. In some instances, the loss of pigment could be attained on diphenylamine-containing medium in the first transplant, while others required several transfers. Turian (1950a, b, c) found that carotenoid production in *M. phlei* was strongly inhibited by diphenylamine at concentrations which did not affect growth. Later, Turian and Haxo (1952) employing diphenylamine inhibition, detected two compounds in *M. phlei* which were absent in cultures grown under normal conditions. It was first felt that the two polyenes detected in the presence of the inhibitor, were intermediates in the biosynthesis of the carotenoids, which in the uninhibited cells did not occur in sufficient quantity for detection. A more recent interpretation considered the possibility that
compounds formed and accumulating during diphenylamine inhibition may not be normal biosynthetic intermediates, but rather side-products of abnormal biosynthesis resulting from the diphenylamine inhibition (Jensen, 1962).

Dihydrostreptomycin, puromycin and chloramphenicol as well as diphenylamine all inhibited pigment formation in *M. lacticola* (Rilling, 1962). Phenol inhibited carotenogenesis in *M. phlei*, while dinitrophenol, in contrast, enhanced the production of carotenoids (Turian, 1951).
III. MATERIALS AND METHODS
MATERIALS AND METHODS

1. MATERIALS

A. Strains of mycobacteria

The majority of chromogenic mycobacteria used in the early studies was isolated by the candidate from clinical specimens submitted to the Provincial Public Health Laboratory, St. John's, Newfoundland. Prior to the present study almost 50 strains had been cultured from patients suspected of having tuberculosis, and during the past three years an additional 31 strains have been isolated. All isolated strains were slow growing mycobacteria and with the exception of 11 pigmentless strains, all were capable of producing pigment in the dark. Thus, they were identified as scotochromogenic (Group II) and non-photochromogenic (Group III) mycobacteria.

A photochromogenic strain was obtained from Dr. C.O. Siebenmann, Connaught Medical Research Laboratories, University of Toronto. The culture was termed "Chadwick". It had been isolated by Mr. A. Hambleton from a case of pulmonary tuberculosis at the Beck Memorial Sanatorium in London, Ontario. Dr. Siebenmann advised that the strain would kill mice, but not guinea pigs. It produced tuberculin sensitivity closely resembling that produced by typical tubercle bacilli. Dr. Siebenmann also supplied an additional five strains which he suspected were scotochromogens. These were labelled "Steiner", "Foster", "Moore", "Rosenberg" and "Lange".

Dr. E. Runyon, Veteran Administration Hospital, Salt Lake City, Utah, kindly supplied representatives of each Group of mycobacteria as classified by
himself. Strains received, along with pertinent data, were as follows:

Strain P1, isolated from the sputum, gastric lavage and resected lung tissue of patient R.F. in Kansas City. It was isolated before administration of chemotherapy and was identified as a photochromogen (Runyon Group I).

Strain P2, isolated from resected lung tissue of patient A.W. in Georgia. This culture was identified as a nonphotochromogen, "Battey" type (Runyon Group III).

Strain P5, isolated from the sputum and resected lung tissue of patient G.W. in Ohio (identified as a scotochromogen; Runyon Group II).

Strain P6, isolated from the sputum and resected lung tissue of patient L.C. in Pennsylvania (identified as a scotochromogen; Runyon Group II).

Strain P7, isolated from the sputum and resected lung tissue of patient J.G. in Texas (identified as a nonphotochromogen, "Battey" type, Runyon Group III).

Strain P8, isolated from the sputum and resected lung tissue of patient M.B. in Pennsylvania (identified as a photochromogen, Runyon Group I).

Strain P25, isolated from the sputum and lung of patient A.R. in New York (identified as a nonphotochromogen, "Battey" type, Runyon Group III).

The type culture of _M. kansasii_ Hauduroy was secured from the American Type Culture Collection (ATCC 12478). This strain was originally isolated by Pollak from a fatal case and was described as being resistant to streptomycin and PAS. A second strain of _M. kansasii_, also isolated by Pollak, was obtained from the same source. It was designated ATCC 12479.
Stock cultures of *M. bovis* (BCG strain), *M. phlei*, *M. avium* and *M. fortuitum* were obtained from the lyophilized stock cultures maintained by the Department of Bacteriology, McGill University.

Dr. E. Mankiewicz of the Royal Edward Laurentian Hospital, Montreal, supplied cultures of *M. tuberculosis* H37 Rv and *M. smegmatis*. The *M. smegmatis* strain originated from Dr. E. Vendra, Budapest (Mankiewicz, 1961b).

**B. Media**

a) Lowenstein-Jensen inspissated egg medium was employed for the primary cultivation of all clinical specimens and also for the primary cultural and pigment studies. The formula and method of preparation are outlined in the Appendix Section A - 1.

b) Dorset inspissated egg medium with 5 per cent glycerol was found to support the best pigment production of chromogenic mycobacteria. It was used in studies related to photoactivation and pigment synthesis. The formula and method of preparation is stated in Section A - 2 of the Appendix.

c) Dubos medium was used in growth rate studies in which stationary and shaken cultures were investigated. For the formula and preparation see Appendix Section A - 3.

d) A fluid mineral-salts medium containing mineral oil was used in a search for "oil soluble" pigments. This medium was proposed by Haas and Bushnell (1944) and is detailed in Section A - 4 of the Appendix.

e) The synthetic medium of Sauton was the medium of choice for growing mycobacteria in mass for pigment extraction. A modified Sauton medium containing
buffer and serum was used as a base medium to which PAS, INH and streptomycin were added in varying concentrations for the sensitivity testing of all clinical isolates (Diena, Eidus and Greenberg, 1959).

The formulae and preparation of Sauton medium and modified Sauton medium are detailed in Section A - 5 of the Appendix. The type of Sauton medium used for each experiment is stated within the text.

C. Chemicals and reagents

All chemicals used were of reagent grade quality. Where special reagents and equipment are involved, pertinent data will be stated in the text of this thesis.

Solvents used for pigment extraction as well as adsorbents and solvents used in thin-layer chromatography are described within the outlined procedure. All solvents are listed in the Appendix, Section B - 1.

2. METHODS

Only the methods which served as routine procedures are included in this section. Special procedures used in some experiments are described in the next section, Experimental Results.

A. Pretreatment and culture of clinical specimens

Commensal organisms in specimens for TB culture were destroyed by treatment with equal parts of 4 per cent sodium hydroxide. This procedure is the most widely used in clinical laboratories (Rohde and Vera, 1956). The method was first described by Petroff (1915).

The total period of exposure to the digestant was never permitted to
exceed 45 minutes. This time period included shaking, centrifuging and neutralizing with normal hydrochloric acid. The method differed in one respect from the conventional procedure. Following treatment of the specimen with 4 per cent sodium hydroxide and neutralization with normal hydrochloric acid (using phenol red as indicator), the final step was a back-titration to an alkaline pH. This modification was introduced during 1957 after a two year study had shown that the number of specimens yielding positive direct microscopic findings and negative cultures was significantly reduced when the inoculum was alkaline rather than acid (personal unpublished data).

Two tubes of Lowenstein-Jensen medium were each inoculated with two drops of concentrated specimen using a Pasteur pipette. The inoculated media were incubated in a horizontal position for forty-eight hours at 37°C. Tubes were then uprighted to a vertical position for eight weeks incubation. Cultures were read weekly.

B. Identification of M. tuberculosis and chromogenic mycobacteria

Identification of M. tuberculosis was based upon a combination of criteria including cultural appearance, rate of growth, temperature growth studies, appearance of growth in fluid medium and niacin testing.

Colonial appearance, extent of growth and the date on which growth was first observed were recorded for all tubes showing growth. Ziehl-Neelsen stained smears were prepared and examined. Cultures, which microscopically were observed to consist of acid-fast bacilli, were then subjected to the following investigations:

1. Representative colonies were removed from slant(s) and suspensions were pre-
pared in sterile diluent, (Sauton medium is a satisfactory diluent). Grinding in a tissue grinder was usually necessary in order to obtain a uniform suspension.

2. The above suspension was subcultured to four tubes of modified Sauton medium (Diena et al., 1959) for incubation at each of the following temperatures: 25°C, 32°C, 37°C and 45°C. In addition the suspension was subcultured to two Lowenstein-Jensen slants. Both slants were incubated at 37°C, one in the presence of light, and one wrapped in tin foil, or black paper.

3. After 2 weeks the subcultures in Sauton medium were examined and the presence or absence of growth for each temperature was recorded as +, ++, +++ or ++++. The type of growth was described as "granular", "flaky", or "bacterial", the latter meaning uniform dispersed growth.

Pigment development was recorded for cultures grown in light and dark on Lowenstein-Jensen medium. If the culture grown in the light was pigmented and the culture grown in the dark was nonpigmented, photochromogenicity testing was performed on the nonpigmented culture.

Photochromogenicity testing consisted of 1 hour exposure, room temperature, 45 cm distance from a lamp fitted with two 15-Watt Westinghouse fluorescent "cool white" tubes (F 15 T 8/CW). Cultures were reincubated in the dark for a period of 24 hours following exposure to light, and then inspected for pigment content. The development of yellow pigment during this 24 hour period was considered a positive test.

4. Testing for niacin (Appendix C-1) was performed on one of the fresh Lowenstein-Jensen subcultures prepared at the time that temperature studies were initiated. The niacin test was not performed if there was any evidence of contamination
either on Lowenstein-Jensen slants or in the liquid medium cultures.

5. Sensitivity tests were performed using modified Sauton fluid medium, the test materials were sterilized by filtration before being added to the sterile medium. This procedure was proposed by the Laboratory of Hygiene, Ottawa, for sensitivity testing of M. tuberculosis (Dr. Greenberg, personal communication). For details see Section C-2 of the Appendix.

 Cultures which conformed to the following criteria were reported as "TB Culture Positive":

 i) Dry, wrinkled, cream-colored colonies which reached maturity in not less than two weeks on Lowenstein-Jensen primary culture and which consisted of acid-fast bacilli.

 ii) Growth within two weeks in liquid media at 32° C and at 37° C, but no growth at 25° C or at 45° C.

 iii) Flaky growth in modified Sauton medium which settled rapidly on standing, leaving a clear supernatant.

 iv) Niacin positive.

 v) No marked primary resistance to PAS, INH and streptomycin.

 Acid-fast cultures which failed to conform to one or more of the above criteria were subjected to further investigation including animal virulence studies.

 Chromogenic mycobacteria were identified by:

 i) Development of smooth pigmented colonies.

 ii) Ability to grow at room temperature.
iii) Development of uniform, dispersed growth in modified Sauton fluid medium.
iv) Negative niacin test.
v) High levels of resistance to PAS, INH and streptomycin.
vi) Failure to cause progressive disease in guinea pigs after subcutaneous inoculation.

C. Growth of M. kansasii for pigment studies

It was essential that a simple defined medium be used when growing M. kansasii for pigment studies. Pigmented ingredients such as egg yolk, serum, malachite green and "Tween 80", normally found in various media for mycobacteria would prevent qualitative and quantitative pigment analysis.

Sauton medium was considered most appropriate for these experiments. Maximum yield per unit volume of medium was obtained when cultures were grown in 20 mm x 125 mm loosely capped test tubes containing 10 ml of medium per tube. Using this method thousands of tubes were prepared. Growth developed in the form of a pellicle if the inoculum were "floated" on the surface. As the pellicle thickened it settled to the bottom leaving sufficient growth at the meniscus to support development of a second pellicle which in turn settled. This formation and settling of pellicle would repeat two or three times.

Incubation was at 37°C for 4 weeks in continuous light for maximum pigment development. Light exposure was approximately 30" from a 40-Watt General Electric "cool white" fluorescent lamp. Unpigmented cultures were incubated at the same temperature in the dark. These were sealed within light proof cardboard boxes.
Experiments using Erlenmeyer flask cultures yielded less growth per unit volume of medium than did test tubes, however, it was observed that a relationship existed between the size of flask and the volume of medium used. Best results were obtained when 50 to 100 ml of medium were used in 500 ml flasks. All cultures were incubated in a stationary position.

Following 4 weeks incubation the organisms were killed by adding an equal volume of 5 per cent formalin. Formalinized cultures were permitted to stand for 24 to 48 hours at room temperature in the dark before being harvested.

Killed cultures were pooled into 250 ml centrifuge bottles, and centrifuged at 2000 r.p.m. for 20 minutes. The supernatant was discarded and the bacterial mass was washed with three changes of distilled water. Each wash water was permitted to stand for 1 hour before centrifuging. This removed residual formaldehyde.

Washed packed cells were weighed, transferred to Petri dishes and dried over phosphorus pentoxide in a vacuum desicator. Dried bacteria were kept under nitrogen in the dark to await pigment extraction.

D. Pigment extraction and partition

a) Extraction

Methanol (b.r. 64.6 - 64.8°C) was chosen for extracting pigments from dried M. kansasii. It was considered desirable to avoid grinding or sonic rupture of bacterial cells since excessive amounts of extraneous cellular fractions might possibly interfere with subsequent division of the pigments into categories by partition between immiscible solvents. Methanol has been found satisfactory
for extracting pigments from various types of bacteria including mycobacteria (Sobin and Stahly, 1942; Schlegel, 1959; Starr and Stephens, 1964).

For complete pigment extraction approximately 60 ml of methanol were used per gram of dry bacteria. This volume was added step wise in several aliquots, each portion being removed when colored, and replaced with fresh methanol until no further pigment was being eluted. To speed pigment extraction, cells and methanol were heated to 50°C in a water bath. Other workers report finding no injurious effects on pigments if methanol is brought to the boiling point (Sobin and Stahly, 1942; Starr and Stephens, 1964), however, in these experiments the temperature was not permitted to exceed 50°C. Extraction was further accelerated by periodic shaking and the extraction process could be completed in approximately 2 hours. The pooled methanol extract was cooled, and freed of bacterial cells by centrifugation. Following extraction, the residual cellular mass was almost void of color. No visible difference could be detected between the cells originally pigmented, from growth in continuous light, and those nonpigmented which had been grown in the dark.

b) Partition of pigments

The carotenoid pigments most commonly found in bacteria can be separated into the following four groups by partition between immiscible solvents:
1. Hydrocarbons, such as beta carotene;
2. Esters;
3. Alcohols, such as xanthophyll;

The basis of the partition procedure employed was first described by Kuhn and Brockmann (1932), and later modified by Sobin and Stahly (1942), and Starr and Stephens (1964). The scheme is diagrammatically outlined in Figure 1 and the details are as follows:

Petroleum ether (b.r. 37.8 - 58.2°C) was added to the methanol cell extract. This formed a monophasic system which was broken by the addition of enough distilled water to give a 90 per cent alcoholic concentration. Two layers developed and the mixture was gently shaken in a separatory funnel. Severe agitation causes the formation of an emulsion. With separation of the petroleum ether and the 90 per cent methanol layers, further and final separation of the pigments contained therein was achieved by repeatedly extracting the petroleum ether layer with fresh portions of 90 per cent methanol, and likewise repeatedly extracting the methanol phase with fresh portions of petroleum ether. These reextracts, by partition, were appropriately pooled. The petroleum ether epiphase contained "hydrocarbons" and "esters", and the methanol hypophase contained "alcohols" and "acids".

The epiphase petroleum ether was repeatedly washed with distilled water, dehydrated with anhydrous sodium sulfate and evaporated to dryness in a small flash evaporator. Saponification was performed by the addition of 50 ml of 3 per cent KOH in methanol, and heating to 40°C for 3 hours. Following saponification, 50 ml of
METHANOL EXTRACT OF BACTERIA

Add an equal volume of petroleum ether (b.r. 37.8 to 58.2°C), followed by enough distilled water to give a methanol concentration of 90%. Shake gently in a separatory funnel.

PETROLEUM ETHER EPYPHASE
(contains "hydrocarbons" and "esters")

Evaporate to dryness. Saponify with 3% methanol KOH (40°C for 3 hr.). Add an equal volume of petroleum ether and enough water to give a 90% methanol concentration.

METHANOL POST-SAPONIFICATION EPYPHASE
(contains "hydrocarbons")

PETROLEUM ETHER POST-SAPONIFICATION EPYPHASE
(contains "hydrocarbons")

METHANOL HYPOPHASE
(contains "alcohols" and "acids")

Add methanol KOH to final concentration of 3% KOH, hold at 40°C for only 5 min. (mild saponification). Add an excess of diethyl ether, followed by water, and shake gently in a separatory funnel.

DIETHYL ETHER EPYPHASE
(contains freed "alcohols")

AQUEOUS HYPOPHASE
(contains salts of "acids")

Acidify, and extract freed "acids" with diethyl ether.

FIGURE 1 - Scheme for separation of bacterial carotenoid pigments
petroleum ether were added and then sufficient distilled water to obtain a 90 per cent alcohol concentration. This resulted in two layers which were gently shaken, and then separated. The petroleum ether epiphase was washed repeatedly with distilled water, dehydrated with anhydrous sodium sulfate, and reduced to dryness in a flash evaporator. This fraction contained hydrocarbons.

Diethyl ether was added to the alkaline methanol layer containing the freed carotenols. These de-esterified carotenoid alcohols were forced into the diethyl ether by the addition of distilled water. The ether layer was repeatedly washed with distilled water to remove methanol-KOH. It was then freed of water by preliminary treatment with a saturated solution of sodium chloride and secondly, anhydrous sodium sulfate. When free of water the fraction was reduced to dryness in a flash evaporator.

The original methanol hypophase obtained in the first treatment of cell extract contained alcohols and carotenoid acids. This mixture was subjected to mild saponification by adding enough KOH in methanol to give a final 3 per cent KOH concentration and then heating to 40°C for 5 minutes. When cooled, an excess of diethyl ether was added, followed by sufficient distilled water to form two layers. The mixture was gently shaken and the diethyl ether layer was removed. To insure complete separation the hypophase was repeatedly partitioned against fresh samples of diethyl ether. The diethyl ether, containing the carotenoid alcohols, was washed several times with distilled water, freed of water by treatment with a saturated solution of sodium chloride and then anhydrous sodium sulfate. It was evaporated to dryness in the flash evaporator.
Carotenoid acids, when present, remain in the aqueous layer in the form of their salts. These "acids" can be recovered by the addition of dilute hydrochloric acid followed by extraction with diethyl ether, drying, and flash evaporation.

Extractions and partition of pigments were performed in subdued light. In addition, separatory funnels were covered with black cloth.

All pigment fractions after being reduced to dryness in the flash evaporator were dissolved in small volumes of petroleum ether and transferred to three or four 4 ml vials. These were then placed within a dessicating jar containing both phosphorus pentoxide and shavings of paraffin. The jar was evacuated and filled with nitrogen three times. Fractions were maintained under these conditions until further studied.

E. Thin-layer chromatography

Pigments extracted from *M. kansasi* and partitioned between immiscible solvents were further subdivided by thin-layer chromatography. The thin-layer equipment used was manufactured by DESAGA, Heidelberg, Hauptstrasse 60, Germany. In North America this equipment is available through C.A. Brinkmann, Cantigague Road, Westbury, Long Island, N.Y. Canlab, (Canadian Laboratory Supplies) is a distributor in Canada.

The standard conditions for thin-layer chromatography which Stahl (1962) developed and outlined were normally adhered to. Whenever it was necessary to deviate from the standard conditions the modification is specifically stated.
a) Adsorbents

Three adsorbents served, either singly or in combination, as the basis for thin-layer preparations in these studies. The adsorbents were Silica Gel G, Kieselguhr G (diatomaceous earth), and calcium hydroxide.

Silica Gel G and Kieselguhr G are manufactured by E. Merck, Darmstadt, Germany, in accordance with Stahl's specifications. The calcium hydroxide is manufactured by Fisher Scientific Company.

Silica Gel G contained approximately 13 per cent calcium sulphate and had an average particle size of 5 - 25 microns. It was of the modified type produced by Merck since 1962 and differed from earlier preparations in both composition and particle size. It also possessed a faster setting time than did the original product.

Kieselguhr G had a particle size of not greater than 10 microns and a calcium sulphate content of approximately 15 per cent.

b) Spreading of layers

A DESAGA Model S-11 applicator was used to spread the adsorbents on glass plates. This model is equipped with an adjustable gate which permits regulation of the thin-layer thickness up to 2 mm. Two thicknesses of layers were used. For preliminary tests, layers of 250 microns were employed. When a suitable system of solvents and adsorbents was found to permit good separation of a specific fraction, greater quantities of the unknown could be harvested for spectrophotometric analysis when layers of 500 microns were prepared. Layer thickness refers to the gate setting of the applicator, and thus to the layer before
drying. Silica Gel G layers when dried are reduced to approximately one-half the wet thickness.

Glass plates of either 200 x 200 mm or 50 x 200 mm and uniform thickness were used. For spreading they were held in position by the DESAGA plastic aligning tray which could accommodate five 200 x 200 mm plates. Adhesion of the plates to the plastic tray was improved by placing a drop of water under each plate. This prevented movement of the plates during the spreading process and contributed to a more uniform layer.

The slurry for coating the plates was prepared in either a 500 ml or 1000 ml Erlenmeyer flask. Silica Gel G, Kieselguhr G, calcium hydroxide or mixtures of Silica Gel G and calcium hydroxide were added to the flask followed by distilled water in a ratio of 1:2 (weight:volume). Mixing within the flask was achieved by swirling the flask in a circular horizontal plane of approximately one foot diameter. It is essential to avoid bubble formation. The complete operation of mixing and spreading was completed within two minutes.

c) Drying and activation

A fan, at room temperature, was used to pre-dry freshly prepared layers. The plates changed in appearance from watery to opaque to white. For final drying and activation of the layer, the plates were arranged in a vertical position and placed in a preheated oven for 30 minutes at 110°C. The oven was equipped with forced air circulation.

DESAGA test mixture containing Butter Yellow, Sudan Red G and Indophenol was used to evaluate each batch of thin-layer plates prepared. Known
standards of alpha and beta carotene were also used to check the activity of layers.

d) Storage

Activated plates were protected from laboratory fumes and moisture. They were stored within a large glass jar containing phosphorus pentoxide and used within three days.

e) Application of sample

The sample was applied with a 1, 2 or 5 lambda pipette depending upon the requirements, and the point of application, unless otherwise stated, was 15 mm from the lower edge of the plate. The distance between points of application was 10 - 15 mm except when harvesting was anticipated from the developed layer. In these instances the points were only 5 mm apart.

f) Development

Chromatographic tanks were fitted with ground-glass covers. The chamber was saturated with the solvent by lining the inner surface of the tank with heavy filter paper. A small window was cut in one side of the paper lining to permit viewing of the developing layer.

Unless otherwise stated the solvent in the base of the tank was 5 mm in depth. The length of run from start to finish was 100 mm. The solvent systems will be described in the individual experiments.

g) Detection of fractions on developed layers

Spots were detected on developed layers by one or more of the following methods:
i) Visual examination for colored areas,

ii) Examination under ultraviolet light for fluorescent areas,

iii) Special staining techniques (vapor or spraying).

In these experiments the only visible color encountered was yellow. It varied in intensity and shade but was usually well defined against the white background. When only small trace amounts were present, they could be detected by employing layers containing a fluorescent indicator (lead-manganese-activated calcium silicate). The nonfluorescing carotenoids were then contrasted against the fluorescing background when viewed under a 2600 A ultraviolet light. These special layers will be discussed more fully later in this section.

Fluorescent fractions could be detected on regular layers prepared from Silica Gel G, Kieselguhr G and calcium hydroxide. Two wavelengths were used for the examination of layers; 2600 A and 3660 A.

Staining of chromatograms permitted detection of otherwise invisible fractions. It also increased the intensity, and gave a better outline to the faintly colored visible fractions. Two methods of staining were routinely employed; iodine vapor and spraying with a saturated solution of antimony trichloride in chloroform (Carr-Price Reagent).

To stain with iodine, plates were placed within a small airtight chamber containing a crucible of hot sand upon which a few crystals of iodine had been dropped. The position of the hydrocarbons was revealed as brown spots against the white background (Davies et al., 1961; Davies et al., 1963).
The second method of staining involved the spraying of plates with a saturated solution of antimony trichloride in chloroform and heating at 110°C for 15 - 20 minutes. Although less sensitive than the iodine stain, antimony trichloride had the advantage that different color reactions were given by different hydrocarbons. The sensitivity of the method was increased by viewing the stained plate under ultraviolet light (3660 Å). The stained hydrocarbons gave an orange fluorescence (Davies et al., 1963).

h) Recovery

Fractions were recovered from developed plates by carefully scraping the adsorbent from the desired areas. Scrapings were collected on clean glass plates and transferred to test tubes for elution of the fraction from the adsorbent. The adsorbents were removed by centrifugation and the supernatant was reduced to dryness in a flash evaporator. If less than 5 ml of a highly volatile solvent was involved it was evaporated under a stream of nitrogen. The concentrate was then rechromatographed, or if pure, was dissolved in an appropriate solvent for determination of its absorption spectrum.

When fractions were visible only under ultraviolet light, the plate was marked with the tip of a finely pointed instrument and harvested under visible light. For fractions which could only be detected on stained plates, narrow plates 50 x 200 mm were developed in parallel, stained, and placed adjacent to 200 x 200 mm plates to determine areas for "blind" harvesting. Staining of the plate from which a fraction had been harvested revealed the success or failure of the attempt. Usually bands were of constant Rf value on parallel plates. Furthermore, sufficient
visible or fluorescent fractions were normally present on plates to provide an adequate basis for accurately judging the position of specific bands on parallel plates.

Harvesting of bands from plates was performed as quickly as possible to prevent oxidation of fractions. It was noted that prolonged delay resulted in fading of visible yellow areas.

i) Impregnated thin-layers

Thin-layers of Kieselguhr G impregnated with liquid paraffin were prepared for the study of some fractions. In this procedure plates were first thoroughly dried at 100°C for 6 - 8 hours, and then impregnated by development of a blank run in an 8 per cent solution of paraffin oil (Fisher; white heavy, of 335/350 viscosity) in light petroleum (b.r. 60 - 80°C). Plates were removed when the solvent front was within 3 - 4 cm of the upper edge, and were allowed to dry for 24 hours. These layers were 250 microns in thickness. Test solutions were applied 10 - 15 mm from the edge of the plate in the unimpregnated region.

j) Commercially prepared thin-layers

Distillation Products Industries, Division of Eastman Kodak Company, in recent months manufactured a thin-layer under the trademark "Chromagram". The sheets are of standard size (200 x 200 mm) and are supported on a flexible poly (ethylene terephthalate) base over which an adsorbent layer of Silica Gel is deposited. Polyvinyl alcohol serves as a binder, and the layers are approximately 100 microns in thickness. Special layers are available (type K301R) containing lead-manganese-activated calcium silicate as a fluorescent indicator.
"Chromagram" layers, in the present research, were time-saving, convenient and economic in certain applications.

Firstly, these layers can be conveniently cut, with scissors, to any desired size. Strips, 1 inch in width and 4 inches in length were used in preliminary test runs to determine suitable solvent mixtures for the separation of unknown fractions. The small test strips were developed in test tubes 3 cm in diameter and lined with filter paper to obtain a saturated atmosphere. Test tubes were closed with small glass Petri dish bottoms. This procedure eliminated the preparation of numerous plates, and also proved a very economic, rapid and convenient way to investigate scores of solvents and solvent mixtures. Only 5 ml of solvent were required per test.

Secondly, the "Chromagram" layer type K301R containing a fluorescent indicator provided an additional rapid means of contrasting the nonfluorescing carotenoids against the fluorescent layer background when viewed under ultraviolet light of 2600 Å. The sheets were stable when protected from laboratory fumes and stored over a desiccant. Prior to use they were activated at 110°C for 30 minutes.

Commercially prepared layers were too thin to permit harvesting of fractions for spectrophotometric analysis.

F. Spectrophotometric analysis

Pigments isolated by thin-layer chromatography were examined spectrophotometrically. A Beckman Model DK-2A Ratio Recording spectrophotometer was used for scanning the spectrum from 600 μm to 270 μm.
Unless otherwise stated the following instrument settings were used to determine absorbance:

- Pathlength: 1 cm
- Cells: matched, standard silica, glass stoppered.
- Reference: same solvent as used in test; usually light petroleum ether (b.r. 37.8 - 58.2°C).
- Sensitivity control: 12
- Time constant selector: 0.2
- Light source: tungsten lamp above 340 μm
  hydrogen lamp below 340 μm
- Detector: photomultiplier X1
- Scanning time selector: 2
- Operating range: 0.1 absorbance (0 - 100% T)
- Chart paper: Beckman 12977 and 12978.

A Beckman DU spectrophotometer Model 2400 was used to determine absorbance of crude extracts or carotenoids at specific wavelengths. Standard curves and quantitative measurements of carotenoids extracted from M. kansasii were measured by extinction, and calculated on the basis $E_{1\text{cm}1\%}$ (450 μm) equalling 2500 (Goodwin and Jamikorn 1956; Schlegel, 1959).

Known standards of alpha and beta carotene (Appendix Section B-2) were used to check both spectrophotometers. The DK-2A accurately reproduced the per cent transmission curve of a standard Beckman 5 mm didymium filter.
IV. EXPERIMENTAL RESULTS
EXPERIMENTAL RESULTS

PART I

STUDIES ON CHROMOGENIC MYCOBACTERIA ISOLATED
FROM CLINICAL MATERIAL

1. GENERAL ASPECTS

A. Introduction

As mentioned in the general introduction, a considerable number of chromogenic mycobacteria had been encountered by the candidate prior to the present study. Almost 50 strains had been isolated from patients suspected of having tuberculosis, but it had not been possible to establish species or strain identity with the methods suggested for their classification. Frequently, repeat or duplicate cultures, under apparently identical conditions, displayed such marked differences as to affect their grouping within Runyon's scheme. All isolates had been maintained as stock cultures for possible further study, and when it was later suggested by Professor Reed that these strains serve as the basis for the present research project, they were revived culturally, reviewed clinically, and subjected to further study. The intensive search for chromogenic mycobacteria in clinical specimens was continued, and all pertinent data were tabulated.

During the period of this study (1962 - present) an additional 31 strains of chromogenic mycobacteria were isolated from clinical specimens. It is interesting that 11 of these recent isolates represent a variety which had not previously been recognized in Newfoundland. The manner in which they were
detected and how they differ from other mycobacteria will be discussed.

B. Incidence

During the eight year period 1957 - 1964 a total of 69,556 specimens were cultured for *M. tuberculosis* at the Provincial Public Health Laboratory, St. John's, Newfoundland. Of this total 3,044 yielded a growth of acid-fast bacilli, 2,964 of which were identified as *M. tuberculosis*. The remaining 80 cultures, representing 2.63 per cent of the positives, were slow growing chromogenic mycobacteria (Table III).

**TABLE III**

CULTURES POSITIVE FOR MYCOBACTERIA OVER AN EIGHT YEAR PERIOD

<table>
<thead>
<tr>
<th>Year</th>
<th>Number of specimens cultured for <em>M. tuberculosis</em></th>
<th>Number of specimens positive for typical <em>M. tuberculosis</em></th>
<th>Number of specimens positive for chromogenic mycobacteria</th>
</tr>
</thead>
<tbody>
<tr>
<td>1957</td>
<td>7,888</td>
<td>373</td>
<td>4</td>
</tr>
<tr>
<td>1958</td>
<td>8,832</td>
<td>514</td>
<td>10</td>
</tr>
<tr>
<td>1959</td>
<td>8,942</td>
<td>417</td>
<td>7</td>
</tr>
<tr>
<td>1960</td>
<td>9,893</td>
<td>377</td>
<td>15</td>
</tr>
<tr>
<td>1961</td>
<td>8,545</td>
<td>351</td>
<td>13</td>
</tr>
<tr>
<td>1962</td>
<td>8,812</td>
<td>335</td>
<td>12</td>
</tr>
<tr>
<td>1963</td>
<td>8,920</td>
<td>316</td>
<td>7</td>
</tr>
<tr>
<td>1964</td>
<td>7,734</td>
<td>281</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td><strong>69,556</strong></td>
<td><strong>2,964</strong></td>
<td><strong>80</strong></td>
</tr>
</tbody>
</table>
C. Age and sex of patients

Figures for the age of patients are available for the years 1961 - 1964 only. The average age is calculated to be 44 years for females and 52 years for males.

A break down of the patients according to sex, and including all eight years indicates that of the total 80 cases, 61 (76%) were male and 19 (24%) were female (Table IV).

**TABLE IV**

<table>
<thead>
<tr>
<th>Year</th>
<th>Female</th>
<th>Male</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>1957</td>
<td>1</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>1958</td>
<td>1</td>
<td>9</td>
<td>10</td>
</tr>
<tr>
<td>1959</td>
<td>1</td>
<td>6</td>
<td>7</td>
</tr>
<tr>
<td>1960</td>
<td>4</td>
<td>11</td>
<td>15</td>
</tr>
<tr>
<td>1961</td>
<td>1</td>
<td>6</td>
<td>7</td>
</tr>
<tr>
<td>1962</td>
<td>2</td>
<td>10</td>
<td>12</td>
</tr>
<tr>
<td>1963</td>
<td>4</td>
<td>3</td>
<td>7</td>
</tr>
<tr>
<td>1964</td>
<td>4</td>
<td>8</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td>19</td>
<td>61</td>
<td>80</td>
</tr>
</tbody>
</table>

D. Type of specimens yielding chromogenic mycobacteria

Practically all the chromogenic mycobacteria were isolated from specimens of sputum and urine. The two exceptions were single isolations
from a gastric washing and an endometrial biopsy (Table V). The percentage of positive cultures due to chromogenic mycobacteria, as related to specimen type, is presented in Table VI.

**TABLE V**

**TYPES OF SPECIMENS YIELDING GROWTH OF CHROMOGENIC MYCOBACTERIA**

<table>
<thead>
<tr>
<th>Year</th>
<th>Sputum</th>
<th>Urine</th>
<th>Gastric washing</th>
<th>Misc.</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>1957</td>
<td>1</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td>4</td>
</tr>
<tr>
<td>1958</td>
<td>6</td>
<td>3</td>
<td>1</td>
<td>0</td>
<td>10</td>
</tr>
<tr>
<td>1959</td>
<td>3</td>
<td>4</td>
<td>0</td>
<td>0</td>
<td>7</td>
</tr>
<tr>
<td>1960</td>
<td>13</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>15</td>
</tr>
<tr>
<td>1961</td>
<td>6</td>
<td>7</td>
<td>0</td>
<td>0</td>
<td>13</td>
</tr>
<tr>
<td>1962</td>
<td>9</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td>12</td>
</tr>
<tr>
<td>1963</td>
<td>5</td>
<td>1</td>
<td>0</td>
<td>1*</td>
<td>7</td>
</tr>
<tr>
<td>1964</td>
<td>11</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td>54</td>
<td>24</td>
<td>1</td>
<td>1</td>
<td>80</td>
</tr>
</tbody>
</table>

*Endometrial biopsy

**TABLE VI**

**SPECIMENS POSITIVE OVER AN EIGHT YEAR PERIOD**

<table>
<thead>
<tr>
<th>Specimen</th>
<th>Number</th>
<th>Positive for M. tuberculosis</th>
<th>Positive for chromogenic mycobacteria</th>
<th>Percent chromogens</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sputum</td>
<td>46,711</td>
<td>1,901</td>
<td>54</td>
<td>2.76</td>
</tr>
<tr>
<td>Urine</td>
<td>15,073</td>
<td>741</td>
<td>24</td>
<td>3.14</td>
</tr>
<tr>
<td>Gastric washing</td>
<td>1,360</td>
<td>105</td>
<td>1</td>
<td>0.94</td>
</tr>
<tr>
<td>Misc.</td>
<td>6,422</td>
<td>217</td>
<td>1</td>
<td>0.46</td>
</tr>
<tr>
<td></td>
<td>69,566</td>
<td>2,964</td>
<td>80</td>
<td>2.63</td>
</tr>
</tbody>
</table>
E. Rate of growth on primary isolation

All of the 80 chromogenic mycobacteria isolated were slow growing organisms. Not one "rapid grower" was encountered in the examination of 69,566 specimens.

Records for the 44 chromogenic strains isolated during the past 4 years show that the average time of incubation before growth could be detected was greater than 6 weeks. This is considerably longer than the period required for the primary growth of M. tuberculosis. Nine of the 44 strains required a full eight weeks to show visible growth. A further nine grew following seven weeks of incubation, ten required six weeks, nine five weeks, six four weeks, and the fastest growing strain required three weeks of incubation.

The primary growth rate was checked for 100 consecutively positive TB cultures. The average culture of M. tuberculosis developed in a period of four weeks and two days.

F. Animal virulence

Each strain of chromogenic mycobacteria isolated was tested for pathogenicity. Young, actively growing cultures were suspended in saline and inoculated subcutaneously into guinea pigs. The inoculum consisted of 1 ml of a suspension of organisms standardized to McFarlane tube No. 4. The guinea pigs were retained for two months before being sacrificed. At autopsy all organs were found to be normal. On several occasions acid-fast bacilli were observed in smears prepared from small abscesses in the omentum or from the site of injection. Only on one occasion were the organisms recovered on
cultures routinely prepared from material collected at time of autopsy. None of the strains led to progressive infection.

G. Sensitivity to antituberculosis agents

Chromogenic mycobacteria generally possess significant levels of resistance to PAS, INH and streptomycin. This even applies to primary isolations from untreated patients. In this respect these organisms differ from _M. tuberculosis_ which is usually inhibited by considerably lower levels of these agents.

Sensitivity tests were routinely carried out for each primary isolation of acid-fast bacilli. Three strengths of each drug, all within the range of clinical interpretation, were used. These concentrations (Appendix C-2) thus aided in the detection of resistant chromogenic varieties.

Chromogenic mycobacteria were usually resistant to as much as 100 ug/ml of streptomycin, 25 ug/ml of PAS, and 100 ug/ml of INH. The results of sensitivity tests for fifteen strains subjected to wide ranges of all three agents are presented in Table VII.

2. RECOGNITION AND GROUPING OF CHROMOGENIC MYCOBACTERIA

A. Introduction

The recognition of chromogenic mycobacteria, in some instances may be simple and obvious. However, highly pigmented smooth colonies differ so noticeably from _M. tuberculosis_ that they are frequently mistaken for contaminants and the cultures are discarded without microscopic examination.
TABLE VII
RESULTS OF SENSITIVITY TESTS USING STREPTOMYCIN, PAS, AND INH ON FIFTEEN STRAINS OF CHROMOGENIC MYCOBACTERIA

Resistant to ug/ml (Sauton medium)

<table>
<thead>
<tr>
<th>Strain No.</th>
<th>Streptomycin</th>
<th>PAS</th>
<th>INH</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5 10 25 50 100 250 500 &gt;750</td>
<td>10 25 50 100 200 300 &gt;400</td>
<td>25 50 100 &gt;200</td>
</tr>
<tr>
<td>1</td>
<td>x</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>x x x x x x</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>x x x x x x</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>x x x x x x</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>x x x x x x</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>x x x x x x</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>x x x x x x</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>x x x x x x</td>
<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>x x x x x x</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>x x x x x x</td>
<td></td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>x x x x x x</td>
<td></td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>x x x x x x</td>
<td></td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>x x x x x x</td>
<td></td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>x x x x x x</td>
<td></td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>x x x x x x</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Even the experienced bacteriologist may encounter difficulty due to the fact that photochromogenic and scotochromogenic mycobacteria may or may not produce pigmented growth depending upon heretofore unrecognized factors. It will be shown that members of Runyon's Groups I and II frequently produce nonpigmented growth when cultured by conventional bacteriological methods.

A second problem concerns the recognition of nonphotochromogenic mycobacteria (Group III). Members of this Group are described by Runyon as producers of little or no pigment. At times these strains closely resemble *M. tuberculosis*. The advantages of a method which would facilitate the recognition and differentiation of all chromogenic mycobacteria are obvious.

The factors influencing pigment development are discussed in Part II of Experimental Results.

### B. Inconstant pigmentation of individual strains

#### a) Isolates from clinical specimens

Prior to this study the candidate had become aware that considerable variation occurred in individual strains of pigmented mycobacteria. On occasions highly pigmented colonies, on repeated subcultures, produced all levels of pigmentation varying from deep orange to buff, or even so-called pigmentless growth similar to that normally produced by *M. tuberculosis*. The reverse had also been noted. Subcultures of nonpigmented colonies on occasions yielded all levels of pigment development ranging up to deep orange.
Using Runyon's scheme, repeated attempts were made in the beginning of this investigation to group some of the clinical isolates on hand. Fifteen strains which had been isolated during 1960 and had been maintained on Lowenstein-Jensen medium were studied. Each strain was inoculated on three different occasions onto 6 Lowenstein-Jensen and 6 Dorset medium slants. Half of the cultures, three of each strain and each type of medium, was incubated in the presence of light at 37°C for 14 days. These cultures were positioned 45 cm from two 15-Watt Westinghouse "cool white" tubes (F15T8/CW). The remaining half was incubated at the same temperature but in the absence of light.

So much variation in pigmentation occurred in the cultures of the same strain, whether grown in the light or in the dark, that the effect of light on pigmentation was impossible to assess. It was noted, however, that the majority of cultures, both those grown in the light and in the dark, when left standing at room temperature following the 14 day incubation period showed a gradual deepening of their color. Some cultures, on the other hand, including members of various strains, remained a pale buff similar in color to the control tubes of _M. tuberculosis_ H37Rv. It appeared that two strains under study, on two of three occasions, produced more pigment in the dark than in the presence of light, but this finding was not consistently reproducible.

These first results of a systematic study, coupled with the recollection of earlier experiences involving the same strains, made it easy to understand why the grouping of specific strains, based on pigmentation, at times differed in the hands of different investigators. From reports in the literature it was
also evident that other workers had experienced similar difficulties.

b) Stock cultures

When the present study started, stock cultures representing members of each of Runyon's groups of chromogenic mycobacteria were obtained from Dr. Runyon, and the Type Culture of *M. kansasii* was secured from the American Type Culture Collection (ATCC 12478, Bostrom). In addition a photochromogen and five scotochromogens were obtained from Dr. Siebenmann, Connaught Laboratories, Toronto. Each of these stock strains was subcultured to 10 tubes of Lowenstein-Jensen and 10 tubes of Dorset media. The cultures were incubated at 37°C in lightproof containers for 14 days before being exposed to light. Photochromogenicity testing was performed as detailed in "Materials and Methods". The photoactivated cultures were reincubated in the dark for a period of 24 hours and then inspected for pigment development.

The extreme variation which frequently occurred in duplicate cultures is depicted in the color photographs. In Figure 2, two cultures of *M. kansasii*, ATCC 12478 are shown following photochromogenicity testing. Both tubes were identical in (a) composition of glass in tube (same make); (b) Lowenstein-Jensen medium (same batch); (c) inoculum; (d) incubation conditions; (e) exposure to light (photochromogenicity test); and (f) age. As can be seen, deep yellow pigment developed in one tube whereas the growth in the other lacked visible pigment. The "Parafilm M" seal was applied to the tubes after completion of tests and was intended only as an added barrier to prevent possible moisture loss during storage.
Figure 2  
M. kansasii (ATCC 12478). Both cultures were prepared on tubes from the same batch of Lowenstein-Jensen medium. They represent identical brand of test tubes, source and size of inoculum, incubation conditions, photochromogenicity testing and age (16 days).

NOTE: The "Parafilm M" seal was applied after growth and photochromogenicity testing, and was intended to prevent drying of the cultures during storage.
Where pigment was absent growth was also appreciably reduced, however, harvest of the growth from the surface of these slants revealed that the growth, even when massed, was practically pigmentless.

From these results one could, on the basis of the subculture involved, assign *M. kansasii*, ATCC 12478 (Bostrom) to either Runyon Group I or Group III. No logical explanation was available to account for this difference. It was known that a World Health Organization-assisted study had found the degree of growth of mycobacteria to be seriously affected by the quality of the glass tubes used (Sula et al., 1960), but this was eliminated in the present study by employing tubes of the same make in parallel tests. There appeared to be a twofold problem; growth was being partially inhibited, and what growth did occur failed to react to the photochromogenicity test. The photochromogenic culture supplied by Dr. Siebenmann ("Chadwick" strain isolated by Hambleton from a case of pulmonary tuberculosis at the Beck Memorial Sanatorium, London, Ontario) also failed to react to the photochromogenicity test when first subcultured. It was observed to produce pigment only if grown under continuous light.

In another series of tubes a scotochromogen provided by Runyon, and designated as strain P-5, was found to give similar variation in pigment production when grown in the dark for 2 weeks. Again identical tubes, media, inoculum and incubation conditions were employed. Figure 3 indicates how this scotochromogen (Runyon P-5) can be assigned to either Group II or Group III. Group I was elimi-
Unclassified mycobacterium, (Runyon P-5 scotochromogen). Both cultures were prepared on tubes from the same batch of Lowenstein-Jensen medium. They represent identical brand of test tubes, source and size of inoculum, incubation conditions and age (16 days).

NOTE: The "Parafilm M" seal was applied after growth, and was intended to prevent drying of the culture during storage.
nated on the basis that growth in the nonpigmented tube failed to give a positive photochromogenicity test. As with M. kansasii, the ratio of pigment to growth for this scotochromogen was much less for the tubes with sparse growth. Scotochromogenic strains provided by Dr. Siebenmann (strains "Foster", "Moore", "Rosenberg", "Lange" and "Steiner") all showed variation in pigmentation as did also the stock strain of M. phlei maintained in the Department of Bacteriology, McGill University.

C. A special problem with nonphotochromogens

Members of this particular group can be quite difficult to recognize and most clinical bacteriologists are still unfamiliar with the pitfalls which contribute to error in the grouping of these strains. There are two distinct problems.

First, and contrary to Runyon's definition, the pigmentation of these strains is not always weak or lacking. Strains of nonphotochromogenic mycobacteria kindly supplied by Dr. Runyon and designated by him as P-2 and P-7, have been found in the present study to produce pigment quantitatively equal with that of certain scotochromogens. In personal correspondence with Dr. Runyon he stated that, "yellow pigment seems to increase in Group III strains after continued laboratory handling." This same situation has, unfortunately, also been the finding with certain strains isolated in Newfoundland. Some strains which were weakly pigmented on primary culture showed increased pigment development within a few subcultures. This prevented differentiation of these strains from members of Group II.
Figure 4 shows the extent of pigment development in Runyon's stock cultures of nonphotochromogenic strains P-2 and P-7. Cultures when photographed were 3 weeks old. In a series of subcultures, P-7 always produced more pigment than did P-2.

The second problem concerns recognition of these strains when the described nonpigmented varieties are encountered. These organisms are occasionally quite rough on primary culture and are easily confused with *M. tuberculosis*. The chance of error is considerably increased when growth occurs in, or adjacent to, water of condensation at the butt of slants. The experienced workers know that the colonial morphology of *M. tuberculosis* when grown on moist slants differs from the textbook description of dry, raised, bread-crumble-like, and frequently appears smooth and less elevated.

Until the present study, nonpigmented strains of mycobacteria other than *M. tuberculosis* had not been recognized in Newfoundland, but the detection of 11 such strains within the past two years would indicate that they had previously been encountered and that they had been considered to be *M. tuberculosis*. This variety was recognized as differing from *M. tuberculosis* because of the type of growth produced in Sauton medium (to be described in the following paragraphs). They were also proven to be niacin negative, nonpathogenic for the guinea pig and resistant to high levels of PAS, INH and streptomycin.

The 11 strains isolated to date in Newfoundland differ from the Group III stock cultures provided by Runyon since all cultures remain nonpigmented despite continuous laboratory subculturing.
Figure 4  Cultures of pigmented nonphotochromogenic mycobacteria on Dorset medium (age 3 weeks).
D. A simple, reliable method for the detection of all chromogenic mycobacteria

In the author's laboratory it had been routine practice for seven years to perform sensitivity tests on all primary isolates of acid-fast bacilli from clinical specimens. Several methods and modifications of sensitivity testing involving the use of solid media, either by incorporating the therapeutic agents or by addition to the surface in the form of sensitivity discs or solutions, had been investigated. Each method and modification possessed shortcomings, and difficulty was frequently encountered in trying to reproduce test results. When a new technique was proposed by the Laboratory of Hygiene, Ottawa, for the sensitivity testing of M. tuberculosis (Dr. Greenberg, personal communication) it was investigated for possible advantages. The new method employed a modified Sauton medium containing buffer and serum (Appendix C - 2).

With this medium it was subsequently observed in the testing of many strains that all chromogenic mycobacteria could be detected, not by their level of resistance, but by the type of growth they produced in the control tubes. M. tuberculosis always showed the formation of heavy flaky growth which settled leaving a clear supernatant, whereas all other mycobacteria encountered produced a fairly dispersed uniform turbidity. All 80 strains of chromogenic mycobacteria isolated since 1957 were subcultured to this medium, as were also all the stock cultures listed under "strains of mycobacteria" in the "Materials and Methods" section of this thesis. Only M. tuberculosis and
M. bovis BCG yielded a flaky sedimented growth with a clear supernatant medium in 14 day cultures. Photochromogenic strains (including the type culture ATCC 12478), scotochromogenic strains, and nonphotochromogenic strains usually produced sufficient characteristic growth of uniform turbidity to differentiate them from M. tuberculosis within 5 - 7 days. Final readings, however, made following 14 days of incubation at 37°C were always sharply defined.

In all instances strains which produced uniform turbidity in Sauton medium were negative in the niacin test and were nonpathogenic for guinea pigs. These strains were also generally resistant to 25 ug/ml of streptomycin, 25 ug/ml of PAS, and 10 ug/ml of INH used as highest levels in routine sensitivity tests.

Over 1000 strains of mycobacteria yielding flaky sedimented growth and a clear supernatant in modified Sauton medium were tested for niacin production (Appendix C - 1). Only two cultures were observed to give negative niacin reactions. These two were possibly either M. bovis or niacin negative M. tuberculosis. The latter is possible according to the reports of other workers.

The subculturing of all isolated acid-fast organisms from clinical specimens into modified Sauton medium will allow the detection of all chromogenic mycobacteria including the nonpigmented varieties. This simple procedure, along with niacin testing and temperature growth studies, constitutes an adequate routine for the diagnostic laboratory. For convenience, temperature growth studies, as outlined in the section on "Methods", can be performed in modified Sauton medium. These tubes may then also serve to reveal the type of growth developing.
3. GROWTH STUDIES

A. Introduction

The slow growth rate of pathogenic mycobacteria seriously limits certain types of studies. Considerable time is involved in securing adequate bacterial harvests, and the metabolic rates of these organisms render impractical many types of experiments normally employed in the study of fast growing bacteria. Furthermore, the risk of exposure to pathogenic mycobacteria prohibits the use of many methods of manipulation routinely used in the study of nonpathogenic bacteria.

It was realized early in preparing for pigment studies that the cultural procedure of choice should be one yielding maximum growth in minimum time, as well as one which would support good pigment production. A review of the literature revealed little agreement concerning growth rate studies on mycobacteria. In addition, one could not assume that the growth characteristics of these unclassified slow growing mycobacteria would in any way parallel those of M. tuberculosis, BCG, or fast growing saprophytic strains.

In determining the cultural method best suited for the study of pigment in unclassified mycobacteria the feasibility of employing fluid or solid media was considered. Since it was known that individual strains could be highly variable in both growth rate and pigment production, it was felt that the simplest and most consistent medium, both qualitatively and quantitatively, would perhaps help to eliminate a number of variables. Both Lowenstein-Jensen and Dorset media, commonly used in the cultivation of mycobacteria, were con-
sidered unsuitable for studies involving pigment characterization. They both contain egg yolk, and Lowenstein-Jensen contains malachite green.

B. Growth in fluid medium

At first it was hoped that a suitable fluid medium could be employed which would support luxurious growth of these organisms, and also permit calculation of bacterial mass (by weight) from optical density readings. Schaefer et al. (1949) had observed such a relationship between optical density and bacterial mass when growing *M. tuberculosis* in Dubos medium, and Dubos (Dubos and Davis, 1946) reported that the Tween 80 in his medium changed the growth of *M. tuberculosis* from that of large compact granules to a dispersed growth in which isolated and microscopic loose clumps prevailed. It was hoped that this would also be true for the unclassified mycobacteria, since in the present study it was originally felt that dispersed growth in liquid medium would be more uniform than surface (pellicle) or aggregative growth. The influence of aeration also had to be considered.

Dubos and Sauton media, both widely used in culturing mycobacteria, were chosen as fluid media for the present growth studies (Appendix A - 3 and A - 5).

a) Dubos medium

Cultures investigated represented Runyon's Groups I, II and III. The strains were those supplied by Dr. Runyon and included two Group I photochromogens (P-1 and P-8), two Group II scotochromogens (P-5 and P-6), and three Group III nonphotochromogens (P-2, P-7 and P-25). In addition two
strains of *M. kansasii* (ATCC 12478 and 12479) were tested.

Dubos medium was prepared and distributed in 50 ml amounts into specially constructed flat bottom 250 ml Florence form flasks fitted with side arm test tubes. These special side arm flasks were constructed so that when 50 ml of medium were added to the flask, the unit could be tilted at right angles without danger of spilling, i.e. 50 ml were retained within the test tube and the adjacent portion of the flask without the level of the medium approaching the neck opening of the flask. This arrangement permitted the reading of optical density in a spectrophotometer (Bausch and Lomb Spectronic "20") at various stages of growth without removing the cultures from the flasks. The test tubes serving as side arms measured 150 x 18 mm O.D. and were optically calibrated using cobalt chloride solution (22 - 23 gm in 1 liter of 1 per cent HCl) as suggested in the Bausch and Lomb manual. The tubes were marked so that, when attached to the flasks, identical positioning could be used throughout a series of optical density readings. (The candidate gratefully acknowledges the technical assistance of Mr. T. Salo in the preparation of these side-arm flasks).

Inocula for these tests consisted of 0.5 ml of an adapted 7 day old culture in Dubos fluid medium. For most strains this gave an optical density reading of 0.010 immediately following inoculation.

Shake cultures were securely fastened to a "New Brunswick" incubator-rotator with a speed of 100 r.p.m., a stroke diameter of approximately 2 inches, and a temperature of 37°C. Stationary cultures were incubated at 37°C and were agitated only as necessary for performing optical density readings.
Both shake and stationary cultures were incubated in the dark and were exposed to light only during readings of optical density. Optical density readings were taken at 660 μ. Uninoculated Dubos medium at this wavelength, when compared with water, was found to have 97.5% transmittance.

**Results**

Optical density readings performed during growth of the various mycobacterial strains are recorded for the shaken cultures in Table VIII, and in Table IX for the stationary cultures.

It became obvious from the early readings that poor correlation existed between optical density and the amount of bacterial growth present. This was particularly true for the shaken cultures. Practically all shaken cultures showed the development of a granular type of growth consisting of cellular aggregates which increased in size to form particles visible to the naked eye. The clumping of cells caused a clearing of the medium with resulting decrease in optical density. In contrast to this, most stationary cultures developed either uniform turbidity, flaky growth, or a very fine type of granular growth. Flaky and fine granular growth in stationary cultures also prevented the use of optical density as an accurate means of determining bacterial growth.

Granular type growth in shaken cultures usually developed in 40 - 48 hours, following which it was not uncommon to record either very slight increases, or even actual decreases, in optical density readings despite the obvious visible increase in growth.

The lack of agreement observed between the amount of visible growth
TABLE VIII

OPTICAL DENSITIES AT 660 μm OF STRAINS OF MYCOBACTERIA IN LIQUID DUBOS-TWEEN 80 MEDIUM (shaken cultures)

<table>
<thead>
<tr>
<th>STRAINS OF MYCOBACTERIA</th>
<th>0 hr.</th>
<th>24 hr.</th>
<th>40 hr.</th>
<th>48 hr.</th>
<th>64 hr.</th>
<th>72 hr.</th>
<th>88 hr.</th>
<th>96 hr.</th>
<th>112 hr.</th>
<th>160 hr.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Runyon - 1</td>
<td>.040</td>
<td>.050</td>
<td>.050</td>
<td>.020</td>
<td>.030</td>
<td>.075</td>
<td>.090</td>
<td>.080</td>
<td>.085</td>
<td>.130</td>
</tr>
<tr>
<td>Runyon - 2</td>
<td>.010</td>
<td>.020</td>
<td>.045</td>
<td>.065</td>
<td>.090</td>
<td>.070</td>
<td>.070</td>
<td>.075</td>
<td>.080</td>
<td>.110</td>
</tr>
<tr>
<td>Runyon - 5</td>
<td>.010</td>
<td>.015</td>
<td>.025</td>
<td>.030</td>
<td>.050</td>
<td>.030</td>
<td>.050</td>
<td>.050</td>
<td>.060</td>
<td>.110</td>
</tr>
<tr>
<td>Runyon - 6</td>
<td>.010</td>
<td>.025</td>
<td>.040</td>
<td>.055</td>
<td>.065</td>
<td>.045</td>
<td>.055</td>
<td>.060</td>
<td>.060</td>
<td>.110</td>
</tr>
<tr>
<td>Runyon - 7</td>
<td>.010</td>
<td>.025</td>
<td>.050</td>
<td>.070</td>
<td>.080</td>
<td>.050</td>
<td>.060</td>
<td>.070</td>
<td>.070</td>
<td>.120</td>
</tr>
<tr>
<td>Runyon - 8</td>
<td>.020</td>
<td>.060</td>
<td>.080</td>
<td>.070</td>
<td>.080</td>
<td>.080</td>
<td>.090</td>
<td>.090</td>
<td>.100</td>
<td>....</td>
</tr>
<tr>
<td>Runyon - 25</td>
<td>.020</td>
<td>.140</td>
<td>.340</td>
<td>.510</td>
<td>.750</td>
<td>.740</td>
<td>.950</td>
<td>1.200</td>
<td>1.500</td>
<td>1.700</td>
</tr>
<tr>
<td>ATCC 12478</td>
<td>.010</td>
<td>.020</td>
<td>.075</td>
<td>.110</td>
<td>.115</td>
<td>.130</td>
<td>.145</td>
<td>.145</td>
<td>.170</td>
<td>.250</td>
</tr>
<tr>
<td>ATCC 12479</td>
<td>.010</td>
<td>.015</td>
<td>.030</td>
<td>.055</td>
<td>.070</td>
<td>.090</td>
<td>.110</td>
<td>.110</td>
<td>.125</td>
<td>.240</td>
</tr>
</tbody>
</table>
TABLE IX

OPTICAL DENSITIES AT 660 \( \mu \) OF STRAINS OF MYCOBACTERIA IN LIQUID DUBOS-TWEEN 80 MEDIUM (stationary cultures)

<table>
<thead>
<tr>
<th>STRAINS OF MYCOBACTERIA</th>
<th>0 hr.</th>
<th>24 hr.</th>
<th>40 hr.</th>
<th>48 hr.</th>
<th>64 hr.</th>
<th>72 hr.</th>
<th>88 hr.</th>
<th>96 hr.</th>
<th>112 hr.</th>
<th>160 hr.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Runyon - 1</td>
<td>.040</td>
<td>.060</td>
<td>.080</td>
<td>.080</td>
<td>.050</td>
<td>.120</td>
<td>.120</td>
<td>.115</td>
<td>.120</td>
<td>.140</td>
</tr>
<tr>
<td>Runyon - 2</td>
<td>.010</td>
<td>.020</td>
<td>.075</td>
<td>.075</td>
<td>.110</td>
<td>.145</td>
<td>.170</td>
<td>.180</td>
<td>.220</td>
<td>.415</td>
</tr>
<tr>
<td>Runyon - 5</td>
<td>.010</td>
<td>.030</td>
<td>.060</td>
<td>.045</td>
<td>.060</td>
<td>.075</td>
<td>.120</td>
<td>.120</td>
<td>.140</td>
<td>.250</td>
</tr>
<tr>
<td>Runyon - 6</td>
<td>.010</td>
<td>.040</td>
<td>.080</td>
<td>.055</td>
<td>.085</td>
<td>.105</td>
<td>.110</td>
<td>.135</td>
<td>.140</td>
<td>.170</td>
</tr>
<tr>
<td>Runyon - 7</td>
<td>.030</td>
<td>.040</td>
<td>.105</td>
<td>.080</td>
<td>.105</td>
<td>.165</td>
<td>.140</td>
<td>.180</td>
<td>.190</td>
<td>.280</td>
</tr>
<tr>
<td>Runyon - 8</td>
<td>.010</td>
<td>.065</td>
<td>.135</td>
<td>.170</td>
<td>.250</td>
<td>.265</td>
<td>.320</td>
<td>.340</td>
<td>.400</td>
<td>.570</td>
</tr>
<tr>
<td>Runyon - 25</td>
<td>.010</td>
<td>.100</td>
<td>.240</td>
<td>.285</td>
<td>.360</td>
<td>.330</td>
<td>.410</td>
<td>.440</td>
<td>.480</td>
<td>.620</td>
</tr>
<tr>
<td>ATCC 12478</td>
<td>.010</td>
<td>.020</td>
<td>.075</td>
<td>.120</td>
<td>.120</td>
<td>.145</td>
<td>.180</td>
<td>.220</td>
<td>.260</td>
<td>.280</td>
</tr>
<tr>
<td>ATCC 12479</td>
<td>.010</td>
<td>.030</td>
<td>.030</td>
<td>.110</td>
<td>.110</td>
<td>.120</td>
<td>.135</td>
<td>.190</td>
<td>.250</td>
<td>.280</td>
</tr>
</tbody>
</table>
developing in shaken cultures and the optical density readings was confirmed by determining dry weights. Following 14 days the cultures were killed with formaldehyde, washed three times with distilled water, and dried over phosphorus pentoxide. Bacterial yields (dry weight) are given in Table X. Optical densities at 160 hours incubation are included for comparison. As previously mentioned, optical density readings beyond 160 hours of incubation were pointless because the aggregation of bacteria in the shaken cultures caused clearing of the medium with reduction in optical density despite increased bacterial growth.

From experimental data it was impossible to differentiate between photochromogens, scotochromogens, and nonphotochromogens on the basis of growth rates. Marked variations were even noted between members within each Group.

b) Sauton medium

The second fluid medium investigated was that of Sauton (Appendix A - 5). It was felt that this medium might have certain advantages over Dubos medium for the pigment studies since it was a synthetic medium lacking albumin or serum, and also lacking the viscous yellow fluid "Tween 80".

Experiments, as already outlined for Dubos medium, were attempted using Sauton medium. It was found that growth of all strains was considerably slower in this medium. To obtain sufficient growth the incubation time had to be extended to four weeks. As with Dubos medium, shaken cultures showed more visible growth than stationary ones. Growth in the shaken flasks was granular
TABLE X

GROWTH OF MYCOBACTERIA IN DUBOS-TWEEN 80 MEDIUM
(shaken and stationary cultures)

<table>
<thead>
<tr>
<th>CULTURE</th>
<th>Optical Density 160 hr.</th>
<th>mg dry wt./50 ml Dubos medium 14 days</th>
</tr>
</thead>
<tbody>
<tr>
<td>Runyon 1 (shaken)</td>
<td>.130</td>
<td>15</td>
</tr>
<tr>
<td>Runyon 1 (stationary)</td>
<td>.140</td>
<td>11</td>
</tr>
<tr>
<td>Runyon 5 (shaken)</td>
<td>.110</td>
<td>17</td>
</tr>
<tr>
<td>Runyon 5 (stationary)</td>
<td>.250</td>
<td>12</td>
</tr>
<tr>
<td>Runyon 7 (shaken)</td>
<td>.120</td>
<td>16</td>
</tr>
<tr>
<td>Runyon 7 (stationary)</td>
<td>.280</td>
<td>13</td>
</tr>
<tr>
<td>ATCC 12478 (shaken)</td>
<td>.250</td>
<td>18</td>
</tr>
<tr>
<td>ATCC 12478 (stationary)</td>
<td>.280</td>
<td>13</td>
</tr>
</tbody>
</table>
but finer than that obtained in Dubos medium.

Stationary cultures in Sauton medium were found to develop a well dispersed type of growth except in instances where the inoculum was "floated" on the surface of the medium. If the latter flasks or tubes were permitted to remain untouched during incubation, a thick pellicle developed which, in the case of photochromogens grown in the presence of light, developed a deep yellow-orange pigment. This pellicle development on Sauton medium was subsequently chosen as the best method to obtain maximum growth of the deeply pigmented photochromogenic strain of *M. kansasii*. Maximum yield of bacterial growth per unit volume of medium, and good pigment development occurred when *M. kansasii* (ATCC 12478) was grown in loosely capped test tubes (20 x 125 mm) containing 10 ml of Sauton medium. Cultures in Erlenmeyer flasks yielded less bacterial mass per unit volume of medium than did the tube cultures. When using flasks, the best yields were obtained when 500 ml flasks contained 100 ml of medium. Typical results for four batches of *M. kansasii* are recorded in Table XI. As great as 6.28 mg of bacterial dry weight were obtained per ml of Sauton medium when test tubes were employed, whereas with Erlenmeyer flasks only 4.22 mg bacterial dry weight were obtained. The dry bacterial weight for *M. kansasii* represented 16.1 - 20.56% of the bacterial wet weight. Harvesting, washing, centrifuging and drying of bacterial cells is outlined in the "Methods" section of this thesis.

C. Effect of pH on growth of *M. kansasii*

Sauton medium was prepared, and portions were adjusted to the following pH levels prior to sterilization: 6.0; 6.5; 7.0; 7.5; and 8.0. To each
### TABLE XI

**GROWTH OF M. KANSASII IN SAUTON FLUID MEDIUM**

(tubes vs. flasks)

<table>
<thead>
<tr>
<th>Method of culture</th>
<th>Total volume of medium (ml)</th>
<th>Bacterial wet weight (gm)</th>
<th>Bacterial dry weight (gm)</th>
<th>Bacterial dry weight per ml of medium (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>400 tubes 10 ml per tube</td>
<td>4000</td>
<td>134.64</td>
<td>25.12 (18.7)</td>
<td>6.28</td>
</tr>
<tr>
<td>400 tubes 10 ml per tube</td>
<td>4000</td>
<td>112.77</td>
<td>18.73 (16.1)</td>
<td>4.68</td>
</tr>
<tr>
<td>40, 500 ml flasks 100 ml per flask</td>
<td>4000</td>
<td>83.28</td>
<td>15.75 (18.91)</td>
<td>3.94</td>
</tr>
<tr>
<td>30, 500 ml flasks 100 ml per flask</td>
<td>3000</td>
<td>61.60</td>
<td>12.66 (20.56)</td>
<td>4.22</td>
</tr>
</tbody>
</table>
of five 250 ml Erlenmeyer flasks were added 25 ml of medium of a specific pH. Following autoclaving at 15 pounds for 15 minutes, one flask of each pH level was used in rechecking the pH. The changes occurring in pH were noted as:

<table>
<thead>
<tr>
<th>pH before autoclaving</th>
<th>pH after autoclaving</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.00</td>
<td>6.02</td>
</tr>
<tr>
<td>6.50</td>
<td>6.50</td>
</tr>
<tr>
<td>7.00</td>
<td>6.85</td>
</tr>
<tr>
<td>7.50</td>
<td>7.30</td>
</tr>
<tr>
<td>8.00</td>
<td>7.64</td>
</tr>
</tbody>
</table>

All remaining 20 flasks (4 at each pH level) were inoculated with 1 loopful (4 mm O.D.) of a 7 day old culture in Sauton medium of M. kansasii ATCC 12478. The method of inoculating differed from that used in other experiments in that the inoculum was mixed with the medium and not "floated" on the surface.

Three of each group of flasks were incubated in the dark at 37°C, and one flask of each group was incubated in a lighted incubator (37°C), the culture being positioned 30 inches from a 40-Watt General Electric "cool white" fluorescent tube. Flasks in the light were observed for the development of growth and aided in determining the time at which the experiment should be terminated.

At the end of 2½ weeks all flasks were examined and were found to show growth. There was a gradual increase in the amount of growth up to a pH
of 7.3, followed by a sharp decline at pH 7.64. All of the flasks grown in the dark were subjected to photochromogenicity testing on the eighteenth day and responded with subsequent production of pigment. A deeper pigmentation occurred in cultures incubated under continuous light. These appeared orange, whereas the cultures grown in the dark, on exposure to light, produced only yellow pigment. Details of the results are presented in Table XII. The findings indicate that maximum growth occurs when Sauton medium is slightly alkaline (pH 7.3). The end point appears critical in the alkaline range since a slight change to pH 7.6 resulted in a marked decrease in bacterial growth. On the other hand, growth is only slightly decreased by more acid pH levels, with only slight changes occurring down to the level of pH 6.5.

TABLE XII
SAUTON MEDIUM AT VARIOUS pH LEVELS, 25 ml PER 250 ml FLASKS, INOCULATED WITH M. KANSASII, AND DRY WEIGHTS DETERMINED FOLLOWING THREE WEEKS GROWTH

<table>
<thead>
<tr>
<th>Flask No.</th>
<th>dry weight bacteria (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pH 6.02</td>
</tr>
<tr>
<td>1</td>
<td>7.9</td>
</tr>
<tr>
<td>2</td>
<td>7.6</td>
</tr>
<tr>
<td>3</td>
<td>9.1</td>
</tr>
<tr>
<td>Average</td>
<td>8.2</td>
</tr>
</tbody>
</table>
D. Unidentified growth stimulating factor for *M. kansasii*

In attempts to determine the ability of monochromatic light of various wavelengths to photoactivate *M. kansasii*, cultures were prepared in Petri dishes containing Dorset egg medium. This procedure permitted the direct exposure of the surface cultures to monochromatic light by opening the Petri dishes within a dark room and positioning them in a beam of light of selected wavelength. During this process Petri dish cultures were open to the atmosphere for periods ranging up to 1 hour. On one such occasion a plate became contaminated, and the following interesting phenomenon was observed when reading the results of the "photochromogenicity" test following 24 hours incubation of the culture in the dark.

The growth of *M. kansasii* on the contaminated plate had mushroomed, not only in the immediate vicinity of the contaminant, but throughout approximately half of the plate. Growth adjacent to the contaminant was heaped up, and it appeared massive compared with growth on the opposite half of the plate. The original contaminated plate, and a parallel control plate from the same experiment are shown in Figure 5.

The contaminating colony was subcultured, and the organism was isolated in pure culture. It was observed to be a large Gram-positive coccus having a tendency toward tetrad arrangement. On blood agar it developed round, smooth, convex, yellow colonies within 24 hours, and it demonstrated an optimum temperature range of 32 - 37°C. No acid or gas was produced in
Figure 5

Left: Culture of *M. kansasii* on Dorset medium accidentally contaminated with *Sarcina lutea*.

Right: Control culture of *M. kansasii*. 
glucose, lactose, sucrose, salacin or mannitol. It was nitrate negative, and showed no action on litmus milk. Gelatin was liquified in less than 10 days. These reactions indicated the organism to be a strain of Sarcina lutea.

Time was not available to identify the growth stimulating factor involved, but by inoculating growing cultures of M. kansasii on hand, the growth stimulating effect was reproduced. A filtrate from a nutrient broth culture of the contaminant, when applied to the surface of a culture of M. kansasii on Dorset medium, failed to stimulate growth. Probably the factor is only produced by the S. lutea when it is grown on Dorset egg medium, or the phenomenon may be one involving symbiotic growth.

Studies will immediately be initiated to investigate this phenomenon and if possible to identify the growth stimulating factor. This could serve as a means of differentiating strains of mycobacteria if the action is limited to M. kansasii. On the other hand, if the factor proves equally as stimulating to M. tuberculosis it could prove even more rewarding.

4. MORPHOLOGICAL OBSERVATIONS

A. Pigmented photochromogens as compared with nonpigmented photochromogens

In the present study the morphology of M. kansasii (ATCC 12478) grown on Lowenstein-Jensen medium was observed to differ depending upon its age, and whether or not the culture had been photoactivated, i.e. pigmented or nonpigmented. This was first observed when smears were prepared from a series of slants which had been photoactivated while standing in a test tube rack. The portions of the cultures located behind the middle crossbar of the rack were
partially shielded during exposure of the culture to light, and in this region the culture failed to produce yellow pigment within 24 hours. Smears prepared from a given area of several parallel cultures showed an identical microscopic picture but differed remarkably depending upon the portion of the slant from which the smears had been prepared. Stained preparations from the nonpigmented portions consisted of organisms which were short (3-4 μ), fairly regular in shape and size, and only slightly irregular in staining (Ziehl-Neelsen method). They compared favorably with the picture normally encountered with \textit{M. tuberculosis} controls. In contrast, smears prepared from the pigmented areas of slants revealed a completely different picture. Cells were 3-4 times longer, and staining was very irregular, with each cell possessing one or more deeply stained granules. These morphological differences in smears prepared from pigmented and nonpigmented areas were consistent in all of 30 Lowenstein-Jensen slants cultured specifically for microscopic studies.

In contrast, the microscopic examination of \textit{M. kansasii} which had been grown in Sauton fluid medium revealed no such differences. In this medium the morphology of the organisms in both pigmented and nonpigmented cultures was similar and showed characteristics intermediate between those observed in pigmented and nonpigmented growth on solid medium. Growth in Sauton medium consisted of cells which were usually twice the length of \textit{M. tuberculosis}. They stained with a definite degree of irregularity, but no deeply stained granules as described for pigmented cultures on solid medium were seen.
B. "Albino" nonphotochromogenic growth of M. kansasii

A series of cultures prepared midway in the present study yielded results which were unexplainable. The batch of Sauton medium involved was prepared as usual, distributed into sixty-six 500 ml Erlenmeyer flasks, and inoculated with *M. kansasii* (ATCC 12478) for mass growth. Following inoculation and incubation at 37°C, half of the cultures were photoactivated but only 3 of the 33 responded with pigment formation. In the three pigmented cultures growth appeared as the typical wrinkled pellicle encountered on all other occasions, but the nonpigmented cultures formed a much smoother pellicle. When disturbed and shaken, the medium and growth in the nonpigmented cultures appeared to be viscous in contrast to the pigmented cultures which were of the normal watery consistency. Despite continued exposure to light no pigment developed in the 30 flasks involved. The remaining 33 cultures, originally intended to serve as dark nonpigmented controls, were also checked for photochromogenicity and all 33 failed to produce pigment. When subcultured to fresh Sauton medium the same type of nonpigmented growth developed, but on further subculture (second), a complete reversion from the "albino" growth to the original type of growth was observed.

A very striking difference was noted in Ziehl-Neelsen stained smears prepared from the pigmented and nonpigmented growths described above. The pigmented growth consisted mainly of fairly evenly stained organisms measuring 2 - 4 microns in length (Figure 6-A). In addition, non-acid-fast bipolar staining bacilli and small coccoid forms were observed (Figure 6-B). The non-acid-fast
Figure 6  Morphology of *M. kansasii* (ATCC 12478) grown in Sauton medium, Ziehl-Neelsen stained.

A. Normal pigmented (photoactivated) culture.
B. Abnormal forms (non-acid-fast) in pigmented culture.
C, D, E and F. Abnormal forms in "albino" culture.
organisms were at first suspected of being a contaminant but this was ruled out after multiple subcultures onto various media.

The morphology of the "albino" cells was distinct from that of the pigmented cells. They were long filamentous forms measuring up to 45 μ in length, and they usually possessed one or more swollen "spore-like" inclusions which stained poorly if at all (Figure 6-C, D, E, F). The swollen areas were positioned either terminally, subterminally or central. In addition these cells, when stained by Ziehl-Neelsen method, were usually studded with a series of deeply stained granules (Figure 6-D, E, F). Branching was suggested in many cells (Figure 6-B, D, F) but the author feels that this could be more apparent than real.

C. "Cord" formation in chromogenic mycobacteria

Some workers closely associate the virulence of mycobacteria with their ability to form "cords". It was therefore of interest to explore the ability, or inability, of chromogenic mycobacteria to produce this form of growth.

Several strains of mycobacteria involved in the present study, including representatives of Groups I, II, III and IV, were subjected to testing for "cord" factor. Strains were grown in Tween-albumin liquid medium containing 0.05 per cent bovine albumin, and 5 per cent human plasma which had not been subjected to heat (Tarshis, 1961).

Various degrees of positive "cording" were recorded for strains of chromogenic mycobacteria belonging to Groups I, II and III, but members of Group IV tested were always negative. It was observed that specific strains
showed varied levels of "cording" in different subcultures, but the "cording" was never equivalent to that observed in *M. tuberculosis* H37Rv.

Although the candidate feels that results of this test were too inconsistent to be of value in differentiating strains, the extent of "cording" noted in one scotochromogen isolated from the sputum of a patient with bilateral moderately advanced pulmonary tuberculosis is worthy of record (Figure 7). "Cording" to a lesser extent was observed in several strains of mycobacteria belonging to Groups I, II and III. It was observed that even in the strain in Figure 7, the "cords" were loose, and they could be easily disrupted if the growth was not gently transferred onto the slide prior to fixing and staining. In this respect the "cording" of chromogenic mycobacteria differed from the tight "cords" produced by *M. tuberculosis* H37Rv.

![Figure 7](image)

"Cording" of a scotochromogen.
PART II
PIGMENT STUDIES

1. INVESTIGATIONS ON FORMATION OF PIGMENT IN
   CHROMOGENIC MYCOBACTERIA

A. Introduction

   For most chromogenicity studies, M. kansasii was chosen primarily
   because of its clinical significance, and also because of its recent status as a
   new species for which a type culture was available. Furthermore, chromogenesis
   is best studied by employing photochromogens; pigment formation occurs rapidly,
   following exposure of a mature culture to light. The type species of M. kansasii
   (ATCC 12478) was used routinely, and was often paralleled by representatives
   of Groups I, II and III kindly supplied by Dr. Runyon.

   Although M. kansasii, as a species, is identified by its distinctive
   characteristic of photochromogenicity, its pigment production per se has never
   been investigated. Studies of light-induced pigment formation in mycobacteria
   have thus far been limited to two reports, both of which concern rapidly growing

   The marked variation in pigmentation often encountered in duplicate
   cultures has already been related. There was no apparent reason or explanation
   for the erratic pigment development in specific strains, and it was considered an
   essential part of this study to investigate the factors responsible for these varia­
   tions. As work progressed the role of moisture, oxygen, temperature, viability,
   age and pH was examined. Some knowledge was also gained concerning media.
New tubes, screw capped unless otherwise stated, were used in all these studies. The egg media were dispensed into slanted tubes to prevent the "clouding" resulting from dispensing of media into upright tubes and then slanting. Optically clear tubes were better for photochromogenicity testing of cultures, and they also permitted better viewing and photographing of the growth and pigment developing within the tubes.

B. Moisture: effect on pigment synthesis in mycobacteria

It is well known that tubes of freshly inspissated egg medium show varying amounts of moisture at the base of slants. In preliminary tests with chromogenic mycobacteria it was observed that tubes which failed to produce pigment usually contained water of condensation. Because of this observation one of the first tests performed involved multiple subculturings of various strains, including *M. kansasii* and representatives of Runyon's Groups I, II and IV onto randomly picked tubes of freshly prepared media. Each strain was inoculated onto 10 Lowenstein-Jensen slants and 10 Dorset slants. The inoculum was standardized to 1 loopful (4 mm O.D.) of a suspension of organisms in saline prepared from growth on the same type of medium to which it was being subcultured. The suspensions were equivalent in density to a McFarlane nephelometer tube No. 1. The cultures were grown for three weeks in light-proof containers at 37°C, following which *M. kansasii* and members of Runyon's Group I were tested for photochromogenicity as outlined in the section on "Methods".

The results, summarized in Table XIII, showed that only 42 of 80 tubes which should have formed pigment were colored. The only detectable
<table>
<thead>
<tr>
<th>STRAIN OF MYCOBACTERIA</th>
<th>10 LOWENSTEIN-JENSEN SLANTS</th>
<th>10 DORSET SLANTS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PIGMENTED</td>
<td>NONPIGMENTED</td>
</tr>
<tr>
<td>M. kansasii (ATCC 12478)</td>
<td>4</td>
<td>6</td>
</tr>
<tr>
<td>P-8 Photochromogen (Group I)</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>P-5 Scotochromogen (Group II)</td>
<td>6</td>
<td>4</td>
</tr>
<tr>
<td>M. phlei (Group IV)</td>
<td>4</td>
<td>6</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>19</strong></td>
<td><strong>21</strong></td>
</tr>
</tbody>
</table>
difference, apart from the color, was the moisture content in the tubes. Tubes showing nonpigmented growth contained varying amounts of visible moisture at the butt of the medium. Tubes showing pigmented growth were either dry or contained very small amounts of visible moisture. It was also recalled that, over several years involving some 100,000 cultures, good luxurient growth of *M. tuberculosis* had never been observed to occur in tubes in which moisture remained throughout incubation. Furthermore, since working with chromogenic mycobacteria there was no recollection of having ever observed good pigment development in moisture-containing tubes. This at first suggested that humidity could be an important factor, a saturated atmosphere inhibiting pigment development. Invariably tubes containing moisture showed less growth and were nonpigmented (Figure 8).

Investigating further, all 38 tubes containing moisture and nonpigmented growth in this test series were opened, the fluid was aseptically withdrawn with Pasteur pipettes, and the tubes were reincubated at 37°C for 24 and 48 hour readings. Again findings resulted which at the time were unexplainable. The 7 cultures of strain P-5 (scotochromogen, Group II) and the 11 cultures of *M. phlei* all developed pigment within 48 hours, but all 20 cultures of the photochromogenic mycobacteria, consisting of 10 tubes of *M. kansasii* (ATCC 12478) and of 10 P-8 (Runyon), remained pigmentless. Still more confusing was the finding a week later that all tubes of *M. kansasii* and P-8 had become pigmented while standing at room temperature. The reason for this delay in pigment development following removal of moisture from cultures of photochromogenic mycobacteria became
Figure 8

Deceptive relationship between moisture and the amount of growth and pigmentation in chromogenic mycobacteria.
obvious only when the experimental results on the role of oxygen in photo-
activation were analysed.

In further attempts to elucidate the influence of the moisture content
of the tube on pigment formation, 20 tubes were selected from a freshly prepared
batch of Lowenstein-Jensen medium; 10 appeared dry and 10 contained visible
amounts of water of condensation. The 20 tubes, before inoculation, were
arranged into four series as follows:

Series A: 5 tubes which appeared dry.
Series B: 5 tubes which appeared dry, but which were made
moist by the addition of 0.5 ml of sterile water per
tube.
Series C: 5 tubes which contained visible moisture at the
base of the slants (averaging 0.5 ml per tube).
Series D: 5 tubes which originally contained moisture but
were aspirated to dryness.

All tubes were inoculated with \textit{M. kansasii} (ATCC 12478), incubated
and tested for photochromogenicity. The results indicated that "dry" slants,
and "dry" slants made "moist", supported better growth and much better pigment
development than did "wet" tubes, or "wet" tubes made "dry". The reason for
this became obvious later.

At the time these results were obtained it was observed in another
experiment involving \textit{M. kansasii} that all cultures grown in tubes plugged with
cotton wool produced pigment when exposed to light. With this added knowledge an experiment was devised to evaluate the effect of both, the type of tube closure and the moisture content of tubes, as related to growth and pigment development.

From a large batch of freshly prepared Dorset medium 100 moist tubes and 100 dry tubes were selected. Each group was divided in half; 50 moist tubes were aseptically aspirated to dryness and 50 dry tubes were made moist by the addition of 0.5 ml of sterile water. Half of each group were capped with conventional screw caps, and the remaining half were capped with "Morton" type stainless steel tube closures. All tubes were inoculated with M. kansasii (ATCC 12478) as outlined above for the earlier experiment, and all were incubated in light-proof cardboard containers at 37°C for 14 days before exposure to light for photochromogenicity testing.

The amount of moisture in the tubes was checked every 48 hours in very subdued light which was not adequate to photoactivate the growth. The moisture content of the "moist" tube series was maintained at approximately 0.5 ml per tube. Screw capped tubes seldom required readjustment of the moisture content.

All tubes capped with "Morton" type closures gave excellent pigment production regardless of the presence or absence of moisture. Tightly sealed screw capped tubes failed to produce good pigment in either series of tubes, and the amount of growth was considerably less than that occurring in the other series. Results clearly indicated that, providing vented caps were used, the presence of moisture had no effect on pigment formation. This finding has also been
demonstrated for the scotochromogen P-5 and _M. phlei_ using smaller numbers of tubes. The earlier assumption that moisture was inhibiting pigment development of P-5 and _M. phlei_ was more apparent than real, since the subsequent pigment development resulted not from the removal of the fluid itself, but because of the atmospheric changes which took place during the process of moisture removal.

The necessity of vented caps for the study of chromogenesis in mycobacteria is a finding which is further elaborated upon in the following section.

C. Oxygen: effect on photoactivation and pigment synthesis

a) Introduction

The observation that cultures of _M. kansasii_ grown in vented tubes regularly produced pigment when exposed to light led to a series of studies designed to establish the factor(s) influencing pigment production.

b) Methods

The first method used to seal tubes was similar to that described by Novy and Soule (1925). Tubes were plugged with cotton, then the plug was cut off even with the lip of the test tube, pressed down into the tube about 1/4 inch, and finally covered with hot melted paraffin. For the vented tubes a small hole less than 1 mm diameter was made through the paraffin cap into the cotton wool with a hot dissecting needle. Later a more satisfactory and convenient means for venting tubes was achieved through the use of "Morton" type stainless steel clip-on tube closures (Morton, 1957). The "Morton" closure has been found most satisfactory since it permits about only one half of the evaporation which
occurs with regular cotton plugs (Ladd, 1963). A more convenient means of hermetically sealing tubes was introduced through the use of conventional screw caps fitted with good cap liners. The cap, when tightened, could be inverted and dipped into a bath of melted paraffin. As an added precaution the join between cap and tube was wrapped with several layers of "Parafilm M" (manufactured by Marathon Division of American Can Co., Menasha, Wisconsin).

For slants, the inoculum consisted of 1 loopful (4 mm O.D.) of culture spread over the entire surface. The medium used in these tests was Dorset with 5 per cent glycerol. Incubation was carried out for 14 days at 37°C in the dark followed by photochromogenicity testing. All tests were performed in triplicate and at least on two occasions.

c) Results

The results for the tests involving air-tight and vented tubes are presented in Table XIV. From test No. 1, in which no pigment developed when sealed unopened tubes were exposed to light, it was concluded that, in sealed tubes, either bacterial growth was:

i) Not photoactivated during exposure to light, or

ii) If photoactivated, subsequent pigment formation was inhibited.

Possible explanations for such findings would include:

i) Depletion of some factor(s) occurring within the sealed tube during growth, which is essential for photoactivation and/or pigment formation.

ii) Accumulation of some factor(s) within the sealed tube during growth, which inhibits photoactivation and/or pigment formation, or
### TABLE XIV

**MYCOBACTERIUM KANSASII**

(ATCC 12478 cultured on Dorset glycerol egg slants, 14 days at 37°C)

<table>
<thead>
<tr>
<th>CULTURED IN</th>
<th>TEST</th>
<th>RESULT</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. AIR-TIGHT TUBES</td>
<td>UNOPENED CULTURE EXPOSED TO LIGHT</td>
<td>NO PIGMENT</td>
</tr>
<tr>
<td>2. AIR-TIGHT TUBES</td>
<td>CULTURE AERATED AND RESEALED 1 HOUR</td>
<td>PIGMENT</td>
</tr>
<tr>
<td></td>
<td>BEFORE EXPOSURE TO LIGHT</td>
<td></td>
</tr>
<tr>
<td>3. AIR-TIGHT TUBES</td>
<td>CULTURE AERATED AND RESEALED</td>
<td>NO PIGMENT</td>
</tr>
<tr>
<td></td>
<td>AFTER EXPOSURE TO LIGHT</td>
<td></td>
</tr>
<tr>
<td>4. VENTED TUBES</td>
<td>EXPOSED TO LIGHT</td>
<td>PIGMENT</td>
</tr>
</tbody>
</table>
iii) A combination of depletion and accumulation of factors, which together, inhibit photoactivation and/or pigment formation.

Since in the second test (Table XIV) the opening and aerating of the cultures one hour prior to light exposure permitted pigment development, it was apparent that whatever was either depleted and/or accumulated during growth was associated with the atmospheric conditions within the tube. The absence of pigment formation in test 3, where air-tight tubes were aerated following exposure to light, suggested that it was photoactivation which did not occur in the air-tight tubes.

Test No. 4, considered as control, has been used routinely in several hundreds of tubes prepared for other experimental purposes. Cultures grown in vented tubes have never failed to produce pigment following exposure to light (Figure 9).

To gain further knowledge concerning photoactivation and pigment synthesis, additional experiments were devised for cultures grown in vented tubes.

Five sets of tests were performed to determine the influence of atmospheric conditions on pigment synthesis (Table XV). In the first test the cultures were made anaerobic before exposure to light. This was achieved by inserting a 1 inch absorbent cotton wool plug well into the tubes and adding three ml of 20 per cent NaOH and 1.2 ml of 40 per cent aqueous pyrogallic acid. These volumes were retained within the absorbent cotton plugs. The tubes were then immediately sealed with melted paraffin. Control tubes of dextrose broth containing methylene blue indicator were always reduced by this procedure. Lack
Figure 9

Increased growth and pigmentation of M. kansasii (ATCC 12478) when grown in tubes fitted with "Morton" type (vented) caps, as compared to parallel cultures in conventional screw capped tubes.

NOTE: The "Parafilm M" tape on the screw capped tubes was applied after incubation and photochromogenicity testing. Tubes were being retained for other studies.
<table>
<thead>
<tr>
<th>TEST</th>
<th>RESULT</th>
<th>CONCLUSION</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. CULTURE MADE ANAEROBIC BEFORE EXPOSURE TO LIGHT</td>
<td>NO PIGMENT</td>
<td>EITHER PHOTOACTIVATION, PIGMENT FORMATION, OR BOTH, REQUIRE OXYGEN</td>
</tr>
<tr>
<td>2. CULTURE MADE ANAEROBIC BEFORE EXPOSURE TO LIGHT, AND AERATED FOLLOWING EXPOSURE TO LIGHT</td>
<td>NO PIGMENT</td>
<td>PHOTOACTIVATION REQUIRES OXYGEN</td>
</tr>
<tr>
<td>3. CULTURE MADE ANAEROBIC FOLLOWING EXPOSURE TO LIGHT</td>
<td>NO PIGMENT</td>
<td>PIGMENT FORMATION REQUIRES OXYGEN</td>
</tr>
<tr>
<td>4. CULTURE PLACED IN CO$_2$ ATMOSPHERE BEFORE EXPOSURE TO LIGHT</td>
<td>NO PIGMENT</td>
<td>PHOTOACTIVATION REQUIRES OXYGEN</td>
</tr>
<tr>
<td>5. CULTURE PLACED IN CO$_2$ ATMOSPHERE FOLLOWING EXPOSURE TO LIGHT</td>
<td>NO PIGMENT</td>
<td>PIGMENT FORMATION WILL NOT OCCUR IN CO$_2$ ATMOSPHERE</td>
</tr>
</tbody>
</table>

**TABLE XV**

MYCOBACTERIUM KANSASII

(ATCC 12478 cultured on glycerol egg slants in vented tubes, 14 days at 37°C)
of pigment production in an anaerobic atmosphere indicated that either photoactivation, pigment formation, or both, require oxygen.

In the second test, anaerobic conditions were created as in test No. 1, but the tubes were aerated following exposure to light. Lack of pigment formation demonstrates the need of oxygen for photoactivation.

Test No. 3, where cultures were made anaerobic following exposure to light, demonstrates the need for oxygen in pigment formation following photoactivation. Thus oxygen is essential for both photoactivation and subsequent pigment formation, each of which is a separate step.

Tests 4 and 5 were performed to determine if a CO₂ atmosphere would support photoactivation and pigment formation. For this purpose the tubes were appropriately fitted with rubber stoppers and glass cotton plugged air passages to accommodate flushing of the tubes with carbon dioxide. In the absence of free molecular oxygen photoactivation and pigment synthesis did not occur.

To determine the amount of atmosphere (oxygen) required to support growth, photoactivation and subsequent pigment formation for a tube culture of M. kansasii, a series of tubes were inoculated and then hermetically attached to various size Erlenmeyer flasks. There was a clear cut off point between the 125 ml and 250 ml flasks. Up to a volume of 125 ml no pigment developed and growth was sparse, whereas a 250 ml volume or greater always gave excellent growth and pigment production. These volumes are in addition to the 25 - 30 ml air volume contained within the tubes themselves. Control tubes, both vented and air-tight, were included (Figure 10).
M. kansasii (ATCC 12478) cultured in tubes hermetically sealed to Erlenmeyer flasks of varying sizes (500 ml; 250 ml; 125 ml; 50 ml; and 25 ml). Controls in test tubes sealed with screw cap and vented "Morton" closure. Photographed following photochromogenicity testing.
Slow growing chromogenic mycobacteria other than *M. kansasii* also require the presence of oxygen for pigment formation. As mentioned earlier, stock cultures of Runyon's P-5 scotochromogenic strain when subcultured into screw capped tubes often developed pigmentless growth (Figure 3). The same was often experienced with clinical isolates. With these organisms it appears that rapid primary growth within air-tight tubes utilizes all the available oxygen or maintains it below the critical level required for pigment synthesis. This has been proven by venting tubes containing colorless growth of strain P-5 as well as several other scotochromogens. On repeated occasions, and without exception, pigment developed within 48 hours following aeration of such pigmentless growth provided the cultures were young.

Pigmented mycobacteria (photoactivated and incubated) retain their pigment when held for 48 hours under anaerobic conditions.

D. Temperature: effect on photoactivation and pigment synthesis

In order to gain information on the nature of the reactions occurring, the effect of temperature was investigated. Six separate sets of tests were performed (Table XVI).

In the first test, 14 day old cultures were chilled in an ice bath to a temperature of 0°C. They were exposed to light while being held at that temperature, and then incubated in the dark at 37°C. Good pigment development within 24 hours revealed that photoactivation had occurred at 0°C. Other cultures chilled to 0°C following exposure to light failed to produce pigment (test 2). Thus pigment formation per se, following photoactivation, is temperature dependent.
<table>
<thead>
<tr>
<th>TEST</th>
<th>RESULT</th>
<th>CONCLUSION</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. CULTURE CHILLED AND EXPOSED TO LIGHT AT 0°C, THEN INCUBATED AT 37°C</td>
<td>PIGMENT</td>
<td>PHOTOACTIVATION OCCURS AT 0°C</td>
</tr>
<tr>
<td>2. CULTURE CHILLED AT 0°C FOLLOWING EXPOSURE TO LIGHT</td>
<td>NO PIGMENT</td>
<td>PHOTOACTIVATED BACTERIA WILL NOT FORM PIGMENT AT 0°C</td>
</tr>
<tr>
<td>3. CULTURE INCUBATED AT 45°C FOLLOWING EXPOSURE TO LIGHT</td>
<td>PIGMENT</td>
<td>PIGMENT FORMATION WILL OCCUR IN NON-MULTIPLYING BACTERIA</td>
</tr>
<tr>
<td>4. CULTURE INCUBATED AT 45°C IMMEDIATELY PRIOR TO AND DURING EXPOSURE TO LIGHT</td>
<td>NO PIGMENT</td>
<td>PHOTOACTIVATION WILL NOT OCCUR AT 45°C</td>
</tr>
<tr>
<td>5. CULTURE HEATED TO 56°C FOR 1 HOUR PERIOD BEFORE EXPOSURE TO LIGHT</td>
<td>NO PIGMENT</td>
<td>EITHER PHOTOACTIVATION, PIGMENT FORMATION, OR BOTH, DEPEND UPON HEAT LABILE FACTOR(S)</td>
</tr>
<tr>
<td>6. CULTURE HEATED TO 56°C FOR 1 HOUR PERIOD FOLLOWING EXPOSURE TO LIGHT</td>
<td>NO PIGMENT</td>
<td>PIGMENT FORMATION DEPENDS UPON HEAT LABILE FACTOR(S)</td>
</tr>
</tbody>
</table>
In the third test, cultures incubated at 45°C following exposure to light at room temperature produced pigment at the elevated temperature. Photoproduction itself, however, was not found to occur at 45°C (test 4). These cultures were incubated in the dark at 37°C following exposure to light.

Cultures held at 56°C in a water bath for 1 hour before being exposed to light failed to produce pigment (test 5). This indicates that either photoproduction, pigment formation, or both, depend upon heat labile factors. In the sixth test, involving cultures which were heated to 56°C for 1 hour following exposure to light, failure to develop pigment indicates that pigment formation depends upon heat labile factors.

Once photoproduced, cultures held for 48 hours aerobically at 0°C, anaerobically at 37°C, or under CO₂ at 37°C, will produce pigment when they are incubated aerobically at 37°C. Heating, however, does cause a change. Following 48 hours exposure to 50°C, pigment formation will not occur during subsequent aerobic incubation at 37°C.

Controls were included in both increased and decreased temperature tests (45°C and 0°C) to show that pigment formation was not stimulated merely by thermal shock.

E. Age and viability of cells

Tests were performed to determine what effect age and viability of cells may have upon photoproduction and pigment formation.

Thirty cultures of M. kansasii were prepared on Dorset medium in vented tubes. They were incubated at 37°C in a moist atmosphere in the dark.
Tubes were removed in triplicate starting after 14 days of incubation and at weekly intervals thereafter, for purposes of photochromogenicity testing. Good pigment resulted when cultures of 2, 3 and 4 weeks of age were exposed to light, but only a slight response was noted in 5 weeks old cultures, and no change occurred in 6 weeks old cultures. Cultures less than 2 weeks of age were usually unsatisfactory for photochromogenicity testing because of the limited bacterial growth.

Similar results were obtained with cultures in Sauton fluid medium. Beyond a period of 5 weeks incubation little or no pigment developed following exposure to light.

It was also found that the cultures which had been heated to $56^\circ$C for 1 hour, either before or after exposure to light in the temperature studies, were nonviable when subcultured. It will be recalled that these cultures failed to produce pigment (Table XVI, tests 5 and 6). The cultures chilled to $0^\circ$C in the temperature studies were viable on subculture (Table XVI, tests 1 and 2) and did produce pigment when placed under appropriate conditions.

Active multiplication of M. kansasii would appear not to be necessary for pigment synthesis. This was demonstrated when pigment was formed at $45^\circ$C, a temperature at which the organism does not multiply.

When young actively growing cultures were killed with formalin prior to light exposure, pigmentation did not occur. Likewise, no pigment developed if the cells were formalinized immediately following photoactivation.
F. pH

In growth studies already related in "Experimental Results" Part 1, Section 3, cultures of *M. kansasii* in Sauton medium had been adjusted to pH levels from pH 6.0 - pH 8.0 at 0.5 intervals. Photoactivation and pigment formation occurred in all cultures throughout this pH range indicating that inhibition of pigment formation does not occur at practical pH levels. Since growth of the organisms is significantly reduced at pH 7.64 it would be pointless to investigate the ability of cultures to produce pigment in a more alkaline medium. Concerning the lower pH range for pigment production, media for the cultivation of mycobacteria would not normally be lower than pH 6.0, and at that level photoactivation and good pigment development occurred.

G. Medium

The media used in these studies were all capable of supporting pigment formation of *M. kansasii* when cultures were incubated under specified conditions, i.e. light, oxygen, temperature and pH. Both Lowenstein-Jensen and Dorset media were satisfactory, but the latter generally supported better growth of chromogens, and the growth was more deeply pigmented.

It was not possible, within the time available, to assess the merits of a multitude of media and media ingredients in relation to pigmentation. Undoubtedly many differences would be detected if such a study were undertaken.

For the intended characterization of *M. kansasii* pigment, the author became interested in the mineral salts medium containing mineral oil (Appendix A - 4) which Haas and Bushnell (1944) had described as supporting the growth
of several strains of mycobacteria with the production of oil-soluble pigments. According to these workers the pigments were formed and eluted from \textit{M. lacticola} to color the supernatant oil layer of the medium yellow or orange. It was felt that if this medium would function in the same manner with chromogenic mycobacteria, it would provide a convenient way of securing pigment for analysis. Despite repeated attempts, however, this could not be demonstrated for any of the Group I, II or IV strains used in this study. Why \textit{M. smegmatis} failed to produce oil-soluble pigment is unexplained. It is synonymous with \textit{M. lacticola}, a species for which Haas and Bushnell reported obtaining good results.

Concerning media, it was determined that the compound serving as the source of nitrogen had no effect on pigment development, at least in Runyon's photochromogenic strain P-8. Good pigment developed if 10 gm of sodium glutamate was used instead of the regular 4 gm of asparagine per liter of Sauton medium. This is in agreement with what Ingraham and Steenbock (1955) found for \textit{M. phlei}.

2. "\textit{ALBINO}" AND "\textit{SCOTOCHROMOGENIC}" GROWTH OF \textit{M. KANSASII}

One instance involving growth of \textit{M. kansasii} (ATCC 12478) which could not form pigment has already been related (Part I, Morphological Observations). This is of major importance, since failure of an organism to show what is described as its "distinctive character" (Subcommittee on Mycobacteria, 1962) will result in mistaken identification. Whether or not photoactivation or pigment synthesis itself was involved remains undetermined. In the instance recorded the change was phenotypic and transient, with reversion within two
subcultures to the form capable of producing pigment.

On another occasion cultures of the same strain on Dorset medium were observed to have well defined colonies which failed to respond to the photochromogenicity test and remained buff while adjacent colonies formed deep yellow pigments. These pale varieties were stable for 3 or 4 subcultures and then reverted to the characteristic type.

Still another appearance, which was quite contrary to the species definition, was observed in the form of well defined deeply pigmented yellow areas in the pellicle of M. kansasii grown on Sauton medium in the dark. Three of 31 flasks, all representing the same batch of medium, showed these pigmented areas 3 - 4 mm in diameter. On subculture the pigmented areas were uniformly nonpigmented prior to light exposure. No explanation for these phenomena is possible at this time.

3. CHARACTERIZATION OF PIGMENTS

A. Extraction and partition

Securing large masses of pigmented mycobacteria was the first problem encountered in the studies concerned with the characterization of the pigments. Cultures required a month to grow, and precise bacteriological manipulation was essential since all of the strains employed in this study had been isolated from either fatal cases, or from the resected lung tissue of patients exhibiting "tuberculous-like" disease. The strains also possessed high levels of resistance to all known therapeutic agents. These factors combined to prevent comparative studies of strains within the various groups of chromogenic mycobacteria. Studies
on the pigments per se had to be limited to the type culture of *M. kansasii* (ATCC 12478). Nonpigmented bacteria, from cultures incubated in the dark, were used as controls.

The procedure and materials used to obtain bacterial harvests have been outlined in Section C of "Methods". The results to be presented are based upon the analysis of pigments extracted from a total bacterial mass of 549.33 gm wet weight (96.64 gm dry weight).

At least 20 gm, dry weight, of bacteria were used for each extraction (Section D of "Methods"). The extracted pigments, after partition between immiscible solvents, were maintained as multiple aliquots of each fraction. This permitted the study of a portion of a particular fraction without exposing the bulk of the material to light and oxygen.

Partition of the original methanol cell extract between petroleum ether and 90 per cent methanol revealed the presence of both epiphasic and hypophasic yellow pigments. It will be recalled that the epiphasic in this system contains "hydrocarbons" and "esters", and the hypophase contains "alcohols" and "acids" (Figure 1 in "Methods").

It was noted from the start that practically all the yellow pigments were contained in the epiphasic, however, the hypophase retained sufficient pigment(s) to give it a definite yellow appearance. Partition was complete because fresh aliquots of petroleum ether added to the hypophase did not remove further pigments.

Following saponification, repeat partition of the epiphasic fraction
between petroleum ether and 90 per cent methanol resulted in a further division. The "hydrocarbons" remained as epiphasic pigments in the petroleum ether, and the de-esterified carotenoid alcohols (freed "alcohols") were contained in the KOH-methanol hypophase. The "alcohols" were transferred into diethyl ether for harvesting. Quantitatively they appeared to represent a very small portion compared with the epiphasic "hydrocarbon" fraction.

The methanol hypophase of the original cell extract, when saponified and partitioned with diethyl ether, was found to contain only epiphasic pigments ("alcohols"). Salts of carotenoid "acids" could not be detected. The hypophase was colorless and concentrated diethyl ether extracts failed to yield a color reaction with either concentrated sulphuric acid, fuming nitric acid, or concentrated aqueous hydrochloric acid. Likewise, no blue coloration was observed when antimony trichloride in chloroform (Carr-Price reagent) was added to this portion. These color reactions are reviewed in a monograph by Karrer and Jucker (1950).

Throughout the remainder of this thesis the three pigment fractions will be referred to as a) "hydrocarbons"; b) "esters" (freed "alcohols"); and c) "alcohols".

In the methanol extraction of 20 - 25 gm dry weight of bacteria, some 1200 - 1500 ml of methanol were involved. This amount was partitioned in 1 liter separatory funnels, and on an occasion when the first aliquots of methanol cell extract were being partitioned while extraction was still proceeding with additional methanol, it was observed that all of the "hypophasic" pigments were
contained within the first 200 – 300 ml of methanol extract. Following the first or second aliquot of methanol used in extraction, only epiphasic pigments were eluted. This observation permitted shortening of the partition procedure and also a saving of solvents, since the bulk of the methanol cell extract required a single partition to separate the "hydrocarbons" from the freed "alcohols".

B. Quantitative determination of carotenoids

Total carotenoids were measured by extinction and calculated on the basis of $E_{1\text{cm}}^{1%} (450 \text{ mu})$ to equal 2500. A standard curve was prepared by using 100 per cent beta carotene in petroleum ether (Appendix B - 2). Figure 11 indicates the straight line relationship when per cent transmittance is plotted against concentration of beta carotene. A similar curve, plotted for 100 per cent alpha carotene under the same conditions, was practically identical and did not vary more than 1 per cent at any one point from that plotted for beta carotene.

From 91.78 gm, dry weight, of pigmented M. kansasii 36.95 mg of carotenoids were extracted. This represented an average of 403 ug/gm dry weight. Practically all (96.87 per cent) of the carotenoids were contained in the "hydrocarbon" fraction. The remaining portion was distributed 1.34 per cent in the "ester" fraction and 1.78 per cent in the "alcohol" fraction. Although these last two percentages are low, the fractions were deeply pigmented in appearance. It will be seen later that a large portion of the pigment retained in the "alcohol" fraction was proven to be epiphasic to petroleum ether and 90 per cent methanol, and was identified as alpha carotene by both thin-layer chromatography and spectrophotometry.
Figure 11  Per cent transmittance for beta carotene in petroleum ether (b.r. 37.3°C - 58.2°C) at 450 mu.
C. Thin-layer chromatography and spectrophotometric analysis

Each of the three partitioned fractions was examined spectrophotometrically (in petroleum ether, b.r. 37.8°C - 58.2°C) using a Beckman DK2A ratio recording spectrophotometer. Figure 12 is a composite chart showing absorbance of each fraction between wavelengths of 370 μm and 530 μm. There was no significant difference in extracts of each fraction prepared at different times.

It was obvious from the absorption maxima in the first spectrophotometric readings that most carotenoids were contained in the "hydrocarbon" fraction (line 1 of Figure 12). The readings for the crude "ester" (freed alcohol) fraction showed a hump at 394-400 μm, and a slight rise at 420 μm (line 2 of Figure 12). The third fraction ("alcohols") showed a broad elevation from 396-420 μm with shoulders at 450 μm and 475 μm (line 3 of Figure 12).

A harvest consisting of 11.20 gm, dry weight, of nonpigmented *M. kansasii* (grown in complete darkness) was processed and partitioned in the same manner as extracts of the pigmented culture. The spectra for the nonpigmented fractions, presented in Figure 13, show no absorption maxima from 370 μm to 550 μm. There were no peaks of absorption in either fraction below 370 μm. The gradual increase in absorption at lower wavelength was due to a fluorescent fraction which on analysis showed no peaks in absorption down to the "cut-off" for the solvent used (270 μm).

As explained in the "Methods" section, it was originally thought that too drastic an extraction process might extract cellular components, other than carotenoids, which would interfere with partition of the pigments between immiscible solvents. Later in the study a trial extraction was run using a mixture of methanol:
Figure 12  Extracts from pigmented *M. kansasii*; 1. "Hydrocarbons"
2. "Esters" (freed "alcohols")
3. "Alcohols"

(All fractions in petroleum ether)
Figure 13: Extracts from nonpigmented *M. kansasii*; 1. "Hydrocarbons"
2. "Esters" (freed "alcohols")
3. "Alcohols"

(All fractions in petroleum ether)
chloroform (1:1) instead of pure methanol. This procedure had been used by others to extract pigments from mycobacteria (Ebina et al., 1962). The methanol:chloroform was found to extract the pigments much faster than methanol alone, and the separation of "hydrocarbons" by partition was no different from that experienced when working with methanol extracts. The "hydrocarbon" fraction from the methanol:chloroform was spectrophotometrically identical with the "hydrocarbon" fraction of the methanol extract (Figure 14). Whether or not the small portions of "esters" and "alcohols", which represented approximately 3 per cent of the total carotenoids, were extracted at the same rate with methanol:chloroform (1:1) was not determined. The batch in question was small and the primary interest was whether or not the major pigment (97 per cent) could be partitioned in the same manner when methanol:chloroform (1:1) was used to extract the dried bacterial cells.

The most suitable thin-layer absorbent and solvent for the separation of a specific fraction was determined by running test plates in many systems. During the early work, these trial runs were made using various materials spread on 200 x 200 mm glass carrier plates. In more recent work, after it had been determined that Silica Gel G was the most appropriate absorbent for most solvent systems, considerable time and expense (solvents) were spared in trial runs by using commercially prepared Eastman Chromagram Silica Gel layers. This procedure has been outlined in "Methods".

For convenience, each of the three pigmented fractions derived from partition of the crude methanol extract will be dealt with in the following order: a) "hydrocarbons", b) "esters", c) "alcohols".
Figure 14  
1. Carotenoid "hydrocarbons" extracted from *M. kansasii* with methanol.
2. Carotenoid "hydrocarbons" extracted from *M. kansasii* with methanol:chloroform (1:1).
(Both fractions in petroleum ether)
a) "Hydrocarbons"

As already stated, this fraction contained almost 97 per cent of the total carotenoids produced by *M. kansasii* when grown in continuous light in Sauton medium. Numerous trial tests involving many combinations of solvents, adsorbents, and mixtures of solvents and adsorbents, were performed on this fraction before systems were detected for the isolation of each component. A total of four visible pigments were detected in the "hydrocarbon" fraction.

The major pigment, yellow in color, was best separated on Silica Gel G layers developed in 40 per cent chloroform in petroleum ether (b.r. 37.8°C - 58.2°C). In this system the major pigment moved with an Rf value of 0.78. In addition a very pale barely discernible yellow fraction had an Rf value of 0.35; a salmon colored fraction had an Rf of 0.10; and a deeply pigmented yellow fraction remained at the point of application (Rf 0.00). All four fractions stained with both iodine vapor and Carr-Price reagent.

An additional three fluorescent fractions were visible under ultraviolet light, and all fluoresced better under ultraviolet light of 2600 A than with light of 3660 A. These fractions had Rf values of 0.35, 0.65, and 0.80. They did not stain with either iodine or Carr-Price reagent. When harvested, eluted with petroleum ether and run through the spectrum, neither fluorescent band showed peaks of absorption. All three showed gradual increasing absorption with decreasing wavelength down to the "cut-off" point of the solvent (270 mu). These fluorescent fractions were not studied further.

Returning to the visible pigments of the "hydrocarbon" fraction, they
will be discussed in sequence.

i) The main yellow pigment was harvested from the thin layer and eluted with petroleum ether. When examined spectrophotometrically it showed absorption maxima at 446 μm and 473 μm, with a shoulder at 415 μm to 428 μm (line 1, Figure 15). Since both alpha and beta carotene were suspected from the readings made on the primary crude extract (line 1, Figure 12), pure solutions of each were prepared in petroleum ether for checking in the spectrophotometer and for comparison with the unknown. Figure 16 shows the standard curve for alpha carotene with absorption maxima at 446 μm and 473 μm in light petroleum ether. These values are correct for alpha carotene (Miller, 1961). Figure 17 compares the curves for the extracted pigment (line 2) with that of 100 per cent beta carotene (line 1). The unknown showed absorption maxima at 446 μm and 473 μm, whereas the beta carotene standard showed absorption maxima at 450 μm and 476 μm. These values are correct for beta carotene (Miller, 1961). The unknown possessed absorption maxima identical with that of alpha carotene.

On thin-layer the extracted pigment showed Rf values identical with those of alpha and beta carotene in a variety of systems involving different adsorbents and solvents. Thin-layers consisting of Silica Gel G; Kieselguhr G; calcium hydroxide - Silica Gel G (6 + 1); calcium hydroxide - Silica Gel G (4 + 1); and special layers of Kieselguhr G impregnated with paraffin oil (Randerath, 1962) were investigated. Included in the solvent systems employed were petroleum ether - benzene (49:1 and 1:1); acetone; petroleum ether - chloroform (60:40); ethyl acetate; petroleum ether - N-propanol (99:1);
Figure 15

1. Main yellow pigment (i) from "hydrocarbon" fraction.
2. Yellow pigment (iv) from "hydrocarbon" fraction.

(Both pigments in petroleum ether)
Figure 16  Alpha carotene standard in petroleum ether.
Figure 17  
1. Beta carotene standard. 
2. Main yellow pigment (i) from "hydrocarbon" fraction. 
(Both pigments in petroleum ether)
undecane - methylene dichloride (4:1); benzene; carbon tetrachloride; dioxane; and ethylene chloride. The Rf value varied with systems, but for each system the extracted pigment along with both alpha and beta carotene had the same Rf value.

One thin-layer solvent system, however, showed a separation of these hydrocarbons. It consisted of calcium hydroxide layers developed in a hydrocarbon mixture - methylene dichloride (95 + 5). The hydrocarbon mixture was made up of pentane-hexane-heptane-octane-undecane (60 + 20 + 10 + 6 + 4). In this system the alpha carotene standard, as well as the extracted pigment, had an Rf value of 0.81, whereas the beta carotene standard had an Rf value of 0.74. There was also a visible difference in the color of the spots. Both the extracted pigment and alpha carotene were pale yellow on the calcium hydroxide layer, but the beta carotene possessed a slight orange tint. This thin-layer chromatographic system involving the hydrocarbon mixture - methylene dichloride and calcium hydroxide is described by Stahl (1962). The Rf values and appearance of the extract fraction in this test confirm the spectrophotometric finding which indicated the extracted pigment to be alpha carotene. Co-chromatography also indicated this. Mixtures of the extracted pigment and alpha carotene were not separated, but mixtures of the extracted pigment and beta carotene were separated by this system.

ii) The second pigment of the "hydrocarbon" fraction, earlier described as a barely discernible yellow spot with an Rf of 0.35 on Silical Gel G layers developed in 40 per cent chloroform in petroleum ether, could not be harvested in sufficient amounts for spectrophotometric analysis.
iii) The third pigment in the "hydrocarbon" fraction, salmon colored and with an Rf of 0.10 on Silica Gel G layers developed in 40 per cent chloroform in petroleum ether, was harvested and eluted with acetone. When reduced to dryness and run through the spectrum in petroleum ether, it showed maximum absorption at 426 mu (line 1, Figure 18). The same fraction in chloroform showed a shift of the absorption maximum to the right (higher wavelength) as demonstrated by line 2 of Figure 18.

iv) The fourth and final pigment in the "hydrocarbon" fraction had remained at the point of application when Silica Gel G layers were developed in 40 per cent chloroform in petroleum ether. This pigment was found to move on Silica Gel G layers developed in acetone. Separation by this method however, was complicated because acetone also moved the main pigment (alpha carotene) at the same rate. The only alternative was to secure a portion of the "hydrocarbon" fraction freed of the alpha carotene. This was accomplished by applying 30 spots of the "hydrocarbon" fraction (5 lambda per spot) in a line 1 cm from the base of 200 x 200 mm Silica Gel G layers and developing them in either 40 per cent chloroform in petroleum ether (b.r. 37.8°C - 58.2°C), or petroleum ether alone (b.r. 60°C - 80°C). Petroleum ether, b.r. 37.8°C - 58.2°C alone was not satisfactory for this separation. Development of the layer in either of the appropriate solvents moved the alpha carotene upward and left the other visible yellow pigment at the point of application. By permitting the plate to develop to the 15 cm mark and then removing it, the same layer could be dried for 1 minute at room temperature, and then developed in a second system containing
Figure 18  Salmon colored pigment (iii) from "hydrocarbon" fraction.
1. In petroleum ether.  2. In chloroform.
acetone as the solvent. The second step moved the desired yellow pigment from the point of application upward 3 - 4 cm into an area of the layer through which the alpha carotene had previously passed in the first system. By stopping the second system before the front of the acetone reached the level of the alpha carotene it was possible to position the desired yellow fraction above the point of application. It was desirable to harvest this fraction at some point other than the point of application because several complexes might have been present in the original extract.

This two-step system using different solvents would have worked had the pathway of the alpha carotene in the first system been left "clear." Unfortunately a fluorescent tail was left behind the alpha carotene and it was collected with the yellow pigment as the latter proceeded upward in the acetone of the second system.

The only way thus far described to purify such a mixture would be to harvest the points of application after the first system had removed the alpha carotene and fluorescent fraction. The harvested material would then have to be eluted, freed of adsorbent, reduced to dryness and redissolved in a suitable solvent before application to a second thin-layer for development in acetone. To avoid this time consuming and wasteful procedure which would require preparation of additional layers and manipulation of very small amounts of the unknown fraction, the author developed a new chromatographic technique. The method, in addition to satisfying the present need, may well be applied to any thin-layer or paper chromatography involving the separation of small amounts
of material present in complex mixtures. The term "one dimensional-reverse-
two solvent chromatography" is proposed for this technique. It was performed
as follows:

Thin-layer plates, 200 x 200 mm, were spotted with 30 applications
of the crude "hydrocarbon" fraction along a line which divided the plate in half -
i.e., directly across the plate 10 cm from the bottom edge, and 10 cm from the
top edge. Spots were positioned 5 mm apart. The plate was then placed in an
upright position in a tank containing the first solvent mixture (petroleum ether,
b.r. 60°C - 80°C) to a depth of 9 cm. The solvent ascended, picked up the
alpha carotene plus the fluorescent fraction from the points of application and
carried them toward the top of the plate. When the front of the solvent was within
1 cm of the top of the plate, i.e. 9 cm above the points of application, the plate
was removed. A straight line was cut through the layer at a point 1 cm above
the points of application and parallel to them. The spots of alpha carotene which
were well above this level, along with all of the Silica Gel G adsorbent layer
more than 1 cm above the points of application were scraped from the plate, and
the exposed glass surface was wiped clean using acetone-saturated absorbent tissue.

The plate was then inverted so that facing the worker the upper 11 cm of
the plate contained a layer of Silica Gel G with the original points of application
running across 1 cm from the lower edge of the layer. The plate in this position
was placed in a tank containing acetone to a depth of 9.5 cm. Development in
this system moved the yellow pigment from the points of application up into the
unused portion of the thin-layer from which it was harvested for spectrophotometric
analysis.
When regular developing tanks are used for this technique it is not necessary to use large volumes of solvent to fill the tanks to depths of 9 - 9.5 cm. The volume of the tank can be reduced by displacement by simply standing a series of clean glass plates within the tank and leaving only enough space for addition of the plate to be developed. In this manner less than 200 ml of solvent is adequate to fill the tank to the desired level. One should calculate the rise in the solvent level caused by immersion of the lower half of the plate to be developed since the final level of the solvent is critical. This is especially true of the "reverse" system when the solvent level to begin with must reach the adsorbent layer but not the points of application which are only one cm above. It is best to start with inadequate solvent and add the necessary amount after the layer to be developed has been placed inside the tank.

When run through the spectrum the yellow pigment harvested by the above procedure showed absorption maxima at 378 mu, 401 mu and 422 mu (line 2, Figure 15). The three peaks suggested a mixture, but repeated attempts with scores of adsorbent-solvent combinations failed to separate the material into two fractions. In one system employing Silica Gel G layers developed in petroleum ether (b.r. 37.8°C - 58.2°C) and N-propanol (87:13) there was slight evidence to suggest two fractions. Developed layers revealed only one oval shaped spot, but when stained with iodine fumes or Carr-Price reagent it appeared that the spots were not uniform throughout. The upper half of an Rf 0.73 stained slightly different from the rear portion which, if the spot were divided in half, would have an Rf value of 0.69. This fraction remains unidentified.
b) "Esters" (freed alcohols)

This portion of the extract contained four detectable pigments, but only one could be isolated in sufficient amounts to permit determination of its absorption spectrum.

As with the "hydrocarbon" fraction, this fraction was run on several adsorbents in many solvent systems. Best separation of the main yellow pigment was obtained when using Silica Gel G layers developed in acetone, or calcium hydroxide layers developed in a hydrocarbon mixture of pentane-hexane-heptane-octane-undecane \( (60 + 20 + 10 + 6 + 4) \) mixed with methylene dichloride in a ratio of 95:5.

Using Silica Gel G layers in acetone a single oval spot was obtained having an Rf of 0.91. However, when stained with Carr-Price reagent, the spots appeared as mixtures staining green in the uppermost portion, blue in the middle and purple at the bottom. The material was harvested from several plates and rerun in additional systems but without successful further separation. The absorption spectrum of this material showed maximum absorption at 394 - 398 mu (Figure 19).

On calcium hydroxide layers developed in the hydrocarbon mixture - methylene dichloride (95:5) the main yellow pigment had an Rf value of 0.12. When harvested it gave the same absorption spectrum as did the material harvested from Silica Gel G layers.

The remaining three fractions, in the hydrocarbon solvent system outlined above moved very slowly and, although they were distinct and clear, the amounts were very small. All three had Rf values of less than 0.10. When stained
Figure 19  Unidentified pigment from "ester" (freed "alcohol") portion of extract, in petroleum ether.
with Carr-Price reagent they appeared as pink, blue, and pink arranged between the uppermost main yellow fraction (Rf 0.12) and the point of application. The Rf values of the three small fractions were too close together to be of any significance.

c) "Alcohols"

This fraction was found to contain 9 components, besides a fair amount of alpha carotene which for some reason was carried over into the "alcohol" portion of each partitioned cell extract. Why this carry-over occurred is unknown, but it may have been caused by the presence of fats, lipids, waxes or other matter contained in the primary crude methanol cellular extract. At any rate, on thin-layers of Silica Gel G developed in petroleum ether (b.r. 37.8°C - 58.2°C) and N-propanol (87:13), the alpha carotene was carried up front and separated from the other components. It was demonstrated as alpha carotene both by co-chromatography and by determining its absorption spectrum with absorption maxima at 446 mu and 473 mu.

In the same system described above, three additional fractions were detected as follows:

i) a fluorescent spot with an Rf value of 0.70 which stained a dull blue-grey with Carr-Price reagent,

ii) a visible yellow spot with an Rf value of 0.57 which stained a dark grey with Carr-Price reagent, and

iii) a visible faint pink spot with an Rf of 0.40 which stained blue with Carr-Price reagent.
The visible yellow portion was harvested from several 200 x 200 mm layers and the absorption spectrum was determined (Figure 20). It showed an elevated plateau at 405 - 425 μ without any peaks. The other two fractions were too weak in concentration to permit harvesting of sufficient amounts for spectral studies.

Eight fractions, including alpha carotene, and excluding the fluorescent fraction, were detected on layers of calcium hydroxide developed in the hydrocarbon mixture - methylene dichloride system previously described in studies of the "ester" fraction. In this the alpha carotene had an Rf of 0.84, the second visible yellow fraction had an Rf of 0.35 and stained blue with Carr-Price reagent. Below this in very fine well defined pencil line fractions were six more components, all with very close Rf values. In the order of their occurrence, below the visible yellow fraction (Rf 0.35) and reading toward the points of application, these 6 fractions when stained with Carr-Price reagent appeared as pink, blue, purple, brown, olive and olive. Despite the keeness of these individual components, it was not possible to sculpture out sufficient material for spectrophotometric analysis.
Figure 20  Yellow pigment from "alcohol" fraction; in petroleum ether.
V. DISCUSSION AND CONCLUSIONS
V. DISCUSSION AND CONCLUSIONS

It is not surprising that considerable interest has developed in the chromogenic mycobacteria during the past ten years. The clinical significance of specific varieties has been well documented. Basically the following needs have been apparent:

a) a speedy and reliable method for the recognition and classification of these organisms,

b) the discovery of suitable drugs for the effective treatment of diagnosed cases, and

c) the detection of the source(s) or reservoir(s) of these organisms, which in turn would explain the epidemiological aspects.

The first part of this study was directed towards more reliable means for the recognition and classification of chromogenic mycobacteria, whereas the latter part was primarily concerned with the pigments produced by M. kansasii, the type culture of Group I organisms.

Preliminary studies and the present investigation on chromogenic mycobacteria suggest that these organisms are much more common in Canada than is generally believed. The fact that 80 isolates, representing 2.63 per cent of cultures positive for mycobacteria at the Newfoundland Provincial Public Health Laboratory over the past eight years have been chromogenic appears to be significant. All strains originated from patients suspected of having tuberculosis. The only other Canadian incidence figure reported was based upon observations over a three year period at the Royal Edward Laurentian Hospital in Montreal, where approximately
4 per cent of the cultures positive for acid-fast bacilli were chromogenic (Mankiewicz, 1958). The author feels that the immediate detection of these strains in clinical specimens following the development of a personal interest, and the subsequent continued isolation at a constant rate over an eight year period, indicate that these organisms had previously existed, but had remained unrecognized. The prompt findings of chromogenic mycobacteria following the awareness of their existence suggest a beginning discovery rather than a beginning occurrence. What effect the incidence of chromogenic mycobacteria within a population has upon sensitization to Old Tuberculin, or upon the development of immunity towards typical M. tuberculosis is at present unknown. It has recently been shown, however, that immunization of mice with mycobacteria representing Runyon's Groups I, II, and III gives partial protection against infection with M. tuberculosis.

A photochromogenic strain equalled or surpassed the BCG strain in protective power (Satake, 1963; Siebenmann and Barbara, 1964).

The proportions of chromogenic strains in Newfoundland are much lower than those reported from many parts of the United States, but they are in agreement with the general trend that shows a decrease in the incidence from the South to the North along the Atlantic seacoast. It is perhaps unwise to make direct comparisons of rates of incidence as methods used in the treatment of certain clinical specimens prior to culturing for mycobacteria may seriously influence the isolation rates for various species and strains. In practice one attempts to rid specimens of all commensal organisms without destroying mycobacteria. If the specimens are insufficiently treated one stands to lose a large percentage of all cultures due to
the overgrowth of residual contaminating bacteria, whereas over treatment results in the death of all or part of the mycobacteria present. Although the majority of laboratories employ sodium hydroxide in the pretreatment of specimens (Rohde and Vera, 1956), the number of modifications for this one method alone certainly may influence the findings in different laboratories. When specimens are treated by other acceptable, but less commonly used methods such as mineral acid, oxalic acid, trisodium phosphate, trypsin etc., one should not attempt to compare isolation rates.

Equally significant as a factor affecting the rate of isolation is the culture medium employed. Again most laboratories use the buffered egg-potato medium of Lowenstein-Jensen, but a World Health Organization project on tuberculosis has shown that this medium, prepared under optimal conditions in seven European laboratories, gave marked differences in both the number of tubes supporting growth, and the degree of growth developing in positive cultures inoculated with standard inocula of different strains (Sula et al., 1960).

It is also known that the atmospheric conditions within culture tubes have a marked effect on the growth rate of various mycobacteria. Whitcomb et al. (1962) performed parallel cultures on 4,070 sputum specimens incubated under normal atmospheric conditions, and under 3 - 10 per cent carbon dioxide tension. In his study, 97 of 178 specimens positive for unclassified mycobacteria showed growth only in the presence of increased carbon dioxide tension. Thus, it is evident that specific varieties of mycobacteria may be favored, or inhibited, by the method of pretreatment of the specimen, the type of medium, or by the atmospheric conditions
of incubation. The findings in the present study substantiate the important role played by atmospheric conditions.

The problem of recognition and grouping of the chromogenic mycobacteria has not been limited to the author. Runyon's grouping, based on quantitative differences in pigmentation and growth rate, under uncontrolled conditions, is inadequate. The experience of Chaves (1960), when he forwarded 114 strains to Dr. Runyon for confirmation, is a good example of the incongruous findings resulting from the use of this classification. To quote from Runyon's reply to Chaves, "Only one photochromagen was identified. This was not one of those recognized by you. Indeed none of the nine strains designated on your cards as 'Photo' were photochromogens according to our testing and definitions." Quoting further, "Three cultures were designated 'atypical' by you, but we identified them as true tubercle bacilli."

In another study 64 strains isolated from patients and described as ranging in color from buff to light orange, when submitted to Runyon for confirmation were all classified as nonphotochromogens (Crow et al., 1957).

Hartwig (1960) found that upon repeated incubation in the light and in the dark an occasional culture originally classified as a nonphotochromogen produced pigment of the type associated with Group II scotochromogens (and vice versa). It has also been reported that freshly isolated photochromogens, more often than not, show no pigmentation (Nassau and Hamilton, 1957). Rogul et al. (1957), as well as the author, both in earlier work and in the beginning of the present study, found strains which produced more pigment when grown in the dark than when grown in the presence of light. Runyon's scheme of classification does not provide for
such "photophobes". Mallmann et al. (1962) described intermediates between Group I and II which they called "pseudochromes". In our hands repeat, or duplicate cultures, under apparently identical conditions, have frequently displayed such marked differences as to affect their grouping within the suggested scheme (Butler, 1962, 1963, 1964–65). Others have reported similar observations and have also stressed that pigmentation is unsatisfactory as a basis for the classification of these mycobacteria (Rodda and Singer, 1963; Xalabarder, 1961; Gordon and Rynearson, 1963).

It is recognized that even the identification of M. tuberculosis may frequently pose serious difficulties. There is unanimous agreement among those widely experienced that one cannot, with certainty, identify primary cultures of M. tuberculosis on the basis of colonial and microscopic morphology. Many cultures appear "atypical" especially in tubes where growth develops in or adjacent to water of condensation at the butt of the slant. Such cultures must be subjected to further investigation before being positively identified. Certainly the importance of the current problem is attested when experienced and qualified laboratories openly admit difficulties and errors encountered in identifying mycobacteria.

It is felt that the results of the present study should aid in and simplify the identification of M. tuberculosis and the sub-grouping of chromogenic mycobacteria. The method which is proposed as a result of this study to differentiate M. tuberculosis and M. bovis from all other mycobacteria is simple, reliable and rapid, and does not require difficult media or special equipment. The uniform turbidity produced in modified Sauton's medium by the growth of all mycobacteria other than M. tuberculosis and M. bovis is in sharp contrast to the flaky sedimented
growth with a perfectly clear supernatant which develops for the latter two strains. *M. tuberculosis* and *M. bovis* can be easily differentiated by the niacin test which is positive only for *M. tuberculosis*. It is interesting that a group of workers (Karlson et al., 1964), in recent months, proposed the use of a similar test employing a modified Proskauer and Beck medium. Their results however, were not as convincing. With their medium they encountered strains of *M. tuberculosis* which produced uniform turbidity, and strains of *M. avium*, scotochromogens, photochromogens and nonphotochromogens which produced a granular type of growth with a clear supernatant. Such exceptions have not been encountered by the author while using the modified Sauton medium over a three year period.

In the past the grouping of chromogenic mycobacteria by the use of Runyon's scheme has been most confusing, but with the knowledge gained from our studies on the "oxygen" requirements, it is now possible to explain many of the seemingly contradictory findings reported for specific strains in the hands of different workers. It likewise can account for the marked variations so frequently experienced in duplicate cultures.

Our results have shown that the presence of oxygen is essential for both photoactivation and pigment formation in *M. kansasii*, and for pigment synthesis in scotochromogenic mycobacteria. This finding can now be applied to the practical identification of these organisms.

In the cultivation of all slow growing pathogenic mycobacteria it is customary to employ inspissated egg-containing medium, prepared in test tubes
equipped with tightly fitting screw-type cap closures. Loosely fitting caps, caused by broken or poorly fitting gasket liners, or chipped lips of test tubes, result in appreciable loss of moisture and drying of the medium during several weeks of incubation. In instances where the caps are grossly misfitted, drying of the culture renders the medium unsatisfactory and tubes have to be discarded before being legitimately considered negative for mycobacterial growth. In other tubes appearing satisfactory in moisture content, one must realize that the extent of closure maintained will include that of absolute sealing, as well as all degrees of minor leakage. Slight leakage is the rule where re-use of test tubes and caps is practiced. Air exchange is also enhanced when inoculated tubes are reduced from incubator temperature to room temperature during weekly readings.

Mycobacteria are aerobic organisms which require enormous amounts of air (oxygen) for maximum growth. Novy and Soule (1925) demonstrated that a single tube culture of tubercle bacilli consumes from 153 - 183 ml of oxygen, an amount calculated by them to be present in 732 - 874 ml of air. Thus, an actively growing tube culture of tubercle bacilli consumes all the oxygen from three-quarters of a liter of air.

It was determined in this study that a volume of 250 ml of air will support excellent growth, photoactivation and pigment synthesis for a tube culture of *M. kansasii* (Figure 10). Although evidence indicates that chromogenic mycobacteria are possibly not as exacting as tubercle bacilli in their oxygen requirements, they are aerobic organisms (Tarshis and Frisch, 1952; Tarshis, 1959). Cabelli et al. (1954), using a fluid medium, have reported that the critical factor for the
rapid development of the chromogenic mycobacteria is apparently the oxygen tension of the medium.

This demand for a large volume of oxygen on the part of actively growing mycobacteria rapidly depletes all available oxygen within the limited confines of sealed tubes containing only 20 - 30 ml of air. One may further conclude, logically, that under such large demands oxygen tension would be reduced to a low level even where minor leakage occurs in screw caps, and lack of oxygen may still be the limiting factor of growth. The same may occur when cultures are grown within sealed Petri dishes.

If cultures grown within such sealed containers remain unopened prior to being subjected to a test requiring the presence of oxygen, it is natural that all levels of reaction will occur, depending on the existing oxygen tension. This supposition agrees with the experimental findings: unopened cultures of $M. \text{kansast}i$ failed to react to the photochromogenicity test. Below a critical level of oxygen tension no pigment was formed and such cultures were improperly grouped as nonphotochromogens (Group III). It is suggested that if photochromogenicity testing must be carried out on a culture grown in a screw capped tube or vial, adequate aeration must be performed at least 1 hour prior to light exposure. This period apparently conditions the culture within the oxygen containing atmosphere so that photoactivation can occur. Cultures grown in sealed tubes and aerated immediately prior to light exposure show only slight pigment development in the photochromogenicity test.

Scotochromogens may likewise be improperly classified as members of
Group III, when grown in unopened air-tight tubes. This situation is more complicated, and depends upon both growth rate and the actual time of pigment formation for the particular strain. If the bacterial growth utilizes all available oxygen, or maintains it below the level required for pigment synthesis, the result will be scanty growth with marked reduction or absence of pigment. At what phase of growth scotochromogens start to form pigment, and what level of oxygen tension is required, remains undetermined. In certain fungi it has been found that major pigment formation occurs only after the cultures are fully grown (Goodwin and Willmer, 1952; Mase et al., 1957; Goodwin, 1959).

The author considers the term "nonphotochromogen" for organisms of Group III unfortunate, since Runyon described the members of this Group as having little or no pigment. The name "nonphotochromogen" might suggest, to those unfamiliar with the field, the meaning of "scotochromogen" rather than "nonchromogenic". The terminology "nonphotochromogenic" has ushered in the use of "Chromogenic Mycobacteria" which again is misleading since it includes by definition nonpigmented varieties. The concept that "chromogenic" mycobacteria are always pigmented is a fallacy held by many bacteriologists, and was undoubtedly created in part by this improper terminology originally applied to Runyon's Group III organisms.

The special problem concerning the recognition of nonphotochromogens has been presented. It is difficult to accept the presence of both pigmented and nonpigmented organisms within the same Group. Nevertheless, it has been demonstrated that Runyon's stock strain P-7 produces a good yellow pigment. The
eleven strains isolated from clinical specimens in Newfoundland differ, since
they have not shown pigmentation despite repeated laboratory subculturings.

The early observation made in this study which suggested that the
presence of visible moisture within tubes affected both the growth and pigmen-
tation of chromogenic mycobacteria, eventually found its correct explanation when
the results of the "oxygen" studies were analyzed. It will be recalled that cultures
in tubes with moisture showed less growth and were nonpigmented (Figure 8). This
could be correlated with the demonstrated oxygen requirements. Tubes with
leaking caps permitted both evaporation of moisture as well as diffusion of gases
with resulting entrance of oxygen. This also explained why, in the "moisture"
studies, the "dry" tubes and the "dry" tubes made "moist" supported better growth and
pigment development than did "wet" tubes and "wet" tubes made "dry". The tubes
which were originally "dry" had leaking cap liners whereas "wet" tubes or "wet"
tubes made "dry" were equipped with tightly fitting caps. That moisture content
as such was unrelated to growth and pigment formation was proven by growing
cultures in vented tubes with and without moisture.

In one series of our studies there was a delay in pigment development
of photochromogenic strains following the removal of moisture from "wet" tubes.
This delay occurred because the cultures had not been photoactivated in the
oxygen depleted atmosphere within the tubes. Air exchange took place during
removal of the moisture, and only then were the cultures capable of being photo-
activated, but as at that time they were standing on the bench under subdued
light, pigment developed slowly.
It is strange that although the species \textit{M. kansasii} is identified by its distinctive characteristic of photochromogenicity, its pigment production \textit{per se} had not been investigated. It is evident from this study that if pigmentation is to be a criterion, cultures must be grown in loosely closed containers (tubes, vials, bottles, Petri dishes etc.) which will permit air exchange. It has been shown that no pigment is formed and growth is seriously limited when chromogenic mycobacteria are cultured in small tightly sealed vessels (Figures 2, 3, 9, 10). On the other hand, when adequate air is provided either by culturing these organisms in vented tubes or in hermetically sealed large containers (Figure 10), good growth and pigment development will regularly occur. In the case of \textit{M. kansasii} (photochromogens) it has also been shown that photoactivation and pigment synthesis are separate steps, and that each requires the presence of oxygen. The results on the role of oxygen in photoactivation of photochromogenic mycobacteria have been confirmed, in part, by the work of Wayne and Doubek (1964) who studied Runyon's P-1 strain and reported on the necessity of aerating cultures before subjecting them to photochromogenicity testing. They did not however, determine the principle involved, i.e. whether it was the removal of accumulated by-products of growth from the atmosphere of the tube, or whether added oxygen was the main factor. Furthermore, since their study was limited to the effect of aeration on photoactivation and did not consider the role of aeration as related to pigment synthesis following photoactivation, their findings bear no relation to the synthesis of pigments in either photo- or scotochromogenic mycobacteria.

Thermolabile factors are involved in both photoactivation and pigment
synthesis. The former will occur at temperatures as low as 0°C, and as high as the lower 40's, but not at 45°C. Pigment synthesis does not occur at refrigeration temperature, but will occur at 45°C, a temperature at which the organism does not multiply. A temperature of 50°C prevents pigment synthesis. An hypothesis was proposed by Rilling (1962) based on studies on a photochromogenic saprophytic species of mycobacteria (M. lacticola). He suggested that pigment synthesis may depend on enzymes induced by exposure to light. The findings with M. kansasii also support this hypothesis. Certainly from the temperature studies pigment synthesis appears to be enzymatic. Photoinduction (or photoactivation) occurring at 0°C is not likely to be enzymatic and appears to be a photochemical reaction, the end products of which seem to be relatively stable.

Several workers investigated the effect of shaking or aeration upon the growth rate of mycobacteria in liquid media and reported different findings which suggest that the results are governed by both the type of fluid medium involved and the particular strain of organism investigated. Lyon et al. (1962; 1963) demonstrated that aeration of Dubos broth cultures stimulated growth of M. tuberculosis in the presence of both glycerol or glucose provided Tween 80 was deleted. This finding was contrary to that of Weiss (1959) who had obtained large yields of well dispersed growth of M. tuberculosis H37Ra in mechanically agitated cultures in Dubos broth medium regardless of whether or not Tween 80 was present.

Our experimental results for representative strains of photochromogenic, scotochromogenic, and nonphotochromogenic mycobacteria showed a substantial increase in growth of all three types when cultures in Dubos-Tween 80 medium were shaken. The actual yield had to be determined by dry weight because the
optical density of the cultures, due to bacterial aggregates, was not proportional
to bacterial mass. These findings suggest to us that the effect of aeration on the
growth of these organisms must be great, because despite the formation of bacterial
aggregates, the total bacterial mass was significantly increased. One may assume
that bacterial multiplication within aggregates is limited primarily to the bacteria
located on the surface of the clumps. The entrance of oxygen and essential
nutrients, as well as the release of bacterial waste products from the bacteria
located towards the center of the aggregates would tend to favor a dying or "resting"
population rather than an actively multiplying one.

The current findings for chromogenic mycobacteria appeared to be
independent of the type of medium used. Shaken cultures showed greater rates
of bacterial growth than did stationary cultures, regardless of whether Dubos
Tween 80 medium or Sauton medium was employed.

In the course of this study it has been established that the media
commonly employed for the cultivation of mycobacteria are of suitable pH for
both photoactivation and pigment synthesis in chromogenic mycobacteria. When
the pH is more alkaline than 7.3 there is a marked decrease in the rate of growth
of M. kansasii. Although Sauton medium, at the time of preparation, is adjusted
to a pH of 7.4, it decreases to approximately 7.2 during autoclaving. Optimum
growth of M. kansasii occurs near neutrality.

Results showed that photoactivation of M. kansasii can occur over a
wide range of temperatures which is well beyond the variations which may occur
in room temperatures within most laboratories. It should be pointed out, however,
that since photoactivation can occur at incubator temperature (37°C), care should be taken to shield growing cultures from light. Even intermittent access of light into the incubator by merely opening the door, as is necessary throughout the normal working day, has been found adequate to support low levels of photoactivation. In this manner photochromogenic cultures may incorrectly be considered scotochromogens. All cultures for pigment assessment should be grown in complete darkness.

The somewhat accidental and unexpected discovery of a potent growth stimulating factor for *M. kansasii* has been reported in Part I of this thesis. From the manner in which the growth of *M. kansasii* was stimulated throughout half of the originally contaminated Petri dish culture (Figure 5), it appeared that the factor involved was water soluble. Whether or not the strain of *Sarcina lutea* produces the growth stimulating factor only when grown on Dorset medium, and whether or not it is a product of symbiotic growth remains to be determined. To our knowledge no observation of a similar nature has ever been reported. If the factor can be isolated, identified, and incorporated into media, it may prove to be of importance. It is hoped to investigate this phenomenon further.

Marked pleomorphism was observed in subcultures of *M. kansasii*. The elongation of cells on Dorset medium following photoactivation and pigment synthesis, along with the change from regular to irregular staining, closely resembles the morphology of a photochromogen which was isolated from a patient with "pulmonary tuberculosis", and described by Gale (1961). The peculiar forms which developed in Sauton medium, consisting of filaments up to 45 microns in length
and containing one or more cellular "spore-like" inclusions which stained poorly and caused the cells to swell to double or triple their diameter, have not previously been reported for chromogenic mycobacteria. These structures along with clubbing and heavily stained beading resemble the forms encountered by Juhasz (1961) during conjugation studies on the BCG strain.

It is possible that these various morphological forms represent different stages in the life cycle of the organism. Such complex cycles have been described for strains of mycobacteria including the avian tubercle bacillus (Mellon et al., 1932-1933) and M. tuberculosis (Csillag, 1962; 1963a,b; 1964). The "spore-like" inclusions seen in M. kansasii (Figure 6, C, E) appear to be identical with a form of M. tuberculosis described by Csillag (1964). It would be interesting to investigate the possible role of mycobacteriophages with respect to these morphological features. The most drastic changes occurred in the "albino" strain of M. kansasii which could not be photoactivated and induced to form pigment.

Artificially induced pigmentless mutants of M. kansasii and scotochromogenic mycobacteria have been reported, but their morphology had not been described (Tsukamura, 1964). Naturally occurring nonpigmented strains of M. kansasii were reported at the latest research conference in pulmonary diseases of the United States veterans administration – armed forces (Rothstein, 1964), but their morphology was not described.

In support of some workers, and in disagreement with others, good "cord" formation in a scotochromogenic strain of mycobacteria has been observed and recorded (Figure 7). Some investigators still link this "cord" factor with virulence.
Oddly enough, this particular strain was isolated from an active, chronic, bilateral moderately advanced case of "pulmonary tuberculosis".

The second part of this investigation involved the isolation and characterization of pigments extracted from large harvests of *M. kansasii*. The growth of organisms in test tubes containing 10 ml of Sauton medium was both slow and tedious, but it had been determined to be the best method in securing sufficient amounts of pigmented bacteria.

Neither the original method of Kuhn and Brockmann (1932) for the extraction and partition of bacterial pigments, nor the modified procedures suggested by Sabin and Stahly (1942) and Starr and Stephens (1964) had been used for members of the genus *Mycobacterium*. In the present study, the modification by Starr and Stephens was employed and it was found to be satisfactory. It was shown however, that by substituting a mixture of methanol:chloroform (1:1 v/v) for methanol as the extracting solvent, the pigments were extracted faster and at lower temperatures than with methanol alone. Parallel extractions performed with methanol:chloroform (1:1 v/v) and methanol alone gave identical spectrophotometric absorption curves. The fact that the mixture of methanol:chloroform does extract the pigments at lower temperatures could be significant. Some fractions under extraction may be heat labile, or may be involved in internal systems in which excessive heat may precipitate undesirable reactions. For these reasons the temperature of extraction in this work was maintained below 50°C. This is in contrast to the original method in which the methanol was brought to the boiling point.
The cell extract of \textit{M. kansasii}, when partitioned in petroleum ether and 90 per cent aqueous methanol revealed the presence of both epiphasic and hypophasic pigments. This is in agreement with the observations of Costello (1963, personal communication). While studying chromogenic mycobacteria he detected, but failed to identify, a fraction with this partition characteristic. It gave a bell shaped absorption spectrum with a single broad peak at 452 \textmu m in petroleum ether, and 462 \textmu m in ethanol. He pointed out that these characteristics were the same as those described by Turian (1950b) for a carotenoid pigment similar to astacine, and named chrysoflein. This pigment however, is not the same as the one isolated from \textit{M. kansasii} in this study. Our results showed that the hypophasic fraction had a single peak in petroleum ether, but it was located across a wide wavelength, ranging from 405 \textmu m to 425 \textmu m (Figure 20). The only two other studies concerning pigments in unclassified mycobacteria both failed to detect pigment which was hypophasic in petroleum ether and 90 per cent aqueous methanol (Tsukamura, 1962b; Ebina et al., 1962). Both reports state that all pigments isolated were epiphasic to this system, which suggested that they were without hydroxyl groups. Rilling (1964) detected, but did not identify, a hypophagic fraction in extracts of \textit{M. lacticola}. The absorption spectrum was similar to beta carotene, but its solubility characteristics indicated it was a polyhydroxy compound. This is unlike the hypophagic fraction isolated from \textit{M. kansasii}.

It was noted that the pigments of \textit{M. kansasii} which were hypophasic to petroleum ether and 90 per cent aqueous methanol were eluted from the bacteria early during the extraction process. This could be due to differences in their
solubility, location, or charge. It was possible to take advantage of this finding since only a small portion of the several liters of methanol extract required more than the single partition to separate the "hydrocarbons" from the "esters" (freed "alcohols").

There were no traces, either visible or chemical, of carotenoid "acids" which would have remained in the aqueous hypophase when the original methanol hypophase was partitioned with diethyl ether.

No figures are available for comparison concerning the total amount of carotenoids produced by chromogenic mycobacteria. It appears, nevertheless, that the present finding of 403 ug/gm dry weight is a good yield. Carotenoid production has been reported for the saprophytic strain _M. phlei_ by Goodwin and Jamikorn (1956) and by Schlegel (1959). The former workers studied three different strains and found a total content of 144 ug, 61 ug, and 106 ug. Schlegel in a study of four strains found a wide variation from 10,015 ug/gm dry weight down to 71.1 ug/gm dry weight. Undoubtedly these values are dependent upon many variable factors. To quote the above merely indicates that higher and lower total carotenoid yields than that found in _M. kansasii_ have been reported for other strains of mycobacteria.

No carotenoid pigments were detected by our methods in extracts prepared from cultures of _M. kansasii_ that had been grown in the dark (Figure 13). This would imply that if they are present the amounts are negligible since the method employed, thin-layer chromatography, was far more sensitive than either column or paper chromatography. With iodine staining of thin-layers, amounts of 0.05 ug
of carotenoid could be detected. The absence of carotenoids in cultures of *M. kansasii* (ATCC 12478) grown in the dark suggests a possible difference among photochromogenic mycobacteria. Ebina et al. (1962) studied the photochromogen P-8 strain of Runyon's stock culture collection and reported finding 4 visible pigments in both light and dark grown cultures. Three of the four pigments were quantitatively equal in both cultures, but the fourth pigment was much greater in the cultures grown in light and was considered by them to be the pigment which developed when cultures grown in the dark were exposed to light. Rilling (1962) in studying extracts of the saprophyte *M. lacticola* also found the synthesis of small quantities of carotenoids in dark grown cultures. He stated that the light independent carotenogenesis was quantitatively less than the photoinduced synthesis, and also qualitatively different. Also interesting was the finding that photoinduced carotenogenesis included an unidentified hypophagic fraction which was absent in cultures grown in the dark.

Ebina et al. (1962) reported the presence of a fluorescent fraction in extracts of a photochromogen grown in the dark. They worked with Runyon's strain P-8, and the strongly fluorescing purple fraction showed absorption maxima of 348 mu and 368 mu in petroleum ether. It was identified as phytofluene, and because it was present in greater amounts in the "dark grown" than in the "light grown" culture they suggested it as a possible precursor for the beta carotene pigment which developed when cultures were exposed to light. Comparable fluorescent material could not be detected in the present work. The findings of Ebina et al. cannot be directly compared with our results since their photochromogenic
strain was first grown in the dark for one month and then exposed to light for only three days. In the present study the cultures were grown in continuous light.

For the intended work on the characterization of the pigments produced by *M. kansasii* a choice of the methods and procedures to be employed had to be made. After due consideration of the available systems thin-layer chromatography was chosen as best suited for our purposes. Commercial equipment for this method was introduced in North America at the end of 1960. Since that time the technique has been used with increasing frequency. This system of micro adsorption chromatography supplements the previously known techniques of column and ion-exchange chromatography. The advantages of thin-layer include speed of separation, sharpness of separation, sensitivity, ease in the manipulation of small quantities, and simplicity in harvesting separated fractions. The technique had been used by a few workers to investigate the pigments of bacteria and algae, and it was hoped that it would also prove adequate in the detection of pigment fractions in the extracts of mycobacteria.

This expectation has been warranted; thin-layer chromatography permitted the separation and harvesting of the major pigment fractions in sufficient amounts for spectrophotometric analysis. Of even greater significance was the ability of this method to detect several smaller fractions which could not have possibly been separated by column chromatography. It was found that once a suitable combination of adsorbent and solvent had been determined for the separation of a particular fraction, no difficulties were ever encountered in reproducing the test results.
The procedure for "one dimensional-reverse two solvent chromatography", as developed and used in the course of this work, possessed the following three distinct advantages:

1. It was time saving by eliminating the laborious steps of: harvesting areas from the developed layers, eluting of material, flashing to dryness, dissolving in appropriate solvent, and reapplication of the material to a second layer.

2. It was economical from the standpoint of materials and equipment. The cleaning, spreading, activation and storage of extra thin-layers is both costly and time consuming.

3. Its greatest advantage was that it eliminated conventional manipulations with the risk of an appreciable loss of a valuable "unknown" present in very small amounts.

By carefully selecting appropriate combinations of adsorbents and solvents, this "reverse" thin-layer procedure can be utilized for the separation of fractions from complex mixtures.

A positive identification of the major pigment of **M. kansasii** was made by co-chromatography and spectrophotometric analysis. As outlined in the "Experimental Methods" known standards were used in both procedures. In petroleum ether the main pigment showed absorption maxima at 446 mu and 473 mu, corresponding to those obtained for the alpha carotene standard (Figure 16; Figure 17 line 2). The absorption spectrum of the extracted alpha carotene is compared with the spectrum of standard beta carotene in Figure 17. Beta carotene has absorption maxima at 450 mu and 476 mu in petroleum ether.
The discovery that the major pigment was alpha carotene is of interest since it is in contrast with the only two reports to date concerning the pigments of slow growing photochromogenic mycobacteria. Both earlier reports claimed beta carotene to be the major pigment developing (Ebina et al., 1962; Tsukamura, 1962). Ebina et al., studying Runyon's photochromogenic strain P-8 found both alpha and beta carotene in cultures whether they were grown in the light or in the dark, but the major pigment in the former was identified as beta carotene. Tsukamura studied two strains of slow growing photochromogenic mycobacteria. He found one to synthesize only beta carotene, while the second possessed beta carotene and lycopene. Lycopene is a red pigment showing absorption maxima at 446 μm, 474 μm, and 506 μm in petroleum ether.

Although a total of 16 visible pigments were noted in the course of this work only the major fraction has been positively identified as alpha carotene. Four further fractions were harvested in sufficient amounts from the thin-layers to permit spectrophotometric analysis.

To recapitulate, the differences in the findings made in this study as compared with the work of others on photochromogenic slow growing mycobacteria are:

1. The detection of alpha carotene as the main pigment rather than beta carotene.
2. The complete absence of beta carotene.
3. The complete absence of a fluorescent fraction with absorption maxima at 348 μm and 368 μm. (This was suggested by others as a possible pigment precursor).
4. The detection of several epiphasic and hypophasic pigments which had not previously been described.

A variety of reasons could be suggested to explain these differences. The present study was the first and only one performed on the type culture (ATCC 12478) of the species M. kansasii.

Qualitative differences in pigment may be due to differences in media, the periods of exposure to light, and extraction methods. There is also the possibility that the bacteria may convert one carotenoid to another. Schlegel (1959) has reported conversion of carotenoids to oxycarotenoids in a strain of M. phlei.

The complete absence of beta carotene in extracts from pigmented cultures of M. kansasii, as well as the complete absence of phytofluene (fluorescent fraction) in the extracts of the nonpigmented M. kansasii suggest differences among strains of photochromogens.

The last of the four major differences, the detection of several epiphasic and hypophasic fractions not previously reported, is probably due to the sensitivity of the thin-layer chromatography as compared with column chromatography used in the other studies.
VI. SUMMARY
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1. A total of 80 strains of chromogenic mycobacteria were isolated from 69,556 clinical specimens cultured for M. tuberculosis. This number represents 2.63 per cent of the cultures positive for acid-fast bacilli. On primary culture growth of the chromogenic mycobacteria was considerably slower than growth of M. tuberculosis.

2. A method is described which will detect all chromogenic (atypical) mycobacteria. It is based upon the appearance of growth developing in modified Sauton's medium. All chromogenic mycobacteria, including the weakly pigmented nonphotochromogenic varieties which often resemble M. tuberculosis on solid media, produce a dispersed uniform turbidity. M. tuberculosis and M. bovis grow as flaky sediments with clear supernatants. The test is simple, reliable and does not require difficult media or special equipment.

3. Pleomorphism, with filaments up to 45 microns including one or more "spore-like" structures has been observed in M. kansasii (ATCC 12478). Non-acid-fast bipolar staining bacilli and small coccoid forms have been seen, as well as "albino" and "scotochromogenic" varieties of this organism.

4. It has been shown that both photoactivation and pigment synthesis in photochromogenic mycobacteria, and pigment synthesis in scotochromogenic mycobacteria, require the presence of oxygen. Chromogenicity tests on cultures, for the purpose of classification within Runyon's scheme, are most unreliable.
when cultures are grown in tightly sealed screw capped tubes or vials.

5. It has been determined that photoactivation and pigment synthesis can occur over a wide range of temperatures and pH. These two factors will not affect the results of chromogenicity studies when commonly employed media at normal laboratory temperature are used.

6. Photochromogenicity tests are best performed on young actively multiplying cultures of \textit{M. kansasii} (2 - 3 weeks).

7. An unidentified growth stimulating factor for \textit{M. kansasii} has been noted. A strain of \textit{Sarcina lutea}, which accidentally contaminated a culture of \textit{M. kansasii} on Dorset medium, markedly stimulated the growth of the organism.

8. Methanol extracts of \textit{M. kansasii} (ATCC 12478) in partition between petroleum ether and 90 per cent aqueous methanol were found to contain both epiphasic and hypophasic pigments. Approximately 97 per cent of the total pigment was epiphasic. An average of 403 ug of carotenoids were extracted per gram (dry weight) of bacteria. A total of 16 visible pigments was detected by thin-layer chromatography. The major pigment was identified as alpha carotene. Four additional pigments were present in sufficient amounts to permit their characterization by spectrophotometry.

9. A new technique has been developed which extends the usefulness of thin-layer chromatography. It is time saving, economical, and it eliminates wasteful manipulations of small amounts of "unknown" material. The term "one-dimensional-reverse two solvent chromatography" is proposed for this technique.
VII. CONTRIBUTION TO KNOWLEDGE AND CLAIM TO ORIGINALITY
VII. CONTRIBUTION TO KNOWLEDGE AND CLAIM TO ORIGINALITY

The factor responsible for the frequent irregularities in pigmentation of chromogenic mycobacteria has been determined. Procedures are suggested to permit reliable chromogenicity testing for the differentiation of photochromogenic and scotochromogenic mycobacteria.

An original method is described which will detect all chromogenic (atypical) mycobacteria including nonphotochromogenic varieties which often resemble M. tuberculosis on solid media.

Pigments of M. kansasii (ATCC 12478) have been studied for the first time. The major pigment has been identified as alpha carotene, and several pigments were detected which were hypophasic to petroleum ether and 90 per cent aqueous methanol. Since our findings differ from those reported for other photochromogenic mycobacteria studied to date, it is suggested that there may be differences among strains of Group I mycobacteria although the Group is considered to be homogeneous.

By employing thin-layer chromatography it has been possible to detect several visible pigments in methanol extracts of M. kansasii. In addition to alpha carotene, four fractions have been characterized spectrophotometrically. A new technique, "one-dimensional-reverse two solvent chromatography" has been developed which extends the usefulness of thin-layer chromatography.

A growth stimulating effect was observed when M. kansasii was grown simultaneously with a strain of Sarcina lutea on Dorset egg medium. The mechanism of this effect has not been determined as yet.
VIII. APPENDICES
APPENDIX A

MEDIA
APPENDIX A

A-1. LOWENSTEIN - JENSEN MEDIUM

Bacto-Lowenstein Medium Base*

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacto-Asparagine</td>
<td>3.6 gm</td>
</tr>
<tr>
<td>Monopotassium Phosphate</td>
<td>2.4 gm</td>
</tr>
<tr>
<td>Magnesium Sulfate</td>
<td>0.24 gm</td>
</tr>
<tr>
<td>Magnesium Citrate</td>
<td>0.6 gm</td>
</tr>
<tr>
<td>Potato Flour</td>
<td>30.0 gm</td>
</tr>
<tr>
<td>Malachite Green</td>
<td>0.4 gm</td>
</tr>
</tbody>
</table>

Eggs

Fresh eggs are broken into a sterile beaker, homogenized, and strained through sterile gauze.

Procedure:

To rehydrate Bacto-Lowenstein Medium Base, 37.2 gm are suspended in 600 ml of cold distilled water containing 12 gm of glycerol. This is heated to the boiling point while being constantly stirred, and then autoclaved for 15 minutes at 15 pounds' pressure. The medium base is cooled to 45 - 60°C and 1000 ml of uniform suspension of fresh eggs is added. After mixing, the medium is dispensed into sterile tubes (20 mm x 125 mm) and inspissated in a slanted position at 85°C for 45 minutes. Prepared media is checked for sterility by incubating it at 37°C for 48 hours. Media is stored at room temperature until used.

* Difco Laboratories, Detroit, Michigan.
A-2. DORSET EGG MEDIUM WITH GLYCEROL*

Fresh eggs are broken, homogenized, and strained. The homogenized eggs are mixed with 0.85 per cent saline in a ratio of 2:1 and glycerol is added in an amount representing 5 per cent of the total volume. The medium is dispensed in 20 mm x 125 mm tubes, and inspissated in a slanted position at 85°C for a period of 45 minutes.

A-3. DUBOS FLUID MEDIUM*

Dissolve 1.3 gm of Bacto-Dubos Broth Base in 180 ml of distilled water. Autoclave at 121°C for 15 minutes. Cool to below 50°C and aseptically add 20 ml of Bacto-Dubos Medium Albumin. Mix and dispense aseptically into tubes, or prepare directly in flasks.

Contents per liter:

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacto-Asparagine</td>
<td>2 gm</td>
</tr>
<tr>
<td>Bacto-Casitone</td>
<td>0.5 gm</td>
</tr>
<tr>
<td>Disodium Phosphate</td>
<td>2.5 gm</td>
</tr>
<tr>
<td>Monopotassium Phosphate</td>
<td>1.0 gm</td>
</tr>
<tr>
<td>Ferric Ammonium Citrate</td>
<td>50 mg</td>
</tr>
<tr>
<td>Magnesium Sulfate</td>
<td>10 mg</td>
</tr>
<tr>
<td>Calcium Chloride</td>
<td>0.5 mg</td>
</tr>
<tr>
<td>Zinc Sulfate</td>
<td>0.1 mg</td>
</tr>
<tr>
<td>Copper Sulfate</td>
<td>0.1 mg</td>
</tr>
<tr>
<td>Tween 80</td>
<td>0.2 gm</td>
</tr>
</tbody>
</table>

* As used in the Department of Bacteriology and Immunology, McGill University.
A-4. **MINERAL SALTS MEDIUM***

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>MgSO(_4),7H(_2)O</td>
<td>0.2 gm</td>
</tr>
<tr>
<td>CaCl(_2)</td>
<td>0.02 gm</td>
</tr>
<tr>
<td>KH(_2)PO(_4),3H(_2)O</td>
<td>1.0 gm</td>
</tr>
<tr>
<td>K(_2)HPO(_4)</td>
<td>1.0 gm</td>
</tr>
<tr>
<td>NH(_4)NO(_3) or (NH(_4))(_2)SO(_4)</td>
<td>1.0 gm</td>
</tr>
<tr>
<td>Distilled water</td>
<td>1000 ml</td>
</tr>
</tbody>
</table>

The medium was adjusted to pH 7.0 to 7.2 with dilute NaOH, and autoclaved at 121°C for 15 minutes. Five per cent sterilized mineral oil was added aseptically to each flask.

A-5. **SAUTON MEDIUM** **

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dipotassium Phosphate</td>
<td>0.5 gm</td>
</tr>
<tr>
<td>Magnesium Sulfate</td>
<td>0.5 gm</td>
</tr>
<tr>
<td>Citric Acid</td>
<td>2.0 gm</td>
</tr>
<tr>
<td>Asparagine</td>
<td>4.0 gm</td>
</tr>
<tr>
<td>Iron Citrate (Ammoniacal)</td>
<td>0.05 gm</td>
</tr>
<tr>
<td>Glycerol</td>
<td>35.0 ml</td>
</tr>
<tr>
<td>Distilled water to</td>
<td>1000 ml</td>
</tr>
</tbody>
</table>

Adjust pH to 7.4 with sodium hydroxide. Autoclave at 15 lbs pressure for 15 minutes.

---

NOTE: In some pigment studies 10 gm of sodium glutamate were substituted for the 4 gm asparagine per liter (see text).

SAUTON MEDIUM (MODIFIED) WITH BUFFER AND SERUM*

1. Sauton medium:

- Asparagine 4 gm
- Magnesium Sulfate 0.5 gm
- Citric Acid 2.0 gm
- Glycerol 60 gm
- Bipotassium Phosphate 0.5 gm
- Ferric Ammonium Citrate 0.05 gm
- Distilled water 940 ml

2. Buffer (pH 7.15)

\[ \text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O} \text{ (179 gm in 1000 ml)} \]
\[ \text{KH}_2\text{PO}_4 \text{ (68 gm in 1000 ml)} \]

The final mixture is prepared by adding 800 ml of distilled water and 200 ml of the buffer (solution 2) to 1 liter of Sauton medium (solution 1). This is autoclaved at 15 pounds' pressure for 20 minutes. After cooling, sterile human serum is added aseptically to a concentration of 5.0 per cent, and the medium is dispensed in tubes in 5 ml quantities.

NOTE: For this study, ten pounds of dehydrated Sauton Medium Base (without glycerol and serum) were prepared by Baltimore Biological Laboratories under special order.

APPENDIX B

REAGENTS
APPENDIX B

B-1. SOLVENTS

Acetone; b.r. 56.1°C - 56.5°C (Fisher).
Benzene; b.r. 80.1°C - 80.3°C (Fisher).
Carbon Tetrachloride; (British Drug House).
Chloroform; (Fisher).
Dioxane; b.r. 101.1°C - 101.4°C (Fisher).
Ether, anhydrous; (Mallinckrodt).
Ethyl Acetate; b.r. 77.0°C - 77.3°C (Fisher).
Ethylene Chloride; b.r. 82°C - 84°C (British Drug House).
Heptane; b.r. 98°C - 99°C (Baker).
N-Hexane; (Fisher).
Methanol; b.r. 64.6°C - 64.8°C (Fisher).
Methylene Chloride; (Matheson, Coleman, and Bell).
Octane; (Matheson, Coleman, and Bell).
Pentane; b.r. 35°C - 37°C (Baker).
Petroleum Ether; b.r. 37.8°C - 58.2°C (Fisher).
Petroleum Ether; b.r. 60°C - 80°C (British Drug House).
N-Propanol; b.r. 96.9°C - 97.4°C (Fisher).
Undecane; b.r. 194°C - 196°C (Matheson, Coleman, and Bell).

B-2. CHEMICALS AND REAGENTS

Alpha Carotene, 100% (Mann Research Laboratories Inc.).
Beta Carotene, 100% (Eastman Organic Chemicals).
Carr-Price Reagent (Fisher).
Paraffin Oil, white heavy, viscosity 335/350 (Fisher).
APPENDIX C

PROCEDURES
APPENDIX C

C-1. PROCEDURE FOR THE NIACIN TEST*

A mature and fairly heavy growth of the organisms on a solid medium
(fresh original culture or 2 week subculture on Lowenstein-Jensen medium) should
be used. The culture must be actively growing.

1. Flood the surface of each culture with 1 ml distilled water and place it
   in a horizontal position for 10 minutes.

2. Transfer water from each culture to a correspondingly numbered screw
   cap tube.

3. Add 1 ml of 4% alcoholic analine solution to each tube.

4. Add 1 ml of 10% aqueous cyanogen bromide solution to each tube.

5. Gently shake tubes and observe for color development (about 10 -
   20 seconds).

A yellow color indicates a positive test. Absence of color indicates a
negative test. Include both positive and negative controls.

Preparation of Reagents:

4% analine in 95% ethyl alcohol should be nearly colorless.

10% aqueous solution of cyanogen bromide is prepared under a chemical
hood.

* REFERENCE: Handbook of Tuberculosis Laboratory Methods, Veterans Administration-
  Armed Forces Cooperative Study on the Chemotherapy of Tuberculosis,
  Veterans Administration, Department of Medicine and Surgery,
  Washington 25, D.C., November 1962, 11 - 12
Both reagents will remain stable for months if kept in a brown bottle and refrigerated when not in use. If crystals form during refrigeration, they must be redissolved before using the reagent.

CAUTION:

Cyanogen bromide is a highly toxic chemical and its vapor is dangerous to the eyes and extremely irritating if inhaled. This chemical must be stored and handled with great care.

C-2. SENSITIVITY TESTING OF MYCOBACTERIA*

Medium

Sauton's medium (modified) with buffer and serum** was used in sensitivity tests. The ingredients and preparation of this medium have been given in Appendix A -5.

The following three antituberculous agents, each in the series of three concentrations indicated, were used routinely in the testing of all mycobacteria isolated from clinical specimens.

a) Streptomycin: 3, 10 and 25 ug/ml

b) PAS: 1, 5 and 25 ug/ml

c) INH: 1, 5 and 10 ug/ml

Medium was prepared from dehydrated Sauton medium base manufactured by Baltimore Biological Laboratories under special order. The antituberculous agents

* Procedure proposed by the Laboratory of Hygiene, Ottawa (Dr. Greenberg, personal communication).

were aseptically added to sterilized Sauton medium. Agents were maintained in concentrate stock solutions and appropriate working solutions were freshly prepared so that the addition of 0.1 ml per tube containing 5 ml of medium yielded the concentrations stated above.

Inoculated tubes were incubated at 37°C and readings were made at the end of two weeks.

Results were interpreted as follows:

**Streptomycin:**
- Inhibited at 3 ug/ml - sensitive
- Inhibited between 3 to 10 ug/ml - moderately sensitive
- Growth at 10 ug/ml - resistant

**PAS:**
- Inhibited at 1 ug/ml - sensitive
- Inhibited between 1 to 5 ug/ml - moderately resistant
- Growth at 5 ug/ml - resistant

**INH:**
- Inhibited at 1 ug/ml - sensitive
- Inhibited between 1 to 5 ug/ml - moderately resistant
- Growth at 5 ug/ml - resistant
IX. BIBLIOGRAPHY
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