A STUDY OF ATYPICAL CHROMOGENIC ACID-FAST BACILLI ISOLATED FROM HUMAN SOURCES AND BLOOD MEDIA FOR THE BACTERIOLOGIC DIAGNOSIS OF TUBERCULOSIS

by

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A THESIS

Presented to the Department of Bacteriology
and the Graduate Division of the University of Oregon Medical School
in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy

September 1950

APPROVED:

[redacted]

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PART I

A STUDY OF ATYPICAL CHROMOGENIC ACID-FAST BACILLI ISOLATED FROM HUMAN SOURCES

INTRODUCTION

During studies with human tubercle bacilli, atypical acid-fast organisms were isolated from time to time from patients with clinical tuberculosis. These organisms are characterized by their pale yellow to deep orange color and the production of smooth colonies. Because they resemble many saprophytic acid-fast bacteria and may be confused with the pathogenic tubercle bacilli they are often difficult to evaluate. Since little is known regarding the exact nature of these strains and their pathologic potentialities in man, further studies are indicated. It is the purpose of this investigation, through cultural and animal experiments to throw some light on the possible position and importance of this group of organisms. These data are presented in Part I of this thesis.

It was further observed that human blood media are capable of growing tubercle bacilli as well as several of the best standard tuberculosis media. Because of the advantages which the blood media appear to offer, it seemed desirable to investigate in detail the use of human blood for the bacteriologic diagnosis of tuberculosis. These studies are included in Part II.

GENERAL DESCRIPTION AND CHARACTERISTICS OF THE MYCOBACTERIA

Classification

The Mycobacteria comprise a large group of strictly aerobic, acid-fast bacilli and are discussed in detail in the following sections. They constitute the genus Mycobacterium of the family Mycobacteriaceae of the order Actinomycetales. The key and classification below is given by Stitt et al.[1] and indicates the various organisms included in this

Key To The Order Actinomycetales[1]

Order II. Actinomycetales. Cultures more like molds.
Family I. Mycobacteriaceae. Very slight branching.
Genus: Mycobacterium. Acid-fast. Cultures usually more or less wrinkled and dry.

I. Grow rapidly on ordinary media at room temperature. Typical species are:
1. Mycobacterium phlei. (Timothy-grass bacillus of Moeller.)
2. Mycobacterium lacticola (M. smegmatis). (The "smegma" bacillus.)

II. Grow only at body temperature and after several weeks of incubation. Primary isolation requires such culture media as solidified blood serum, glycerin agar, glycerin potato, and egg media.
A. Cultures fairly moist, luxuriant, and flat. Optimum temperature 43 C.
1. Mycobacterium avium. (Bacillus of avian tuberculosis.)
B. Cultures scanty, wrinkled, and dry. Appear in 10 to 14 days. Optimum temperature 38 C. Smear from organs of inoculated guinea pig shows few bacilli. Less virulent for rabbits.
1. Mycobacterium tuberculosis var. hominis. (Bacillus of human tuberculosis.)
C. Cultures as above, but even more scanty. Smear from organs of guinea pig shows many bacilli.
1. Mycobacterium tuberculosis var. bovis. (Bovine tubercle bacilli.)

III. Primary isolation requires growth factor from heat-killed acid-fast bacilli.
1. Organism of Johne's disease.

IV. Are not cultivable by ordinary methods.
1. Mycobacterium leprae (B. leprae). Found in man chiefly in nasal mucus and in juice from lepra tubercles. Less often in nerve leprosy.
2. Mycobacterium lepraemurium. (Bacillus of rat leprosy.) Indistinguishable from M. leprae except by inoculation into young rats.

Family II. Actinomycetaceae. Filamentous and branching, forming mycelia which break up into fragments which may function as conidia.
Genus: Actinomyces. No aerial hyphae. Anaerobic. Not acid-fast.
1. Actinomyces bovis (A. israeli). Cause of actinomycosis.
Genus: Nocardia. Short aerial hyphae. Aerobic. Some species acid-fast.
1. Nocardia madurae. Cause of mycetoma. Not acid-fast.
2. Nocardia asteroides. Acid-fast.

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[1]From Stitt et al., 1948. The cold-blooded Mycobacteria are not included.

order and some of their general features. The data in Table 1 indicates more specifically the general character of growth, natural habitat, other sources from which the organisms are isolated and animal pathogenicity of the Mycobacteria.

Since the property of acid-fastness is also possessed by members of the family Actinomycetaceae, a brief discussion of their characteristics and significance is included below. The family Actinomycetaceae is a heterogeneous group of filamentous mold-like micro-organisms, growing in the form of a branched mycelium, and exhibiting characteristics intermediate between the bacteria and molds. Colonies of these organisms are usually dry, tough, and wrinkled. Their mycelial structures are delicate, usually less than 1 micra in diameter, and, therefore, within the realm of bacterial dimension. The mycelium is septate, with marked tendency to branch and fragment into bacillary and coccoid structures. Under special conditions some varieties may develop into diphtheroid forms. Some types are anaerobic or micro-aerophilic, non-acid-fast, and obligate parasites. These are placed in the genus Actinomyces; other types are aerobic, partially acid-fast or non-acid-fast, saprophytic but facultative parasites. These varieties belong in the genus Nocardia. Several species of Nocardia can cause disease in man and animals, but only one species of the genus Actinomyces (A. bovis) is capable of doing so. The diseases which they produce are known as Nocardiosis and Actinomycosis, both of which can simulate tuberculosis. A number of avirulent intermediate types have been classified according to their method of reproduction. There is considerable difference of opinion regarding the exact position of the Actinomyces, and many classifications have been proposed. Some workers consider them as a

TABLE 1

Classification and Characterisation of the Mycobacteria

General Character of Growth	Natural Habitat and Other Sources from Which Isolated	Surv.[1] 60 C.	Growl 47 C.	Representative or Type Species (Mycobacterium)	Experimental Pathogenicity
Grow rapidly (2 to 7 days) on ordinary media at room temperature; usually pigmented; often of smooth colony type	Soil, timothy grass animal skin lesions	+	+	phlei (timothy grass)	Nonpathogenic for warm-blooded animals; although many cause suppuration. Local injection may produce lesions which may be mistaken for true tubercles or leprous granulomas. Large doses intravenously often prove fatal.
	Soil, tubercles of cold-blooded animals, animal lymph nodes or skin, leprosy.	-	-	marinum (fish) ranae (frog) thamnopheus (snake) "leprae" (human)	
	Soil, manure, butter, nasal secretions, smegma, leprosy.	-	+	butyricum (butter) "leprae" (human) lacticola (smegma)	
Primary isolation requires blood, egg yolk, or potato media and several weeks of incubation at body temperatures	Avian tuberculosis, more rarely from cattle, pigs, and sheep.			tuberculosis (avium)	F. +++ R. ++ C. - G.P. -
	Human tuberculosis, also from monkeys, pigs, dogs, and parrots.			tuberculosis (hominis)	- + - +++
	Bovine tuberculosis, more rarely from pigs, man, horses, dogs, cats, sheep.			tuberculosis (bovis)	- +++ +++ +++
Primary isolation requires growth factor from heat-killed acid-fast bacteria	Johne's disease (chronic enteritis of cattle and sheep)			johnei or paratuberculosis	- - + -
Do not multiply on bacteriologic media	Human leprosy			leprae	Not experimentally transmissible.
	Rat leprosy			leprae-murium	May be passed through rats and some strains of mice.

[1] Survival at 60 C. for one hour; growth over wide temperature range, including 47 C.
F = fowls; R = rabbits; C = calves; G.P. = guinea pigs. From Stitt et al., 1948.

primary stock from which both bacteria and fungi have developed, others believe they are higher types of bacteria and still others consider them as degraded fungi. On the recommendation of some systematists, these organisms are placed together with the Mycobacteria in the family Actinomycetaceae.

History

The first organism of this group to be discovered was the leprosy bacillus (M. leprae) by Hansen in 1868(2).

In 1882 Koch described the mammalian tubercle bacilli (M. tuberculosis). During the years 1898 to 1910 through the work of Smith, Vagedes, Ravenel, Kossel, Weber and Heuss, the English Royal commission, Park and Krumwiede, it was demonstrated that the mammalian organisms consisted of two distinct types, the human and the bovine, and that the latter could cause infection in man(2,3,4).

From 1889 to 1891 the discovery of the avian tubercle bacillus (M. tuberculosis var. avium) resulted largely from the work of Rivolta, Maffucci, Cadiot, Gilbert and Roger, Sibley and Straus and Gamaleia(2,3,4).

From 1889 to 1928 a number of cold-blooded tubercle bacilli M. piscium (in snakes, salamanders, lizards and fish), M. marinum (in fish), M. ranae (in frogs), M. chelonei (in turtles), M. tropidonatum (in snakes), and M. thamnopheos (in snakes) were isolated through the investigations of Bataillon, Dubard and Terre, Ledoux-Lebard, Aronson, Küster, Friedman, Sibley, Gibbs and Shurley, Lucas and Scott and Griffith(2,3).

In 1895 Johne and Frothingham reported the isolation of

M. paratuberculosis which is now known as the Johne's bacillus, the etiologic agent of a chronic type of enteritis in cattle, sheep and deer[2,3].

In 1903 the bacillus of rat leprosy (M. lepraemurium) was described by Stefansky, Rabinowitsch and Dean[2,3,4].

From 1885 to 1909 the discovery of a variety of saprophytic acid-fast bacilli M. smegmatis (in smegma), M. butyricum (in butter), M. berolinensis (in butter), M. friburgensis (in butter), M. stercoris (in cow dung), M. phlei (in timothy grass), M. graminis (in plant dust) and M. alluvialum (in soil) was accomplished through the studies of Alvarez and Tavel, Lasear, Czaplewski, Pellegrino, Petri, Rabinowitsch, Moeller, Korn, and Kersten[2,3,4].

In 1937[2] Wells succeeded in isolating M. muris from voles with natural tuberculosis.

Habitat

The human, bovine, and murine varieties are responsible for mammalian tuberculosis. The avian variety is found mainly in various birds, but pigs and cattle can also be infected; the organism has also been isolated from hens' eggs. Some investigators have reported infections in man. This literature has been reviewed by Branch[5]. The cold-blooded types of tubercle bacilli cause disease in a number of cold-blooded animals and fish, and it has been reported[1] that one strain was capable of producing disease in mice and pigeons. The saprophytic strains are ubiquitous, being found in water, milk, butter, grass, fruits, vegetables, smegma, dust, feces and manure. They have also been reported in isolations from human pathological processes, the

skin, tonsils, middle ear, nasal secretions, blood, sputum and even in the intestinal contents of insects. The leprosy bacillus is a specific parasite of man, and the rat leprosy bacillus for rats. Johne's bacillus infects cattle, sheep and deer. Although tubercle bacilli have remarkable capacity to resist cold, heat and drying there is no evidence that these organisms can live outside the animal body.

Resistance

Like most bacteria the acid-fast bacilli are sensitive to heat, desiccation, radiation, and other physical effects. On the other hand, under proper physical conditions they possess a great deal of resistance to such substances as strong acids and bases, numerous chemical disinfectants, and most antibiotic and chemotherapeutic agents. Tubercle bacilli may remain viable in cultures for months. When protected from direct sunlight, they may survive in putrefying sputum for weeks and in dry sputum for months. Smith(6) has shown that droplets of dried sputum adhering to dust particles in the air may remain infectious for 8 to 10 days. Blood, serum, albumin and other proteins also help to protect acid-fast bacilli against the effects of both physical and chemical agents. Rhines(7) has demonstrated that in polluted water kept in the dark at room temperature, tubercle bacilli may remain alive for 3 months. It is believed that their resistance is related to the quantity of waxy components in the organism and to the hydrophobic character of its cell surface. Whether the organisms are in a dispersed phase or in clumps is another important factor, for obviously, survival is greatest in the latter situation, where part of the resistance is due mainly to the physical barrier of clumping. It has been shown by McCulloch(8) that when agents which are capable of lowering surface

tension are added to a bactericidal solution, both the rate of penetration and the bactericidal effect are increased, even though the wetting agent itself is non-toxic to the organism.

Morphology and Staining

The acid-fast bacilli vary in morphology from coccoid to long filamentous forms depending upon the type, the strain, the character of the culture medium and the environment. Branching has been observed in old cultures and in smears from caseous lymph nodes; it has also been produced by special cultural technic. Typical tubercle bacilli are slender, straight or slightly curved rods with rounded ends and more or less parallel sides. Usually they are arranged singly in small bundles or in groups with individual organisms lying at acute angles to each other, like diphtheria bacilli. Their size varies considerably depending on the composition of the medium. When observed from animal sources they are generally longer and thinner than the organisms on culture. They vary in length from 1 to 8 micra, and in width from 0.2 to 0.6 micra.

The bacilli are gram positive and acid-fast. They are non-motile, non-sporogenous and produce no capsules either in susceptible animals or on culture media. The most characteristic property of this group of organisms is their "acid-fastness", the ability when once stained with aniline dyes to resist decolorization by acids or alcohol. The degree of acid-fastness is not uniform for all members. Organisms in the earlier phases of growth, as a rule, are less acid-fast than in the older phases. In young cultures it is not uncommon to find a number of non-acid-fast forms. Saprophytic strains are, in general, less acid-fast

than virulent strains. Staining may be either uniform or granular in character; in the latter type the granules may be limited to the ends of the organism, or they may assume an even distribution--the well known beaded form. Since environmental conditions may alter the character of staining, this quality is not reliable in the differentiation of types. By means of the electron microscope Mudd and Anderson(9) have demonstrated a somewhat thick, presumably rigid cell wall. Beneath the cell wall there is a definite cell membrane which exhibits differential permeability. The older concept that acid-fastness depends upon the presence of a waxy sheath surrounding the cell is no longer maintained by some workers. Mycolic acid, isolated by Anderson(10), has been claimed responsible for this property. Others attribute the staining reaction to a semi-permeable membrane around the organisms allowing fuchsin to diffuse in but preventing acid fuchsin from diffusing out.

The Ziehl-Neelsen method is the one most commonly employed for staining, although numerous other modifications and methods have been described. The technic simply consists in allowing the stain (carbol fuchsin) to act on the smear for about 5 minutes, during which time heat is applied to the point of steaming. The stain is then washed off with water, the smear decolorized with 95 per cent alcohol containing 3 per cent hydrochloric acid, washed again and counterstained with methylene blue. The bacilli are then seen as brilliant red rods against a blue background.

According to Kretschmer(11) Mycobacteria cannot be classified as Gram positive or Gram negative because once they have been stained by basic dyes, they cannot be decolorized by alcohol. Therefore, the staining reaction is independent of the mordant effect of iodine, and

instead appears to depend upon the same factors which are responsible for "acid-fastness".

Metabolism and Cultural Characteristics

There is great variability in the requirements for cultivating acid-fast bacilli. Tubercle bacilli are obligate aerobes and will not grow in the absence of an adequate oxygen tension. The optimum for the human type is about 40 to 50 per cent, and for the avian and saprophytic types about 60 to 70 per cent. About 10 per cent carbon dioxide is also beneficial for growth.

The human, bovine, avian and murine types of tubercle bacilli can grow on a variety of simple synthetic or complex organic media between pH 6.0 to 8.0 with an optimum around 6.5 to 6.8. The optimal pH for maintaining virulence is 6.8 Cultures of human and bovine bacilli grown at pH 6.0 rapidly become attenuated.

The optimal temperature requirements for the isolation of avian strains is 40 C., for human and bovine strains 37 C. and for cold-blooded strains 25 C. The human, bovine, and avian types do not grow below 30 C. The cold-blooded and saprophytic acid-fast types grow readily at 20 C. Many saprophytic strains grow at 45 C. and a few even at 55 C. After a number of generations of growth on culture media, the temperature requirements become less exacting.

Moisture is necessary for growth of tubercle bacilli in vitro. Growth occurs in sealed tubes provided an adequate amount of moisture and air are present. A simple method of supplying moisture consists of passing steam into the tubes before inoculation. When first isolated, acid-fast bacilli may be atypical, but after a number of subcultures

the organisms become stable and manifest their true cultural characteristics. Tubercle bacilli, in general, will not grow on the usual media but require special formulae such as inspissated serum, coagulated egg, potato, or synthetic media. The number of organisms inoculated is a limiting factor for growth on different media. When the inoculum is large the growth requirements are less exacting. On the other hand, small inocula require the more nutritious special media. Failure of small inocula to grow may not necessarily result from nutritional inadequacies, but may be due to the inhibitory components either in the media or from contaminating materials. Tubercle bacilli utilize a large number of carbon compounds as a source of energy, especially glucose and glycerine. Glycerine accelerates the multiplication of most strains of human and avian bacilli; it has little or no effect on the bovine strains, and it inhibits the growth of the vole bacillus. The degree of acceleration or inhibition varies somewhat depending upon the composition of the medium to which glycerine is added. Amino acids are used as a source of nitrogen. Growth is enhanced by the addition of various compounds such as the dicarboxylic acids and asparagine. The role of vitamins in metabolism is not definite. While development on the classic synthetic and complex organic media is, in general, slow compared to other organisms (2 to 6 weeks), newer developments in culture media have made it possible to obtain results much earlier. One of the most interesting of these is the medium developed by Dubos et al.[12,13] who found that "Tween 80", a water-soluble ester of a long chain fatty acid, alters the osmotic conditions in liquid media. Wetting the surface of the organisms by use of this compound has facilitated not only rapid growth, but the organisms are dispersed throughout the medium. The

latter factor has been of value in certain diagnostic and research procedures.

The acid-fast bacilli behave rather characteristically on different culture media, producing various colony types, pigmentation, pellicle formation, distinctive odors and other growth features. The effect of temperature, pH, different chemical components and rate of growth has already been mentioned.

Pigment formation is the chief characteristic of most of the saprophytic strains of the acid-fast bacilli, forming a wide range of colors such as white, pink, lemon, pale-yellow, orange and brick red. Under the proper conditions the other types of organisms may also form pigments. Avian strains may manifest a pink pigment. Bovine strains which are usually colorless may also show a pink pigment. Mycobacteria from cold-blooded animals are generally colorless, but the colonies of these strains may also be white, pink or yellow. Even human strains may be pigmented, and when grown on deeply colored ox serum, the colonies may be yellow or orange-yellow. Of interest also is the report of Reed and Rice[14] who found that a concentration of 0.02 per cent ferric citrate on a glycerol agar medium stimulated the development of chromogenic colonies of human, bovine and avian tubercle bacilli and saprophytic acid-fast organisms. Pigmentation is best seen in cultures which have been incubated at 37 C. and then stored in the dark at room temperature. Most workers believe that pigment production depends upon a number of environmental factors, particularly oxygen supply and the chemical composition of media. In addition to the above are the group of so called "atypical" acid-fast chromogenic bacteria of the variety studied in this investigation. These characteristically show a yellow

to deep orange pigmentation on the usual tuberculosis media. As will be shown later, this property can be altered by changing the environment.

Unlike most of the other bacteria, the biochemical reactions of the acid-fast bacilli are of very limited value in differentiating the various types. All degrees of variation exist between members of the group. From a cultural standpoint, indistinguishability between widely separated types is not at all uncommon. In this connection it is well to mention that serologic procedures have also proved of very limited value in differentiation, and that animal pathogenicity is still the best criterion for identifying types.

Chemical Structure and Metabolites

By growth of large numbers of acid-fast bacilli on Long's[15] synthetic medium and by chemical extraction it is possible to obtain lipoidal material which can be separated into three fractions consisting of glycerides, phosphatides and waxes. A considerable amount of polysaccharide is also obtained. This information is presented in Table 2 after Chargaff et al.[16]. It will be noted that the total lipin yield is highest from the human type of bacillus and lowest from the saprophytic acid-fast bacillus. Of interest is the reverse order of the polysaccharide fraction. Further extractions and analysis have revealed that the phospholipins contain saturated and unsaturated fatty acids, and glycerophosphoric acid. On hydrolysis these yield large amounts of water-soluble carbohydrates, of which mannose and inositol seem to be the most important. The lipoidal fractions constitute about 25 to 40 per cent of the dry weight. The exact amount of the various substances produced differs greatly according to the type of tubercle bacillus and the

TABLE 2

Percentage Fractions of Lipoid and other Material Isolated from Alcohol-Ether and Chloroform Extracts of Acid-Fast Bacilli

	Type of Organism			
	Human tubercle bacillus H 37.	Avian Tubercle bacillus.	Bovine tubercle bacillus.	Timothy grass bacillus.
Phosphatide.....	6.54	2.26	1.53	0.59
Acetone-soluble fat.....	6.20	2.19	3.34	2.75
Chloroform-soluble wax.....	11.03	10.79	8.52	4.98
Total lipins.....	23.78	15.26	13.40	8.37
Polysaccharide.....	0.87	1.02	1.09	3.90
Dried bacterial residue....	75.01	83.71	85.50	87.70

From Chargaff et al., 1931.

composition of the culture medium. In addition to the lipoidal material and polysaccharides, the acid-fast bacilli contain water soluble proteins. These fractions are of interest because they are responsible for skin allergy. The protein derivatives of avian strains, Johne's bacillus and saprophytic acid-fast bacilli can be distinguished from those of the mammalian type by serologic and quantitative skin tests in hypersensitive individuals, but there are cross reactions throughout the genus Mycobacterium.

Catalase is produced regularly and H_2S is formed in varying amounts in cultures of tubercle bacilli. Pope and Smith[17] have shown that both the human H37Rv and the bovine Ravenel strains synthesize all the known B-complex vitamins including biotin and folic acid.

Of special interest are the following organic compounds synthesized by tubercle bacilli:

1. Mycolic acid which is acid-fast[18].
2. Tuberculostearic acid and phthioic acid, the latter being responsible for the appearance of epithelioid cells in tissues[19].
3. Insoluble waxes which induce the formation of giant cells of the foreign body type[19].
4. A polysaccharide which exerts a chemotactic effect on neutrophilic polymorphonuclear leucocytes and precipitates sera of immune animals but which is neither toxic nor antigenic[20,21,22].
5. An undenatured protein which in a dose of 0.02 mg., produces a positive skin test in a tuberculous patient, is antigenic and induces tuberculin sensitivity in animals[22,23,24].
6. Nucleic acid which is neither antigenic nor toxic, even for tuberculous animals[22].

Exotoxins are not produced by the acid-fast bacilli. Endotoxins capable of killing rabbits are elaborated by the avian strains, and several of the saprophytic strains, such as the smegma, timothy grass and mist bacilli.

Antigenic Structure

As a result of the work of Tulloch et al.[25], Cumming[26], Wilson[27], Griffith[28], Fürth[29] and Kauffman[30] it has been shown that by agglutination, agglutinin absorption and complement fixation, the acid-fast bacteria may be separated into four serologic types: (1) mammalian (2) avian (3) cold-blooded and (4) saprophytic. Wells[2] has also found that the human, bovine and murine types from voles are indistinguishable serologically. Wilson[27] has reported that direct agglutination is not adequate to differentiate between the types, but agglutinin absorption is necessary, and by this technic he demonstrated that the avian type not only possesses an antigen in common with the human and bovine forms, but has a specific antigen of its own. The observations of Seibert[31] suggest that group-specificity is determined by the polysaccharide fractions, and type-specificity by the proteins. The investigations of Freund[32] and Mudd and Mudd[33] indicate that the surface of the tubercle bacillus is composed of a lipid-protein-carbohydrate complex which stimulates the production of agglutinins and precipitins when injected into animals. The studies of Mudd and Fürth[34] and Steenken and Gardner[35] have shown that the virulent H37Rv and bovine C strains, the avirulent BCG and R_1 strains and the avirulent dissociates of recently isolated human strains all have essentially the same antigenic structure and are equally effective in producing a relative degree of immunity in the guinea pig. Trudeau et al.[36],

Zinsser and Petroff[37], Zinsser et al.[38], Petroff et al.[39], and Opie and Freund[40] have demonstrated that heat-killed tubercle bacilli produce some immunity but this is never as effective as that produced by the living avirulent BCG and R_1 strains. The work of Olson et al.[41] has shown that virulent human tubercle bacilli, killed by ultraviolet light, are as effective in immunizing guinea pigs as the living BCG strain. Most workers feel that serological methods are less specific in the differentiation of tubercle bacilli than cultural methods.

Dissociation

It has long been known that the mycobacteria exhibit many modifications from their usual characteristics. These have been referred to as dissociation or variation, a phenomenon which results in changes of cellular and colony morphology, in virulence and in a number of other physical and biological characteristics. Oatway and Steenken[42] define dissociation as "the process of separating two or more variants of a microorganism from a parent culture". Thus from the numerous studies reported during the last 23 years[42-65] it has become increasingly evident that for the mycobacteria the dogma of monomorphism and stability can no longer be accepted. To date the underlying factors favoring dissociation are not clearly understood.

The terms "smooth" and "rough" have been used in connection with tubercle-bacillus colonies by a number of the earlier workers. The standard smooth (S) and rough (R) terms were employed in an effort to associate the symbol S with virulence even though the colonies were rough. This scheme caused considerable confusion and, as a result, the terminology was changed in 1936. The standard nomenclature now employs S to designate a smooth colony and R to designate a rough one. Many of

the R strains, but not all, are virulent. In 1935 Steenken proposed a new terminology in which the standard S and R symbols are retained but in addition sub letters are added. For instance, R_v = rough virulent; R_a = rough avirulent; R_{in} = rough intermediate; S_{ch} = smooth chromogenic; S_{cha} = smooth chromogenic avirulent. This classification is now accepted as standard.

Dissociation of tubercle bacilli was first reported by Petroff[43,44] Prompted by the finding of dissociation of R and S colonies in other microorganisms, he studied human bovine and avian strains and succeeded in dissociating them into rough and smooth colonies with different cultural characteristics. Subsequently Petroff et al.[45] found that the BCG strain could be separated into rough and smooth colonies which exhibited different degrees of virulence in guinea-pigs. This organism was then dissociated into three types of colonies, R, S, and intermediate forms. According to Petroff and his associates the S variant of BCG produced progressive disease in animals. The R variant was only slightly virulent, and at times avirulent. It was believed that all organisms contained the R and S components. If the avirulent rough variant of BCG was cultivated on a medium containing anti-R serum the more virulent S form developed. These studies led to a great deal of controversy between Calmette[46] and Petroff[47].

In subsequent studies Petroff and Steenken[48] and Petroff[49] showed that when certain pathogenic acid-fast organisms were cultivated on special media, growth generally appeared white at first, and gradually changed to cream and finally to an orange color. The pigmented variants showed either reduction or loss of virulence. Conversely, when certain pigmented avirulent acid-fast bacilli were cultivated, chromogenicity

was gradually lost, and, after 40 to 50 subcultures, guinea pigs inoculated with the organisms developed visceral lesions. At first the lesions were not very extensive, but after 5 or more animal passages they became more so. From this it was concluded that the development of pathogenicity in the avirulent organisms depends largely upon the environment.

Miller(50) reported the isolation of a non-acid-fast chromogenic variant of the virulent H37Rv strain, which maintained cultural characteristics similar to the virulent organism, but was less pathogenic for guinea pigs.

Winn and Petroff(51), studying the avian tubercle bacillus, were able to dissociate four distinct types: a smooth (S), flat smooth (FS), rough (R) and chromogenic (Ch) type. The S type proved most virulent, the FS less so. The R and Ch types were comparatively avirulent. Histopathologic studies of the tubercles of the S variant produced an acute toxic type of tubercle; the FS a foreign body type; whereas the R and Ch variants a relatively benign type.

Steenken et al.(52) in later studies were able to dissociate the human H37Rv strain into a smooth virulent and rough avirulent variant. Both variants were distinct in colony morphology and cultural characteristics. Some of the R strains were chromogenic.

Alexander(53, 54), in her investigations with human and avian tubercle bacilli, was also able to dissociate these types into rough and smooth variants by the use of Bordet-Gengou medium containing 0.0004 per cent ferric chloride. In pathologic studies she showed that the lesions produced by the smooth strains differed radically from those produced by the rough strains. Tarshis and Frisch(55) made

similar observations of variant forms in cultural studies with human virulent strains of tubercle bacilli on Bordet-Gengou and other blood media.

In his studies relating to the influence of pH on the dissociation of the human H37Rv strain of tubercle bacillus, Steenken(56) found that R variants increased with increase in acidity, whereas the S variant increased and the R variant decreased with increase in alkalinity. Organisms grown at pH 6.0 in special media manifested chromogenicity, and were less virulent than organisms grown at pH 7.6. Smithburn(57,58) reported similar observations, and in addition he obtained smooth virulent variants from freshly isolated human strains of tubercle bacilli. Their colony morphology was similar in appearance to the smooth forms of other bacteria.

Petroff and Steenken(59) were also able to dissociate the saprophytic M. phlei acid-fast bacillus into rough and smooth variants, but only with difficulty.

Extending their studies, Oatway and Steenken(60, 61) reported the dissociation of the avian bacillus into rough, rough stippled, smooth, and smooth pigmented variants, the bovine bacillus into rough and smooth variants and the human bacillus into rough, rough stippled, and smooth variants. They also showed that the qualities of a culture of tubercle bacilli which may be affected by dissociation are colony morphology, chromogenicity, bacterial morphology, immunological properties and chemical composition.

It is of interest here to call attention to the report of Steenken(62) on the confusing effect of egg oil on colony morphology. He observed that when tubercle bacilli are grown on media containing egg, R_v and R_a colonies were converted artificially by the egg oil into S

forms. When subcultured to egg-free media the colonies reverted back to their original forms. Such physical effects must be distinguished from true dissociation phenomena.

Steenken and Gardner[63] have shown that a number of avirulent dissociated variants of tubercle bacilli could confer appreciable degrees of immunity against standard virulent tubercle bacilli, but there was no evidence to suggest a type specificity, since the vaccinating power of the homologous strains was not appreciably greater than that of heterologous strains.

The recent investigations of Middlebrooke et al.[64] on the morphologic characteristics of tubercle bacilli in liquid and on solid media have demonstrated that all virulent eugonic strains of human and bovine tubercle bacilli tend to form microscopic serpentine cords. Avirulent variants of virulent strains of mammalian tubercle bacilli do not exhibit this phenomenon.

Larmola's[65] studies add further evidence to the great versatility of bacterial variability. He has found numerous variations in the morphological, cultural and biological activities of tubercle bacilli.

From the above data there is little doubt that dissociation can be accomplished with a variety of mycobacteria. In addition all grades of pathogenicity are possible from single strains, depending upon the environment and other factors which can be varied either by chance or by accident. The true significance of these variants in relation to human and animal disease is not clear. In experimental work dealing with pathogenicity and cultural characteristics it is evident that variability may become a source of serious error unless rigid precautions are maintained.

Mutation

At the Fourth Conference of the International Union Against Tuberculosis at Lausanne, Switzerland, Maher(66) posed the following question "do there exist in nature or can there be artificially produced saprophytic varieties of the Koch's bacillus which can become virulent tubercle bacilli?" On the basis of numerous studies he concluded that this phenomenon did occur. As one example evidence was presented of the transmutation of the smegma bacillus into virulent bacilli pathogenic for man. He indicated further that many workers as well as himself have effected radical changes in "acid-fast bacilli differing markedly in cellular and colony morphology and in cultural and biologic activity."

In direct opposition to Maher's views are those of Calmette(67) who, after reviewing the work of many previous workers, concluded that, up to the present, there was no experimental evidence to support the problem posed by Maher(66). What positive results were reported Calmette felt were due to the contamination of cultures with virulent forms.

But other workers, not content with the conclusions of Calmette and those of his school, undertook studies in the hope of establishing more conclusive evidence in favor of the transmutation concept. It is only necessary to call attention to some of the more interesting studies. Gay et al.(3) reviewing some of the earlier work point out that numerous attempts have been made to transform mammalian and avian types of tubercle bacilli into the piscine or cold-blooded type. For instance, Combmale fed carp for long periods with human tuberculous sputum, and found that the organisms were not changed morphologically,

but there was a slight decrease in virulence. Hormann and Morgenroth investigated the effect on human strains in goldfish and obtained the same results. They found 7 months later that the organisms were still alive and virulent for guinea pigs. Sorgo and Suess claimed positive results after injecting human strains into the slow worm. Subcultures were avirulent for guinea pigs and exhibited the characteristics of the piscine type. Bertarelli and Bocchia inoculated human, bovine and avian strains into fish and lizards and found that after 8 months the virulence of these types was unaltered. These investigators believed that tubercle bacilli had multiplied in the artificial hosts. On the basis of these and other studies many workers believed that the theory of the transmutation of types had not been proved. Wilson and Miles(2), reviewing the studies on atypical strains of tubercle bacilli, call attention to the opinion of Griffith who believed that these organisms represent no more than modifications of the fixed types and that they are not transitional forms between types. It has been further demonstrated by animal passage that it is often possible to restore the normal characteristics of the standard types, but for the present there is no definite evidence to prove that one type can be made to change into another type.

Developmental Variability

In studies on the developmental stages of the tubercle bacillus Stockwell(68) observed motile gram negative and acid-fast forms.

Sweany(69) isolated non-acid-fast coccoid bodies from patients dying of tuberculosis. By placing sections of spleen containing these structures into celloidin capsules and then embedding them into the

body cavities of guinea pigs, he was able to recover acid-fast bacilli from the tissues after 3 weeks. Cultures of this organism proved to be virulent and possessed no constant morphology or cultural characteristics. Animal passage resulted in typical guinea pig tuberculosis. In a subsequent report(70) he found that granules in certain stages of their life-cycle, isolated from tuberculous patients, could be made to sprout and reproduce acid-fast organisms. In other stages, they could be made to sprout into actively motile non-acid-fast bacilli. Certain selected granules in large doses were also capable of producing tuberculosis in guinea pigs; small doses of granules produced atypical and chronic tuberculosis. A semi-acid-fast mold-like growth was also obtained which manifested tuberculogenic properties and reverted to complete acid-fastness on animal passage. From these studies Sweany concluded that "the most common acid-fast bacillary form of the tubercle bacillus is not the only manifestation of the etiologic agent of tuberculosis; and that the phylogenetic aspects of microorganisms, as well as the influence of the animal host and associated microorganisms upon it, have heretofore not been sufficiently understood or emphasized."

By a special micromanipulator technic Kahn(71) was able to study the growth of a single H37Rv tubercle bacillus or small groups of 2 to 6 organisms in micro-droplets of Long's medium. Under the conditions of the experiment he concluded that this strain of the human tubercle bacillus does not multiply solely by simple fission, but undergoes a complex type of reproduction involving various morphological forms.

Dreyer and Vollum(73), in their studies on non-acid-fast forms, felt that these were resting stages of the tubercle bacillus and possibly its least active form. The morphologic variations they observed were

considered either stages in the life cycle of the organisms, due to varying cultural conditions, different strains of bacilli, the result of experimental methods, or to different degrees of skill in distinguishing between artifacts and organisms. In any case they were of the opinion that the non-acid-fast structures were of great importance in the biology and etiology of tuberculosis, whether experimental or clinical.

Kahn(71,72), Miller(50), Wherry(74) and others have reported the presence of non-acid-fast rods and granules in young cultures of tubercle bacilli. Of interest also is the report of Yegian and Porter(75) who showed that these non-acid-fast forms could be produced artificially by traumatizing the organisms with an inoculation loop or by cutting colonies with a microtome knife. Since organisms from old cultures appeared resistant to this manipulation it was assumed that the cell walls of the younger bacilli were not sufficiently resistant to protect the delicate semipermeable cytoplasmic membranes which are believed responsible for the retention of the stain.

Much(76) reported that under certain conditions tubercle bacilli might be present in the tissues in the form of non-acid-fast granules. In such specimens as pus from cold abscess, granulomatous lesions of lymph nodes, serous exudates and some samples of sputum it was impossible to demonstrate acid-fast bacilli, even though their presence could be proved by culture and animal inoculation. By employing a modified Gram stain(77) he was able to demonstrate fine rods often accompanied by small rounded granules occurring singly, in pairs or in short chains resembling beaded organisms. Much believed that these structures were virulent, non-acid-fast tubercle bacilli. Sweany(70) and Kahn(78) have considered these structures as stages in the life cycle of the tubercle

bacilli, Yegian and Porter(75) feel that many of the non-acid-fast forms are merely artifacts resulting from trauma to the organisms in the preparation of smears, and still others feel that these are merely products of degeneration. Since Much's granules cannot be differentiated with certainty from other granular structures, most workers feel they are of no value in diagnosis.

It has been claimed by numerous workers that under the proper conditions filterable forms of the tubercle bacillus exist, which represent a stage in the life-cycle of the organism and possess a low degree of pathogenicity for guinea-pigs. Pinner and Veldrich(79) Soltys and Taylor(80) have concluded that there is no satisfactory evidence to support the existence of such structures.

Pathogenicity and Experimental Infection in Animals

The first convincing knowledge of the transmissibility of tuberculosis to animals resulted from the work of Villemin[1] who in 1865 reported the successful inoculation of various types of human tuberculous material into rabbits. Later he was able to transmit bovine tuberculosis to rabbits, and made the significant observation that infectious matter from cows produces in the rabbit a disease identical with that which develops when this animal is inoculated with the organism of human tuberculosis. He further observed that the reaction from bovine infection was more rapid and generalised. The final proof of the transmissibility of the disease was achieved by Koch in 1882 who showed that animals could be infected with pure cultures of tubercle bacilli. In 1898 Smith firmly established that the bovine tubercle bacillus was more virulent when

[1] In Villemin's day tuberculosis was known as pommeliere, a term adopted because of the resemblance of some bovine tubercles to a small apple (pomme).

injected into rabbits and calves than the human bacillus. This observation has been confirmed by many subsequent workers.

Table 3 summarizes the range of pathogenicity of tubercle bacilli under natural conditions. Table 4 given by Pierce et al.(81) and Table 5 by Smith et al.(4) summarizes the degrees of pathogenicity of the types of tubercle bacilli for various species of animals.

Saprophytic acid-fast bacilli, though occasionally isolated from various tissues are rarely capable of producing progressive disease. It is also of importance to bear in mind that virulence among all types of tubercle bacilli is variable. These organisms are usually virulent on isolation, but many strains may become more or less avirulent as a result of subculture in the laboratory. The phenomenon of dissociation may also play a role. Though these facts are well accepted, there seems to be little exact information based on statistically adequate numbers of animal tests concerning differences in pathogenicity of freshly isolated strains of the same type, or the various factors responsible for changes in virulence.

Cellular Reactions to the Tubercle Bacillus and Tubercle Formation

The cellular reaction following the inoculation of tubercle bacilli into susceptible animals has been studied in great detail by a number of workers. While there is much controversy in respect to the origin of certain types of cells, their functions and the mechanism of necrosis and caseation, there is agreement on the general features of the reaction.

Organisms in the tissues produce localized minute, translucent, greyish to yellow nodules called tubercles. Single tubercles are not visible macroscopically, but the coalescence of a number of tubercles makes this possible. The characteristic features are a central area of

Table 3

Pathogenicity of Types of M. tuberculosis
Under Natural Conditions

Types of Tubercle Bacilli	Species of Animal Affected
Human.....	man, monkeys, pigs, dogs[1], parrots[1]
Bovine.....	cattle, horses, pigs, man, sheep[1], dogs[1], cats
Murine.....	voles
Avian.....	birds, cattle[1], sheep[1], pigs[1], man(?)
Cold-blooded.....	cold-blooded animals, fish

[1]Disease produced occasionally in these animals.
(?) Disease in man still controversial.

TABLE 4

Pathogenicity of Types of M. tuberculosis

Animal Species	Types of Tubercle Bacilli		
	Bovine	Human	Avian
Guinea pig.....	++++	++++	0
Rabbit.....	++++	+	++++
Mouse[1].....	++++	++++	+(?)
Hamster.....	++	+++	+
Anthropoids and monkey.....	++++	++++	0
Goat.....	+++	0	0
Horse.....	++	0	++
Dog.....	++	+	0
Cat.....	+++	+	0
Cattle.....	++++	0	0
Swine.....	+++	+	+++
Parrot, cockatoo.....	+++	++	+++
Domestic fowl.....	0	0	++++

"0" indicates that natural infection occurs most rarely although temporarily progressive lesions may be produced by the injection of relatively large numbers of living bacilli.

[1]Certain strains of mice are highly susceptible to bovine and human type, others are very resistant. From Pierce et al., 1947.

TABLE 5

Pathogenicity of Tubercle Bacilli for Laboratory Animals

Animal	Human Type	Bovine Type	Avian Type	Murine Type	Johne's Bacillus
Guinea Pig	++++	++++	0	+	0
Rabbit	++	++++	+++	+	0
Calf	+	++++	++	----	++
Fowl	0	0	++++	----	----
Vole	+	++++	+	+++	----

From Smith et al., 1948.

coagulative, caseous necrosis surrounded by a cluster of epithelioid cells, some of which fuse to form different types of giant cells, and a peripheral zone of fibroblasts, lymphocytes, monocytes and plasma cells supported by a retisulum.

Pinner(82) has described two types of lesions in tuberculous infection. One is classed as exudative, the other productive or proliferative. Exudation is characterized by infiltration of tissue with polymorphonuclear leucocytes and plasma constituents, and there may be abundant fibrin formation. There is no replacement of normal tissue, and this type of reaction is most commonly seen in primary infection. However, both lesions may occur in chronic tuberculosis and intermediate types are frequently evident. Proliferation is an infectious granulomatous reaction since it resembles both granulation tissue and a tumor. It is characterized by the formation of new tissue.

The cellular reactions to inoculation of tubercle bacilli have been studied by observations of tissue at intervals following infection. The type of response to infection will depend upon a number of factors such as the animal species, the number of organisms inoculated, their rate of multiplication, and whether the animal is free of tuberculosis or has already been infected (hypersensitive state).

Following inoculation there is an acute inflammatory response during the first twenty-four hours. Exudation of fluid results with rapid accumulation of polymorphonuclear leucocytes, many of which contain bacilli. Though actively phagocytic they are unable to destroy the organisms. These cells play a useful role in focalizing the infection and preventing to some extent the invasion of bacilli through the tissues. They also may serve to disseminate organisms by migration to

new sites. During the next few days exudation diminishes, and the polymorphonuclear leucocytes begin to die. Monocytes (macrophages) then appear, and begin to phagocytize the dead leucocytes and bacilli. In the non-allergic host, the degree of exudation and cellular response is far less intense than in the allergic host.

Depending upon various factors, the exudative lesion follows one of three courses. It may heal by resolution, that is, multiplication of the tubercle bacilli may be inhibited or the organisms may be destroyed and healing takes place without scar; it may undergo necrosis with cavity formation; or it may develop into the typical productive lesion of chronic tuberculosis.

If proliferation results, many of the mononuclear cells are soon transformed into epithelioid cells, so characteristic of the tuberculous reaction. In two or three weeks, providing the lesion does not spread too rapidly, a zone of proliferating fibroblasts, plasma cells and lymphocytes appear at the periphery of the lesions, and near the central part of the older lesions there may be one or more giant cells, which are formed by the fusion of epithelioid cells, or possibly by amitotic division of nuclei. These are large multinucleated cells several hundred microns in diameter, of characteristic structure, and well known as the "Langhan's", "epithelioid" or "rosette giant cell" (because of the rosette of vacuoles in the cytoplasm and the peripheral arrangement of the nuclei). Another type of giant cell with a greater number of irregularly arranged nuclei has also been described but occurs less regularly than the Langhan's type, and is believed to be derived from the fusion of the mononuclear cells. Giant cells may or may not be found within the tubercles. Their chief function is phagocytic.

The tubercles may grow in size, coalesce and undergo a coagulative necrosis. This is believed by some workers to be due to the occlusion of the blood supply to the tubercles, some think it is due to the liberation of endotoxins from the tubercle bacilli, others hold that it is due to enzymes given off by the dying cells, and still others are convinced that certain autolytic enzymes of the host, which normally cause liquifaction are inhibited. Since the necrotic material has the consistency and appearance of cheese, the process is called "caseation necrosis".

When the dosage and virulence of the organisms are low and resistance is high, epithelioid cells are predominant, giant cells may be scarce or absent, and there is little evidence of necrosis. This type of lesion is often referred to as the "hard tubercle". On the other hand, when the dosage and virulence are high and resistance is low, epithelioid cells are scarce. Such tubercles are called soft.

The fate of the tubercle varies greatly. A caseous tubercle may dissect into a bronchiole or bronchus and cause cavitation. Such caseous material may then be coughed up or aspirated into other parts of the lung. The caseous mass may become surrounded by fibroblasts forming a fibrous capsule. Lime salts are deposited, and the tubercles are spoken of as healed. However, living bacilli can often be recovered from so called healed or quiescent lesions. Calcification and even ossification of tubercles may result. This is a frequent occurrence in children. Another alternative is healing with scar formation.

Lesions Caused by the Injection of Dead Tubercle Bacilli

Miller(83) has demonstrated that inoculation into animals of dead tubercle bacilli, preferably heat-killed strains, causes lesions of

varying degrees of severity. Subcutaneous injections of the organisms merely result in local abscess, but intravenous inoculation produce lesions closely resembling true tubercles, at times even manifesting caseation. The reaction is believed to be due to the breakdown of the organisms by the host's cells, with liberation of intracellular toxins. The toxins acting on the tissues result in the lesions. This phenomenon has been called "necro-tuberculosis". Apparently virulent organisms are not necessary. Wilson and Miles(2) reported similar observations in rabbits by intravenous injections of BCG.

Immunity

Humoral Antibodies. The role of humoral antibodies in immunity against tuberculosis is not known. Agglutinins and opsonins appear regularly in low titers in experimental animals but irregularly in man. Specific antibacterial sera agglutinate tubercle bacilli and promote phagocytosis by polymorphonuclear leucocytes and monocytes in vitro. It has also been demonstrated that immune sera fix compliment in the presence of tubercle bacilli and certain of their antigenic components. They also form precipitates with protein and carbohydrate fractions of the organisms. It has not been demonstrated that immune sera possess bactericidal or lytic effects.

Allergy. Allergy in tuberculosis represents tissue sensitization or the mechanism of tissue response to tubercle bacilli and their constituents. It is distinguished from other allergic phenomena such as anaphylaxis, serum sickness or atopy in that it is apparently not mediated by a humoral mechanism, and is of the delayed type, requiring a number of hours for response.

The Koch Phenomenon. This allergic mechanism was demonstrated by

Koch during some of his early work. He found that when a normal guinea pig was inoculated subcutaneously in the inguinal region with virulent tubercle bacilli, there resulted in 10 to 14 days at the site of inoculation a nodule which became necrotic, and persisted until the animal expired. A regional lymphadenopathy also occurred with tubercle formation and caseation, and massive disease in the lungs, liver and spleen. When he injected the same quantity of organisms into a tuberculous guinea pig quite a different series of reactions were observed. After approximately 48 hours, a local inflammation resulted at the site of the inoculation, which became indurated, dark in color, and was soon followed by ulceration and sloughing. Healing occurred rapidly, and there was no evidence of caseation in the regional lymph nodes. This type of response in animals previously infected was interpreted as a manifestation of some degree of acquired resistance which is now known as the Koch phenomenon. In later experiments Koch found that tuberculous guinea-pigs also reacted in a similar way to killed tubercle bacilli or their fractions.

Allergic Mechanisms. Since the report of these observations many workers have attempted to add to our understanding of the allergic phenomenon by studying the fate of inoculated organisms into tuberculous animals. It has already been mentioned that in the infected animal lymphatic dissemination is reduced or checked. Krause(84) injected virulent tubercle bacilli into the skin of allergic and normal guinea-pigs and after a short lapse of time he removed the infected skin. He found that he could prevent tuberculosis in the allergic, but not in the anergic animals. He concluded that the inhibition of lymphatic dissemination was due to the elaboration of fibrin which served as a mechanical

barrier.

Freund and Angevine[85] performed cultural studies of excised cutaneous lesions and showed that at the site of inoculation the organisms may multiply but their dissemination to the regional lymph nodes is inhibited. This again indicates the operation of some local immunologic mechanism. Numerous workers have studied the cellular reaction in tuberculous infection. The general manifestations of cellular activity have already been reviewed. Here we need only mention that in the immune animal it has been observed that tubercle formation is greatly increased, and newly inoculated organisms are either inhibited or destroyed. Cellular infiltration at the site of inoculation is chiefly monocytic, epithelioid and giant in character. In the non-immune animal the cellular response is principally polymorphonuclear, with gradual and limited accumulation of mononuclear cells. If the inoculum in the immune animal has been slight or even moderate, tubercles heal with fibrosis, whereas in the non-immune animal they progress and become caseous.

Lurie[86] has shown that mononuclear cells aspirated from the peritoneal cavities of tuberculous rabbits exhibit an increased capacity not only for destroying tubercle bacilli, but also for a non-specific phagocytosis of carbon particles.

Thus it becomes apparent that the monocytic, epithelioid and giant cells play a significant role in immunity.

Immunization. After the discovery of the tubercle bacillus, not only Koch, but numerous other workers attempted to find some effective immunizing agent against tuberculosis.

In 1922 Calmette and Guerin reported successful results using an

avirulent bovine tubercle bacillus which they were able to attenuate by long cultivation on a glycerine-potato-bile medium. The organism became known as the bacillus of Calmette and Guerin or BCG. This strain has been employed for vaccination in more than 2,500,000 individuals the world over. A description of its properties, the technics used for immunization, the arguments for and against its use and the present status of BCG vaccination may be found in the following excellent reports(87-106).

That man as well as animals can acquire a definite but limited immunity by vaccination has been demonstrated in many experiments with BCG. Because of the questioned safety of this agent numerous studies were undertaken to determine the immunizing potentialities of killed tubercle bacilli. Opie and Freund(107) found that a moderate degree of immunity against virulent organisms could be produced in rabbits. Opie et al.(108) studying the immunizing effect of heat-killed tubercle-bacilli in human subjects found a lower morbidity and mortality in the vaccinated than in the nonvaccinated group.

Other workers conclude from both animal and human experiments that BCG and other living attenuated organisms are more effective than killed organisms.

Wells(109) has utilized the vole bacillus, M. muris, for vaccination. The degree of immunity appears comparable with that produced by BCG, and this organism is now being tried experimentally in humans with the belief that a reversion to virulence is less apt to occur with this type than with BCG or similar attenuated bovine or human strains.

That other organisms might possibly be used for vaccination has recently been reported by Wenkle et al.(110) who found that some chromo-

genic acid-fast bacilli produced a certain degree of immunity in guinea pigs.

It must be stressed that immunity, whether in animal or man, and no matter under what conditions acquired, is at best only limited. Most studies are limited because of the lack of proper controls, and figures on the per cent of active tuberculosis developing in vaccinated versus unvaccinated controls are subject to numerous sources of error.

Tuberculin and Tuberculin Sensitivity

Preparation. Following his discovery of the tubercle bacillus Koch next attempted to find a diagnostic agent, a preventive and possibly a specific cure for tuberculosis. Following a long series of investigations he isolated a substance from liquid culture filtrates of the tubercle bacillus which produced characteristic toxic reactions in tuberculous but no reaction in non-tuberculous animals. This substance he called tuberculin, which later was shown to be a mixture of protein fractions. Koch prepared tuberculin by growing tubercle bacilli in a 5 per cent glycerol broth medium for 6 to 8 weeks at 37 C. after which time he sterilized and filtered the culture and finally evaporated the filtrate at 100 C. to one-tenth of its original volume. The resulting mixture was a clear, dark-brown, viscous fluid containing approximately 40 to 50 per cent glycerine, components of the medium, and metabolites of the tubercle bacillus. This material was spoken of as AT (Alt Tuberkulin) or OT (Old Tuberculin). Since Koch's day many modified preparations have been introduced for diagnostic use. To obviate the danger of false positive reactions due to chemical substances other than the metabolic products of the organism, a control is used. This is made by evaporating some of the uninoculated medium to one-tenth of its volume.

In the newer preparations 0.5 per cent phenol is usually added as an additional precaution.

Standardization. Various standardization methods have been used to test the potency of tuberculin. Koch used a guinea pig test which consisted of tuberculin injections into a number of guinea pigs weighing from 300 to 350 grams each, four weeks after being infected with 4 or 5 mg. of a fresh culture of virulent organisms. If 0.5 ml. or less killed a guinea pig within 6 to 36 hours, the preparation was considered potent. Various other methods of standardizing tuberculin have since been developed including cutaneous, febrile, intracerebral and intratesticular reactions.

Dilution. Since Koch observed that severe reactions could result from concentrated tuberculin, it was found necessary to make various dilutions before administration. Tuberculin is stable in its concentrated form but becomes unstable when diluted. It is generally never used after 2 to 3 days.

Test Dose. The test dose of OT is calculated on the basis that 1 ml. contains 1,000 mg. of tuberculin. It should be pointed out, however, that this is an erroneous assumption, since the active proteins constitute only a very small amount of the tuberculin. The dilutions generally used diagnostically are shown in Table 6. Dilutions are made on a decimal measure, being expressed in terms of milligrams.

The test dose of OT given will vary under different conditions. For checking suspected cases of tuberculosis and for routine case finding surveys the usual dose is 0.1 mg. In patients with tuberculosis of the eye or skin, or in cases of hypersensitivity, 0.001 mg. is usually given. Since some individuals do not respond to smaller doses, it is necessary

TABLE 6

Dilutions of OT Used Diagnostically

Ml. OT	Ml. Diluent	Dilution	Test Dose In Ml.	Mg. OT
1	9	1:10	0.1	10.0
1 of 1:10	9	1:100	0.1	1.0
1 of 1:100	9	1:1,000	0.1	0.1
1 of 1:1,000	9	1:10,000	0.1	0.01
1 of 1:10,000	9	1:100,000	0.1	0.001

to use larger amounts.

Methods of Application. The test is performed in various ways. The subcutaneous test was first employed by Koch[111] and since then other modifications have been used such as the intracutaneous method of Mantoux[112, 113], the percutaneous method of Moro[114], the cutaneous scratch test of Von Pirquet[115, 116], the ophthalmic test of Calmette[117] and the patch test of Vollmer and Goldberger[118,119].

Reading of Reactions. Readings are made in 24 and 48 hours, and graded from + to ++++ on the basis of the extent in millimeters of hyperemia, edema, and necrosis. Persons reacting to tuberculin are called allergic, those failing to react anergic.

Purified Protein Derivative (PPD). Seibert et al.[120] and Long and Seibert[121] found that tuberculin could be obtained in relatively pure form by growing tubercle bacilli on synthetic media and precipitating the protein fractions. When heated their capacity to sensitize could be decreased. This preparation, known as PPD (purified protein derivative) is stable and of uniform strength and is being used more and more in place of the less carefully standardized preparations of OT. Like OT, PPD is not a complete antigen and for this reason false positive reactions will not occur in individuals who are tested repeatedly over long periods of time. The test material is prepared as a dry powder which is diluted with a buffered diluent prior to use. In the dry form it is stable indefinitely, but begins to deteriorate in 2 to 3 days after being dissolved. Diagnostically it is used in two strengths, first, which is approximately equivalent to the 0.01 mg. dose of OT, and second, which is 250 times as strong as the first, and equal to about 1 mg. of OT. The same precautions exercised with OT in

regard to sensitivity applies to PPD.

Character of Sensitivity. Tuberculin sensitivity is distinguished from the phenomenon of anaphylaxis or atopy in that it is apparently not mediated by a humoral antibody mechanism. Reactions following inoculations with tuberculins are delayed, requiring 24 to 48 hours to reach their height, and depend on a sensitization of tissue cells.

Types of Reactions. The reactions produced by tuberculins are of three types:

1. A local typical inflammatory response at the site of injection.

2. A focal inflammation, hemmorrhage or necrosis at the site of the tuberculous process, the severity of which depends upon the dose of tuberculin. Pain, edema, increased sputum, and hemoptysis are characteristic responses. Histologically there is evidence of capillary dilatation and leucocytic infiltration. The spread of organisms to other tissues may occur, tubercle formation increases, and if the process continues, miliary or disseminated tuberculosis results, and the patient may even die.

3. A general reaction characterized by fever, rapid pulse, malaise, prostration and even death, or other reactions typical of those which occur after the inoculation of other toxic agents.

Variations in Sensitivity. There are a number of important variations in tuberculin sensitivity which should be mentioned. Krause(122) has shown that tuberculin sensitivity can not be elicited until approximately 8 to 12 days after infection, when caseation has occurred. With the healing of foci, the intensity of the reaction diminishes. It is also of interest to mention that the cells nearest the focus of infection are more sensitive than the other cells, but Long(123) has shown

that the germinal cells of the testicle are exquisitely sensitive and because of this fact has used the testicle in guinea pigs for standardizing tuberculin. In the terminal stages of tuberculosis or in an acute miliary phase of the disease where the body is overwhelmed by infection, the intensity of the reaction is greatly reduced or may be completely lost. Sensitivity does not manifest itself in the preallergic state. Repeated applications of tuberculin may reactivate a healing focus, and given at too frequent intervals may lead from a state of allergy to one of anergy. Other conditions have been reported to produce anergy. For instance, in experimental work an animal which is too young or emaciated, even though successfully infected, will not react to tuberculin. Intercurrent infection, menstruation, pregnancy and parturition have also been observed to temporarily diminish allergic capacity; but with the return to a normal physiologic state, the allergy again becomes manifest. The varying degrees of reaction in reference to foci of infection has already been mentioned.

Specificity of the Reaction. The question of tuberculin specificity has always been one of great controversy. One group of workers believes that the reaction is specific, another group feels that it is not, and between these two extremes there are all degrees of opinion. There appears to be sufficient evidence to indicate that specificity is only relative. Many investigators have shown that in such types of tuberculosis as advanced pulmonary, skin, genito-urinary, and joint the inoculation of non-specific proteins as egg albumin, milk, sera, vaccines, fungi, bacteria, or their extracts, proteoses, and glycerine can also produce all the tuberculin types of reactions. Other workers have called attention to the non-specificity factor by pointing out that positive

reactions could be elicited in other inflammatory diseases such as arthritis. Petroff[124] showed that large enough doses of heat-killed tubercle bacilli could establish an allergic state. Baldwin[125] demonstrated that slight transitory degrees of hypersensitivity to tuberculin could be produced by repeated inoculations of tubercle bacilli proteins, but water extracts of the saprophytic timothy bacillus, (M. phlei) failed to react in guinea pigs infected with the human strains. Long[126] referring to the work of Krause and Baldwin on cross sensitization and skin allergy pointed out that their experiments clearly indicated that tuberculous animals are hypersensitive to extracts of various non-pathogenic acid-fast bacilli, and conversely that animals infected with the avirulent strains respond to tuberculin in the same manner as tuberculous animals. In his own work he observed that extracts of acid-fast turtle bacilli could elicit an allergic response, though mild, in guinea pigs infected with human strains. In a later study with acid-fast bacilli he and Soyfarth[127] noted that extracts from the frog, grass and smegma bacilli were also capable of producing allergic phenomena in tuberculous guinea pigs. Elder and Lee[128] succeeded in producing local lesions in cattle with the avian tubercle bacillus, and they reported that these animals were able to react to mammalian tuberculin. Calmette[129] showed that inoculations of extracts of paratubercle bacilli in both man and animals gave local or febrile reactions similar to those produced with tuberculin. In an effort to determine the causes for tuberculin sensitivity in non-tuberculous cattle, Crawford[130] demonstrated that the timothy grass, the mist, and hog-skin bacilli isolated from lesions of these animals could produce definite tuberculin sensitivity and since the reactions to mammalian tuberculin was

usually atypical, he felt that this suggested a group rather than a specific mammalian-tubercle bacillus sensitization. The chief importance of this problem is based on the fact that acid-fast bacilli from the soil presumably cause certain types of skin lesions in cattle and can sensitize these animals to tuberculin. Sensitization has even been described in cattle with no macroscopic lesions. Such sensitization is thought to result from ingestion. Crawford[130,131], Van Es[132], Frey and Hagan[133], Daines and Austin[134,135], and Branch[136] have reported similar studies.

In spite of these extensive investigations there are those who still maintain that although it has been demonstrated that non-specific substances, fungi, certain non-acid-fast bacteria (colon, typhoid, brucella and diphtheria) or their products, and many acid-fast bacilli other than tubercle bacilli manifest allergic phenomena, the reactions are never as clear cut or as intense as those obtained using equivalent amounts of tuberculin. The methods of preparing and standardizing various tuberculins have been other factors of considerable debate.

The major evidence that tuberculin allergy is based on tuberculous infection has been derived from the high correlation between tuberculin hypersensitivity and postmortem findings.

Cytotoxic Properties of Tuberculin. Interested in the effects of tuberculin on tissue cells, Rich and Lewis[137] undertook studies of living tissue cultures of washed leucocytes, and reported that tuberculin in adequate concentration could exert a selective toxic effect on cells from tuberculous animals. The same results could be produced with sensitized cells in normal plasma, and for this reason it was believed that the toxic manifestations were not due to humoral anti-

bodies, but to some mechanism within the cells. These observations were extended and confirmed by Aronson[138], Rich and Lewis[139], Moen and Swift[140] and Heilman et al.[141].

Evidence for the cellular mechanism of tuberculin sensitivity was established through some of the work of Chase[142], Cummings et al.[143] and Stavitsky[144] who demonstrated that tuberculin allergy could be established passively in normal guinea pigs by inoculations of cells from peritoneal exudates, lymph nodes, spleen and whole blood of guinea pigs which were sensitized by heat-killed tubercle bacilli suspended in mineral oil. Stavitsky felt that the lymphocytes may play an important role in sensitivity, and emphasized the significance of such experiments for the possible elucidation of the chemical mechanisms responsible for this type of allergy.

In further studies on the cellular mechanisms of tuberculin sensitivity Favour[145] has shown that the protein extracts of the tubercle bacillus can produce marked lytic effects in vitro on leucocytes from tuberculous mice and guinea pigs. Fremont-Smith et al.[146] have reported similar cytotoxic effects on white cells from tuberculous humans. In a subsequent study Favour[147] found that leucocytes from normal tuberculin negative persons, as well as from patients with active tuberculosis, were capable of adsorbing OT from homologous fresh plasma. White cells from normal and tuberculous mice could not do so. This suggested an affinity of tuberculin for the tissues of some species of animals and not for others.

Since normal serum or plasma was used in studying the cytotoxic action of tuberculin, it was believed that the effects were specific for the tuberculous-type leucocytes and was independent of any serum component.

But in later studies[148] it was found that such thoroughly washed cells could not be lysed in normal plasma. On the other hand, washed cells from tuberculous patients, or from normal tuberculin negative persons, when placed in plasma from infected patients in the presence of Old Tuberculin could be lysed. Attention was called to the fact that the results of earlier studies[146] in which the tuberculous cells similarly lysed in normal serum were probably due to traces of tuberculous plasma on the surfaces of unwashed or insufficiently washed cells. These observations indicated that some component in tuberculous plasma was essential for the in vitro lysis of leucocytes by tuberculoprotein. This was proved in subsequent experiments[149]. The factor, present in the euglobulin portion of the plasma, is a non-dialyzable heat labile fraction which is stable for 7 days at 10 C., and can be precipitated with the globulin fraction of the plasma proteins.

Prompted by the observation that a positive tuberculin test in man may be followed by an increase in in vitro tuberculin white cell lysis, Favour et al.[150] studied this phenomenon further in guinea pigs, and noted that the animals, sensitized to tuberculin by the inoculation of heat-killed tubercle bacilli in paraffin, exhibit a similar increase in in vitro tuberculin cytolysis.

THE ATYPICAL ACID-FAST BACILLI

Introduction

A large number of atypical tubercle bacilli and acid-fast organisms other than tubercle bacilli isolated from human, animal and other sources have been described. Many of these strains have been shown to possess varying degrees of virulence. The biologic relationship between the tubercle bacilli, the so-called saprophytic, and the atypical acid-

fast organisms is not well understood. A number of workers have isolated chromogenic acid-fast bacilli from the sputum of patients who presented vague clinical symptoms and roentgenographic evidence simulating tuberculosis and, because of the indefiniteness of these findings, diagnosis was difficult, for chromogenic acid-fast bacilli are generally accepted as saprophytes incapable of producing disease. The ubiquity of these organisms in nature and in the other sources mentioned also emphasizes the necessity for clarifying the position of these strains in diagnostically uncertain cases. Since the classification of the saprophytic and atypical group is still very inadequate, it is difficult to determine whether all the organisms described are different, or, in many instances, merely redescriptions of similar types.

History

Reports describing organisms resembling, more or less, the atypical chromogenic group studied in this investigation have appeared from time to time, and these observations are summarized below.

Eastwood and Griffith(151) isolated a number of chromogenic mycobacteria from tissues, pus and fluid of human bone and joint tuberculosis which differed in one or more respects in cultural characteristics and virulence from the human and bovine types.

Griffith(152) obtained four atypical chromogenic strains from the sputum of tuberculous patients which exhibited standard mammalian virulence, but these did not resemble culturally either the human or bovine types.

Cobbett(153) reported a case with chronic intractable pustules covering the back, buttocks and thighs from which atypical cream-colored acid-fast bacilli were isolated. Peritoneal inoculations into

guinea pigs of pus from the lesions failed to kill the animals or produce visible lesions. Results of culture inoculations into animals were not reported.

Rabinowitsch[154] in her studies of atypical acid-fast bacilli called attention to the fact that occasionally human tuberculosis is caused by strains of tubercle bacilli possessing the biological properties of the avian type, and that a strict division of the several types is not always possible because of intermediate strains. She further pointed out that similar organisms were found frequently in cases of tuberculosis of the skin (lupus), and she could not exclude the possibility that, under certain conditions, a transformation of the primary type may take place in the human body. Similar opinions were held by Pibram[155], Maher[66], Miller[50], Larnola[65], Stodkwell[68], Sweany[69] and Dreyer and Vollum[73].

Mitchell[156] isolated a number of atypical acid-fast bacilli from lesions of cattle simulating subcutaneous tuberculosis. The organisms were difficult to culture, and were of low virulence for guinea pigs and rabbits. About 70 per cent of the cattle reacted less severely to tuberculin than animals in the same herd affected with characteristic tuberculous lesions.

The identification of chromogenic acid-fast bacilli has been further complicated by the fact that avian tubercle bacilli can dissociate into chromogenic strains which culturally and in other respects resemble the types of chromogenic organisms under consideration in this investigation. It is not the purpose at this point to discuss avian infection in man, but in reviewing the history of this problem attention should be called to the work of L'Esperance[157,158] who presented

evidence that avian bacilli may be the cause of Hodgkin's disease. She felt that her observations justified the conclusion that in the chickens which she inoculated with Hodgkin's glands, there were lesions with the histologic features of Hodgkin's granuloma which were also comparable with avian tuberculosis. This obviously suggested the pathogenicity of the avian bacillus for man. She further demonstrated that while avian bacilli are nonpathogenic for the usual laboratory animals, except the chicken and rabbit, they do become infectious if dead human or bovine bacilli are first injected. From these observations she concluded that it is possible to transform a resistant animal into a more susceptible one by previous treatment with dead heterologous tubercle bacilli. The acquisition of virulence for man by some chromogenic acid-fast bacilli might be explained by such a concept. Branch(159) in his studies of tuberculosis concluded that avian infection in man is rare, and that the etiologic role of the avian tubercle bacillus in Hodgkin's disease cannot, for the present, be accepted as proved. In a later report(136) he called attention to the fact that there were a number of instances of non-mammalian acid-fast bacilli infecting man. Some of these organisms were thought to belong to the avian group, while others were believed to be new strains of pathogenic mycobacteria.

Beaven(160) and Beaven and Bayne-Jones(161) reported a case of extensive pulmonary disease resembling tuberculosis in an 11 week old infant due to an atypical chromogenic acid-fast organism which culturally closely resembled the avian tubercle bacillus.

Miller(50) isolated a "variant" from a two-months-old culture of the H37 strain of the human tubercle bacillus which morphologically resembled the parent colony except that it was chromogenic. Microscopi-

cally he found numerous gram-positive non-acid-fast rods and granules, together with a few acid-fast rods and granules. Some of the non-acid-fast rods also contained from one to three acid-fast granules. When inoculated into guinea pigs the chromogen produced fewer lesions than the more virulent H37 strain. Miller believed that this organism was a non-acid-fast mutant derived from the parent culture. Similar observations with the H37Rv strain have been made in this study(162).

Pinner(163) isolated and studied a number of atypical chromogenic acid-fast bacilli which he believed had not been described previously. These organisms varied in their growth intensity, in pigment production, in their growth on liquid media and in their pathogenic effects. In a subsequent investigation(165) he reported the isolation of 4 white, smooth acid-fast strains. He thought that these were probably relatively avirulent variants of human tubercle bacilli. By serial animal passage 3 of these strains were transformed into typical human colonies. The resulting rough-growing organisms behaved entirely like true human strains, and their characteristics remained stable for many generations. In other studies(82,164) Pinner isolated atypical chromogenic acid-fast bacilli in 0.9 per cent of 5,000 diagnostic sputum cultures. He considered it striking that all of these organisms, with one exception, were isolated during, or at the beginning of, inactive phases of tuberculosis. All attempts to obtain them from extraneous sources such as air, media, or any material used in culturing specimens were unsuccessful. These strains, in contrast to the classical saprophytes, were capable of sensitizing guinea pigs to tuberculin and produced self-healing granulomatous lesions. In two experiments serial animal transfer caused progressive tuberculosis from which colonies of typical tubercle bacilli

were isolated. Pinner could not duplicate these results in a second experiment.

Prompted by the suggested immunologic relationship of chromogenic acid-fast bacilli to true tubercle bacilli Wenkle et al.(110) investigated the immunizing capacity of the former organisms in guinea pigs against virulent human tubercle bacilli. The chromogenic organisms were isolated from sputum, nasal secretions, urine, skin and the mucous membranes of patients with active, arrested and cured tuberculosis. From their general description, these strains appear similar to those described by Pinner and the organisms studied in this investigation.

Schiff and Tarshis(166) reported a case of tuberculous meningitis from whom an atypical chromogenic organism was isolated. Two cultures of this organism obtained from the spinal fluid during the third and fourth months after the patient was placed on streptomycin therapy, killed guinea pigs in approximately 6 weeks following 1.0 ml. intraperitoneal inoculations of heavy suspensions. On gross examination of the lungs, liver and spleen of the animals, a typical picture of tuberculosis was found. Microscopic findings were atypical. Stains of direct smears from the lesions of these organs and tissue sections revealed acid-fast organisms. Chromogenic acid-fast bacilli were also found present in 1 per cent of 3,011 diagnostic cultures from patients in various phases of tuberculosis, but chiefly from those in an inactive phase of the disease. Of special interest is the fact that 19 per cent more chromogenic organisms were isolated from patients who were receiving or who had received streptomycin than from patients who had never received such treatment.

Thomson(167) in an endeavor to find criteria for classifying

saprophytic acid-fast bacteria described a group of pigmented "leprae" bacilli which bear some resemblance to those of Pinner[164] and to the strains studied in this investigation.

Daines and Austen[134,135] isolated three different groups of acid-fast bacilli from cutaneous lesions in tuberculin-reacting cattle. Their group III may also bear some relationship to the chromogenic human strains described by Pinner and the ones in this study.

Schwabacher[168] reported the isolation of chromogenic saprophytic acid-fast bacilli from blood cultures of 6 tuberculous patients, 1 patient with Schizophrenia, 6 tuberculous rabbits and 3 normal rabbits. Six strains were recovered in culture from water and slime, 3 from dust, and 1 from a comedo. Some of these organisms had rough, while others had smooth colony characteristics. They were all avirulent in 1.0 mg. doses for guinea pigs.

Cummins and Williams[169] reported a case of acute pulmonary disease in a young female from whom an atypical chromogenic acid-fast bacillus ("M" strain) was isolated. The disease simulated tuberculosis. Animals infected with varying doses of the organism (0.5 to 2.0 mgs.) showed chronic abscesses which were septic rather than tuberculoid in type, and, in this respect, resembled the septic pneumonitis of the patient. Positive reactions to a tuberculin prepared from the organism were obtained in the infected animals and also in one case of human tuberculosis. Normal humans and guinea pigs were negative on test. Griffith[170] in more intensive animal studies with this organism using larger doses (1 to 25 mgs.) found that it was pathogenic for rabbits and mice when inoculated intravenously or intraperitaneally, but had little pathogenicity for the guinea pig and none for the fowl or frog. This chromogenic strain differed from tubercle bacilli both in cultural

characteristics and its effect upon animals, and was not identical with any of the acid-fast bacilli he obtained from tubercle-like lesions in cold-blooded animals or any of the saprophytic acid-fast organisms with which he worked. Steenken and Landau[171] studied the "M" strain further and included an "IP" strain isolated from an almost identical case. The object of their investigation was to determine whether these organisms could be dissociated, to compare the behavior of the variants on artificial media, and to ascertain their pathogenic potentialities. Each strain was dissociated into at least 2 variants possessing distinct topographical and biological characteristics. Some of the variants in the early stages of colony development appeared to be white, but with age at room temperature and exposed to light, they invariably developed chromogenicity. Environmental conditions were shown to play an important part in morphological variation. The strains were non-pathogenic for laboratory animals except in the case of "M" which exhibited some pathogenicity for mice and rabbits. It was felt that larger doses or animal passage might increase the degree of virulence. Specific tuberculins could be prepared from each strain, and animals reacted more strongly to the homologous than to the heterologous extract.

Lester[172] studied the frequency of occurrences of acid-fast saprophytes in cultures from human material to determine their significance as sources of error in cultural examinations. In a five year period (1932 - 1936) she isolated 130 acid-fast saprophytic strains from 26,343 samples of sputa, stomach washings, pleural fluids, urines, and other specimens. Of these 55 had a bright orange color. In routine work four groups of organisms were found, (1) human tubercle

bacilli, (2) bovine tubercle bacilli, (3) atypical strains of tubercle bacilli, including chromogenic organisms which from her descriptions appear similar to those described by Pinner(164) and the ones in this study, and (4) acid-fast saprophytes. Of the 130 acid-fast saprophytic strains none were pathogenic in the doses used (0.01 to 10.0 mgs.), but the most dysgonic of those growing in orange colored colonies were able to produce small abscesses by intracutaneous injections. No propagation was seen, and the abscesses healed spontaneously in a few weeks. One smooth atypical yellow chromogenic strain had the virulence of an avian tubercle bacillus, for a 1.0 mg. inoculum killed a rabbit in 4 weeks, and the postmortem findings corresponded to those described as "Yersin" type tuberculosis. In guinea pigs 0.1 to 1.0 mg. injections only produced local lesions, whereas 2 guinea pigs inoculated with 5.0 mgs. intraperitoneally died in a month with degenerative changes in the organs, but with no nodules, and many caseous glands in the omentum. Here the organisms were abundant, whereas in the organs they were scarce. Lester called attention to the fact that in diagnostic work it should always be remembered that saprophytes may show resemblance to typical tubercle bacilli. She further pointed out that atypical strains are scarce, and if an organism is not easily recognized as a typical human or bovine tubercle bacillus, it should not be discarded until sufficiently examined by cultural and animal tests.

Baldwin(173) reported a case of chronic pneumonitis in a 24 year old female secretary associated with an acid-fast chromogenic organism which he believed to be related to the timothy or grass group of acid-fast bacilli. The organism proved non-pathogenic for guinea pigs, white mice, rabbits and chickens. The patient reacted to 0.000,25 ml.

of OT, and much more strongly to 0.001 ml. of a tuberculin prepared from the infecting strain. The organism was isolated from the sputum on repeated occasions. Following an episode of acute pneumonia and achalasia, the patient developed fibrosis and emphysema. In a subsequent publication[174] he also showed that chromogenic acid-fast bacilli could be isolated from various fresh fruits and vegetables, and pointed out the possibility that patients suffering from cardiospasm might aspirate into the respiratory tract regurgitated food containing these organisms and thus produce pulmonary irritation.

Feldman et al.[175] reported a case of pulmonary disease of long duration in a 53 year old underground miner associated with an atypical chromogenic acid-fast organism which was dissimilar to the bovine and human types of mycobacteria. Although roentgenographic examination of the chest appeared to justify a diagnosis of far advanced, bilateral pulmonary silicotuberculosis, specimens of sputum containing acid-fast bacilli failed repeatedly to produce tuberculosis in guinea pigs. The organisms sensitized guinea pigs, rabbits and chickens to avian tuberculin and less consistently to mammalian tuberculin, and they were not pathogenic for guinea pigs. They did, however, exhibit a relatively low but definite pathogenicity for rabbits. With one exception, all chickens inoculated intravenously did not manifest progressive tuberculosis; intramuscular inoculations of the chickens, however, resulted in some instances in the development of a destructive tuberculosis-like process without extension to the parenchymal organs. The resistance of the organisms to oxalic acid, their tinctorial characteristics, their ability to induce sensitivity to tuberculin, their agglutinogenic features and their ability to establish limited tuberculosis-like lesions

in rabbits and chickens all indicated a strain closely related to true tubercle bacilli. Although characterized by many features suggestive of avian tubercle bacilli, the cultures investigated were not typical of this organism. On the basis of certain biochemical and agglutinogenic characteristics the various cultures were not all identical. This indicated an instability of the organisms present in the pulmonary lesions, although the specific mechanism was unexplained. It was thought conceivable that the silicosis of the patient may have provided a favorable threshold for the parasitism of an organism ordinarily nonpathogenic for human beings.

Larmola(65) in his studies of a large number of typical and atypical tubercle bacilli isolated from sewage, water tanks, and forest ditches in the vicinity of a tuberculosis sanatorium and from sputum and gastric washings of tuberculous patients found great variability in staining, morphologic, cultural, and pathogenic properties of these organisms. For instance, he noted that organisms of low pathogenicity on cultivation in the laboratory for long periods lost their pathogenicity. This was associated with change in the characteristic mode of growth, staining and morphological appearance, and colony pigmentation. Some strains of tubercle bacilli isolated from patients with slight activity exhibited low pathogenicity. Other strains with low pathogenicity for man produced overwhelming disease in guinea pigs, while others avirulent for these animals were still capable of sensitizing them to tuberculin, and by animal passage they could again be made virulent. Still other strains which resembled true virulent human tubercle bacilli in all respects were not always pathogenic for guinea pigs.

Sources of Organisms and Histories of Patients

The chromogenic acid-fast organisms studied in this investigation have been isolated from the following four groups of patients.

1. From those suspected of having tuberculosis.
2. From those in an active phase of tuberculosis.
3. From those in a clinically improved phase of tuberculosis.
4. From those having died of tuberculosis.

The sources of the chromogenic acid-fast organisms and histories of the patients from whom isolated are given in Table 7. The sources of the standard strains of tubercle bacilli and the saprophytic mycobacteria used as controls are shown in Table 8.

General Bacteriological Characteristics

Morphology and Staining. All of the chromogenic organisms are acid-fast, gram-positive, non-motile, non-spore-bearing, non-capsulated rods. They are quite variable in size, ranging approximately from 1 to 15 microns in length by 0.1 to 0.8 microns in width. Most of the organisms resemble tubercle bacilli, but the majority are somewhat longer and in some cases thicker. They are also somewhat pleomorphic, exhibiting clubbing at one or both ends with central body constrictions. Some strains are very tiny and regular in contour, others are elongated, somewhat irregular, and show central arching. Morphology varies considerably according to the medium used for growth and the age of the culture. In general, pleomorphism is more marked in older cultures. Granules are seen frequently in some of the strains and only rarely in others; these are spaced regularly for the most part but may also be found arranged irregularly. Occasionally only one granule can be seen, but several are

TABLE 7

Sources of Chromogenic Acid-fast Organisms

Strains	Source	Specimens from Which Isolated	Therapy Prior to Isolation of Organism	Tuberculosis History
S-1 to S-10 M-1 to M-6 (14 strains)	Mayo Clinic Rochester, Minn.	Sputa and gastric lavages	Unknown	From suspected, active, or inactive cases
W-1 to W-3 (3 strains)	Veterans Hospital Walla Walla, Wash.	Sputa	Streptomycin 4 months	All in clinically improved phase
W-4	Veterans Hospital Walla Walla, Wash.	Sputum	No therapy	Pneumonitis
W-7	Veterans Hospital Walla Walla, Wash.	Pus, lung abscess	Streptomycin 4 months	Active, pulmonary, expired
509	Veterans Hospital Vancouver, Wash.	Sputum	Streptomycin 42 days	In clinically improved phase
786	Veterans Hospital Vancouver, Wash.	Sputum	No therapy	Pneumonitis
886	Veterans Hospital Vancouver, Wash.	Gastric lavage	No therapy	In clinically improved phase
6288	Ore. St. Brd. Health Portland, Oregon	Sputum	Unknown	Suspected
98717	Olive View Sanatorium Olive View, Calif.	Unknown	Unknown	Unknown
Parks, S-4V (2 strains)	Trudeau Laboratory Trudeau, New York	Unknown	Unknown	Unknown

TABLE 8

Sources of Standard Strains of Tubercle Bacilli and Saprophytic Mycobacteria

Strain		Source
TUBERCLE BACILLI		
BCG	*M. tuberculosis* (var. bovis)	Henry Phipps Institute, Philadelphia, Pennsylvania
H37Ra	*M. tuberculosis* (var. hominis) Avirulent variant	Trudeau Laboratory, Trudeau, New York
H37Rv	*M. tuberculosis* (var. hominis) Virulent variant	Trudeau Laboratory
SAPROPHYTIC MYCOBACTERIA		
	M. phlei - a	Trudeau Laboratory
	M. phlei - b	Trudeau Laboratory
	M. smegmatis - a	Tuberculosis Evaluation Laboratory, Communicable Disease Center, Chamblee, Georgia
	M. smegmatis - b	Tuberculosis Evaluation Laboratory
	M. butyricum	Tuberculosis Evaluation Laboratory

generally present. Acid-fast staining may be intense, moderate, or weak. Young colonies often stain weakly acid-fast and occasionally they may not stain at all. One may find only acid-fast granules. Branching has not been observed.

Cultural Characteristics. The strains are aerobic and the majority can be cultured on a variety of media, but all of them multiply best on those used for culturing tubercle bacilli. In liquid media growth rarely occurs on the surface with pellicle formation, in others it takes place at the bottom of the tube, while in still others it is uniform throughout. In some cases one may find a combination of any of these three types. On the most favorable medium colonies may be detected in about 2 days at 37 C., but the average period is about 4 to 6 days. This is chiefly dependent upon the size of the inoculum, colonies appearing earlier with the larger and later with the smaller inocula. Maximum development takes place in from 2 to 4 weeks. At room temperature growth is very much slower than at 37 C. The organisms can also withstand the usual concentration technics used for destroying contaminants prior to culture.

Colony Morphology. With the exception of one strain (M-2), all colonies are convex, smooth, shiny, and soft to tenacious in consistency. In color they range from lemon-yellow to dark orange, the intensity of pigmentation increasing with the age of the culture. Some strains are easily emulsified in water or saline, but others are not. Dispersed growth can be obtained in Dubos-Davis medium(12). At first strain M-2 was like the other organisms, but after repeated subculture on Corper-Cohn medium(178) it acquired rough characteristics similar to the human tubercle bacillus, suggesting possible dissociation.

Pathogenicity. All strains were tested for pathogenicity in guinea pigs and some in rabbits and white Swiss mice. Small doses in general

did not produce any significant macroscopic lesions, but larger doses did, and in some cases death resulted. The details of the pathological manifestations are discussed in the section on pathogenesis.

Methods

The media used in this investigation and the methods of preparation are presented in the section on culture media. To obtain dispersed growth, tubes of Dubos-Davis liquid medium (formula given by Fisher)(176) were inoculated with the various organisms and incubated for 14 days at 37 C. Following incubation the cultures were adjusted to the No. 5 tube of the McFarland(177) nephelometer standard (1,500,000,000 organisms per ml.) and 0.1 ml. amounts (150,000,000 organisms) were used for inoculation in the cultural and streptomycin sensitivity experiments. All washed organisms were prepared by centrifuging the tubes of Dubos-Davis medium, decanting the supernatant fluids, and washing the bacterial sediments three times with 0.85 per cent sodium chloride solution. Following inoculation, all culture tubes of solid media were incubated in an inclined position for the first 6 days, after which time they were placed in the vertical position. The tubes were incubated at 37 C. and observed daily under a bright light for 6 weeks. The day growth first appeared was recorded numerically. After 6 weeks incubation the final degree of growth was indicated from + to ++++. The control media included Corper-Cohn(178) and Dubos-Davis(176). All media were checked for sterility prior to use. Streptomycin sensitivity experiments were done using Youmans liquid medium (formula given by Fisher)(176). The cultures were incubated for 14 days at 37 C. before final readings were made. In other experiments incubation was continued for as long

as 12 weeks. The concentrations of the antibiotic used were 1, 10, and 20 mcgs. per ml. All organisms studied were subcultured on Corper-Cohn medium once monthly, and, in addition, the H37Rv strain was subcultured every two weeks on Youmans medium (without serum albumin). This was done at the recommendation of the Trudeau Laboratory to maintain virulence.

In the animal experiments all strains were tested for pathogenicity in guinea pigs, and in addition, strains M-6, 886 and H37Rv (control) were tested in rabbits and white Swiss mice. Young male animals were selected for study, the guinea-pigs ranging in weight from about 200 to 380 grams, the rabbits from 1440 to 2240 grams and the mice from 10 to 17 grams. Almost all inoculations were made intraperitoneally and a few were made subcutaneously and intravenously. The inocula were made up according to the wet weight quantitative planting method described by Corper and Cohn(178). Samples of all such preparations were cultured at the time of inoculation to check the viability of each strain. All animals were weighed weekly, and tuberculin tested prior to infection. The time of subsequent testing is indicated in the protocols. Second strength PPD (purified protein derivative containing 0.005 mg. of tuberculin)[1] was used, and given intracutaneously in 0.1 ml. amounts.

Tuberculin reactions were graded as follows:

\+ = Reaction more than 5 mm. and not exceeding 10 mm. in diameter, showing some redness and definite edema.

++ = Reaction more than 10 mm. but not exceeding 20 mm. in diameter, with an area of redness, edema and with or without necrosis.

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[1]Parke, Davis and Company, Detroit, Michigan.

+++ = Reactions exceeding 20 mm. in diameter with moderate to marked redness, edema and with or without necrosis.

++++ = Reactions exceeding 20 mm. in diameter with very marked redness, edema and necrosis.

Experimental tuberculins[1] were prepared by growing the organisms in Youmans liquid medium for 6 weeks at 37 C., then centrifuging the cultures, and evaporating the supernatant fluids to one-half of their volumes in a 60 C. water bath. After evaporation 4 ml. amounts were added to sterile vaccine vials and enough 5 per cent phenol, made up in a solution of 0.85 per cent sodium chloride, was added to each vial to give a final concentration of 0.5 per cent. Bacterial suspensions were prepared in the same manner after diluting the centrifuged sediments with 0.85 per cent sodium chloride. A control solution was made of 0.85 per cent sodium chloride and 0.5 per cent phenol. The tuberculins, bacterial suspensions and control solution were placed in the refrigerator for 72 hours prior to use. Intracutaneous inocula of 0.1 ml. of each preparation was used in the tests.

Animals were sacrificed by ether inhalation, and those killed or expired were autopsied and sections of tissue removed aseptically, placed into sterile Petri dishes, cut into small fragments and portions inoculated into tubes of Corper-Cohn(178) and Petragnani(179) media in an attempt to isolate the organisms used to infect the animals. Direct smears were also made of the lungs, livers, spleens, kidneys, and abscesses and stained for acid-fast bacilli by the Ziehl Neelsen technic. Other portions of these tissues were preserved in 10 per cent formalin for microscopic study. The spleens of all the animals were measured.

[1] The term tuberculin as here used refers to bacterial extracts of acid-fast organisms, not necessarily true tubercle bacilli.

Experimental

Cultural Studies.

A. Growth of Chromogenic Acid-fast Organisms and Tubercle Bacilli on Different Media. The initial experiments were designed to ascertain the general cultural characteristics of the chromogenic organisms. Thirteen different media, arbitrarily selected, were observed to determine when growth first occurred, the degree of growth after 6 weeks incubation and which media would or would not favor pigment production. The range and degree of growth of the media and the organisms were also studied to find out which medium supported the growth of the greatest number of organisms and which the least, and which organism grew on the largest number of media and which the least. For comparison the three standard strains of tubercle bacilli were included. These data are presented in Tables 9, 10, and 11.

In Table 9 it will be noted that, in general, the media such as the Loeffler, liver tryptose agar, brain heart infusion broth ascitic fluid, brain heart infusion broth, thioglycollate and extract broth do not support the growth of either the chromogenic organisms or tubercle bacilli very well. The other media do support most of these organisms, especially the Dubos-Davis and Corper-Cohn. Strains W-3 through 509 resemble the tubercle bacilli, since they also grew poorly on the first 6 media. Strains S-9 through W-7 differ in this respect, because they were able to grow with two exceptions, on all of the last 4 of these media. Rather marked selective requirements were exhibited by S-1, M-3, M-1 and 509, which developed on only the liver tryptose agar, whereas W-2 developed on only the extract broth, and 98717 on only the thioglycollate. Outstanding is M-6 which multiplied on all the media, in

TABLE 9

Growth of 25 Acid-fast Organisms on 13 Different Media to Indicate the Day Growth was First Observed, the Degree of Growth, and Pigment Formation

Medium	GAFB																
	W-3	6288	S-7	786	S-3	98717	S-8	M-2	W-1	S-1	M-3	W-2	M-1	509	S-9	886	W-
L.....	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
L-TAg....	0	0	0	0	0	0	0	0	0	7++	8+	0	8++	11++	0	0	0
BHI+AF...	0	0	0	0	0	0	0	0	0	0	0	0	0	0	10+	0	0
BHI.....	0	0	0	0	0	0	0	0	0	0	0	0	0	0	9+	32+	13
T.....	0	0	0	0	0	35+	0	0	0	0	0	0	0	0	10+	32+	15
E-B.....	0	0	0	0	0	0	0	0	0	0	0	17+	0	0	9+	33+	13
B-Ag....	0	0	0	0	0	16+	23+	0	0	0	0	12+	35+	18+	0	0	13
E-Ag....	0	0	12++	0	14+	0	0	12+	12+	13+	7+	15+	15+	14+	0	12+	16
B-G.....	0	12++	0	39+	0	0	0	0	6++C	0	35+	12+C	20+	21+	8++C	11+C	16
B-A.....	0	0	6++	31+C	8+	0	9+	12++C	12+	7+	8+	15+	8+	16+	0	11+	0
T-Ag....	16+	7+C	0	31+C	18++C	13+	9+	12++++	5++C	7++	6+	12+	21+	12+	8+	9++C	13
D-D.....	4+	3++	4+	5+	10+	3+	3+	3+	3+	3+	2++++	3+	3+	3++	3++	3++	3
C-C.....	8+++C	6++++C	10++++C	9+C	8++++C	4++C	4++++C	6++++C	5++++C	3++++C	3++++C	4++++C	5++++C	7++++C	4++++C	4++++C	5

GAFB = chromogenic acid-fast bacilli.
TB = tubercle bacilli (M. tuberculosis: BCG = var. bovis; H37Ra, H37Rv = var. hominis).
C = media showing chromogenic colonies.
L = Loeffler. L-TAg = liver tryptose agar. BHI+AF = brain heart infusion + human ascitic fluid. BHI = brain heart infusion. T = thioglycollate. E-B = extract broth. B-Ag = blood agar. E-Ag = extract agar. B-G = Bordet-Gengou agar base. B-A = blood agar base. T-Ag = tryptose agar. D-D = Dubos-Davis. C-C = Corper-Colm.
Numbers represent the day growth was first observed.
Symbols + to ++++ represent final degree of growth after 6 weeks at 37 C.

TABLE 9

25 Acid-fast Organisms on 13 Different Media to Indicate the Day was First Observed, the Degree of Growth, and Pigment Formation

	CAFB														TB		
	W-1	S-1	M-3	W-2	M-1	509	S-9	886	W-4	S-10	S-5	S-6	W-7	M-6	BCG	H37Ra	H37Rv
	0	0	0	0	0	0	0	0	0	0	0	0	0	7++C	0	0	19+
	0	7++	8+	0	8++	11++	0	0	0	0	0	0	0	7++++C	0	0	0
	0	0	0	0	0	0	10+	0	0	24+	30+	28+	9+	4+++	0	0	25+
	0	0	0	0	0	0	9+	32+	13+	19+	28+	26+	9+	4++	0	0	25+
	0	0	0	0	0	0	10+	32+	15+	26+	30+	29+	9+	6++	0	0	0
	0	0	0	17+	0	0	9+	33+	13+	20+	25+	27+	9+	5++	0	0	21+
	0	0	0	12+	35+	18+	0	0	13+	0	9+	7+	14++	7+	11+	12++	8+++
	12+	13+	7+	15+	15+	14+	0	12+	16+	12+	0	7++C	18++C	7+C	0	0	9++
	6++C	0	35+	12+C	20+	21+	8++C	11+C	16+++C	12++	9+++++C	7++C	12++	5+++++C	13+	11+	13+
+C	12+	7+	8+	15+	8+	16+	0	11+	0	19+	9+	7+	12++C	7++C	16+	17+	17++
+++	5++C	7++	6+	12+	21+	12+	8+	9++C	13+	21+	9++	7+	12+	7+++C	2++	6++	10+++
	3+	3+	2++++	3+	3+	3++	3++	3++	3+	3+	4+	4+	3++	3++	3++	7++	4++++
++C	5+++C	3++++C	3++++C	4++++C	5++++C	7++++C	4++++C	4++++C	5+++C	3++++C	5++++C	5++++C	6++C	6++++C	4++++	6++++	6++++

r. hominis).

+ human ascitic fluid. BHI = brain heart infusion. T = thioglycollate. ordet-Gengou agar base. B-A = blood agar base. T-Ag = tryptose agar.

TABLE 10

Summary of the Results of Table 9 to Indicate the Range of the Media Growing the Smallest and Largest Number of Organisms and Their Degree of Growth

Medium	No. strains showing growth	Degree of Growth +		++		+++		++++	
		CAFB	TB	CAFB	TB	CAFB	TB	CAFB	TB
L.....	2	0	1	1	0	0	0	0	0
L-T Ag.....	5	1	0	3	0	0	0	1	0
BHI + AF.....	7	5	1	0	0	1	0	0	0
BHI.....	9	7	1	1	0	0	0	0	0
T.....	9	7	1	1	0	0	0	0	0
E-B.....	10	8	1	1	0	0	0	0	0
B-Ag.....	13	9	1	1	1	0	1	0	0
E-Ag.....	16	12	0	3	1	0	0	0	0
B-G.....	18	6	3	6	0	1	0	2	0
B-A.....	20	13	2	4	1	0	0	0	0
T-Ag.....	24	13	0	6	2	1	1	1	0
D-D.....	25	15	0	6	2	0	0	1	1
C-C.....	25	1	0	2	0	3	0	16	3

CAFB = Chromogenic acid-fast bacilli (22 strains).
TB = tubercle bacilli (3 strains).
L = Loeffler. L-T Ag = liver tryptose agar. BHI + AF = brain heart infusion + human ascitic fluid. BHI = brain heart infusion. T = thioglycollate. E-B = extract broth. B-Ag = blood agar. E-Ag = extract agar. B-G = Bordet-Gengou agar base. B-A = blood agar base. T-Ag = tryptose agar. D-D = Dubos-Davis. C-C = Corper-Cohn
Symbols + to ++++ represent final degree of growth after 6 weeks at 37 C.

TABLE 11

Summary of the Results of Table 9 to Indicate the Range of the Organisms Growing on the Smallest and Largest Number of Media and Their Degree of Growth

	Strain	No. Media Showing Growth	Degree of Growth +	++	+++	++++
CAFB	W-3	3	2	0	1	0
	6288	4	1	2	0	1
	S-7	4	1	2	0	1
	786	5	5	0	0	0
	S-3	5	3	1	0	1
	98717	5	4	1	0	0
	S-8	5	4	0	0	1
	M-2	5	2	1	0	2
	W-1	6	3	2	1	0
	S-1	6	3	2	0	1
	M-3	7	5	0	0	2
	W-2	8	7	0	0	1
	M-1	8	6	1	0	1
	509	8	5	2	0	1
	S-9	8	5	2	0	1
	886	9	6	2	0	1
	W-4	9	7	0	2	0
	S-10	10	8	1	0	1
	S-5	10	7	1	0	2
	S-6	11	7	3	0	1
	W-7	11	5	6	0	0
	M-6	13	2	6	2	3
TB	BCG	6	3	2	0	1
	H37Ra	6	2	3	0	1
	H37Rv	11	5	2	2	2

CAFB = chromogenic acid-fast bacilli (22 strains).
TB = tubercle bacilli (3 strains).
Symbols + to ++++ represent final degree of growth after 6 weeks at 37 C.

contrast to W-3, which grew on only 3. A reverse effect can be seen with the brain heart infusion broth ascitic fluid, brain heart infusion broth, thioglycollate and extract broth media.

On the whole, the final degree of growth was more luxuriant on the Corper-Cohn than on any of the other media. Of special interest is the fact that the more nutritious media, such as the Loeffler, liver tryptose agar and brain heart infusion broth ascitic fluid were inferior to extract agar.

The most rapidly growing strain was M-6 which could be first detected in 3 to 7 days. The time required for the initiation of growth among the other strains varied from 2 to 39 days.

Pigment production was constant on Corper-Cohn, but variable on the other media. It is interesting to note that although certain strains grew very luxuriantly on some of the media, pigmentation was absent. These findings indicate that pigment formation, in part, is probably dependent upon the nutritional environment.

Of further interest is the fact that the tubercle bacilli are not as restricted in their growth requirements as is generally believed; for example, strain BCG grew on tryptose agar in as short a period as 2 days and H37Rv developed on blood agar in 8 days.

The overall pattern in Table 9 is not necessarily a valid measure of the growth-supporting qualities of the media for either the chromogenic organisms or tubercle bacilli. It is necessary to consider that in the above, and all subsequent nutritional studies, a large inoculum was used (150,000,000 organisms). Subsequent experience has shown that the number of organisms is a limiting factor for growth, and the inoculum used in these experiments was much larger than those employed in critical

evaluation studies of culture media (see Part II).

The data in Table 9 have been rearranged in summary form in Table 10 to indicate the range of the media growing the smallest and largest number of organisms and their degree of growth. It will be noted that all the organisms grew on the Dubos-Davis and Corper-Cohn media, but most luxuriantly on the latter. Tryptose agar, blood agar base, Bordet-Gengou agar base, extract agar and blood agar were outstanding among the remaining media, but did not compare with Corper-Cohn in degree of growth. Loeffler medium, surprisingly, was the poorest of all those tested, and grew only 1 chromogenic organism and 1 tubercle bacillus.

The ability of the various strains to adapt themselves to the different media is summarized in Table 11. Strain M-6 was outstanding, since it grew on all 13 of the media. An unexpected result was that the H37Rv strain grew on 11 media. BCG and H37Ra were more selective in their growth requirements. The remaining chromogenic bacilli varied considerably in this respect. From a review of the data, it can be stated that, in general, the chromogenic group of organisms culturally resemble the control tubercle bacilli.

B. Growth of Chromogenic and Saprophytic Acid-fast Organisms and the Human Virulent Tubercle Bacillus on Different Media. To investigate further the cultural similarity of the chromogenic strains to tubercle bacilli, a comparison of a selected number of these organisms was again made with 5 saprophytic strains[1] on 9 of the media used in the previous experiment. From the data presented in Table 12 it can be seen that the saprophytes grew characteristically on all the media within 24 to 48

[1]On Corper-Cohn medium, the M. phlei strains form rough, orange colonies, while the M. smegmatis and M. butyricum strains form rough, white to slightly yellowish-white colonies.

TABLE 12

Growth of 10 Acid-fast Organisms on 9 Different Media to Indicate the Day Growth was First Observed, the Degree of Growth, Pigment and Pellicle Formation

Medium	CAFB S-7	CAFB S-9	CAFB S-5	CAFB M-6	SAFB M.phlei-a	SAFB M.phlei-b	SAFB M.smeg-a	SAFB M.smeg-b	SAFB M.butyr.	TB H37Rv
T.....	0	0	0	8++	2++++P	1++++P	1++++P	1++++P	1++++P	0
L.....	0	0	0	7++C	1++++	1++++	1++++	1++++	1++++	18+
E-Ag.....	38+	0	0	8++C	1++++	1++++	1++++	1++++	1++++	11+
B-A.....	0	0	10+	8++C	1++++	1++++	1++++	1++++	1++++	19++
BHI.....	0	15+	15+P	3+++	1++++P	1++++P	1++++P	1++++P	1++++P	34+
E-B.....	0	9+	9+	3+++	1++++P	1++++P	1++++P	1++++P	1++++P	15+
T-Ag.....	0	15+C	4+	5+++C	1++++	1++++	1++++	1++++	1++++	10++
D-D.....	4+	3++	3+	2+++	1+++	1+++	1+++	1+++	1+++	4+++
C-C.....	6++++C	4+++C	3+++C	3+++C	1++++C	1++++C	1++++C	1++++	1++++	6++++

CAFB = chromogenic acid-fast bacilli.
SAFB = saprophytic acid-fast bacilli (M. phlei, a-b; M. smegmatis, a-b; M. butyricum).
TB = tubercle bacillus (M. tuberculosis var. hominis).
C = media showing chromogenic colonies.
P = media showing pellicle formation, body of medium clear with varying degrees of growth on bottom of tube.
T = thioglycollate. L = Loeffler. E-Ag = extract agar. B-A = blood agar base. BHI = brain heart infusion. E-B = extract broth. T-Ag = tryptose agar. D-D = Dubos-Davis. C-C = Corper-Cohn.
Numbers represent the day growth was first observed.
Symbols + to ++++ represent final degree of growth after 6 weeks at 37 C.

hours. Their growth was much more rapid and luxuriant than any of the other organisms, and an additional feature was consistent pellicle formation in all the liquid media except Dubos-Davis. A slight pellicle was formed by only one chromogenic strain (S-5). The results of pigment production were the same as in the previous experiment with the exception of strain S-9, which this time formed pigment on tryptose agar. The results in Table 12 again emphasize the fact that the chromogens are distinctive in their manner, rate and degree of growth from the saprophytes, and in this respect they resemble the tubercle bacilli more than the former group.

C. Effects of Washing on the Growth of Chromogenic and Saprophytic Acid-fast Organisms and the Human Virulent Tubercle Bacillus on Different Media. The fact that so many of the chromogenic organisms and tubercle bacilli could develop on such a variety of media was an unexpected result. Theoretically the data could be explained on the basis that the original inoculum (0.1 ml.) carried over a sufficient amount of Dubos-Davis medium to initiate growth. Therefore, it seemed desirable to rule out this factor. For this purpose the last experiment was repeated in duplicate, omitting 3 of the saprophytic strains. In the first part, the procedure was identical to the one used previously. In the second part, however, the organisms from the Dubos-Davis medium were washed 3 times and brought back to the standard turbidity. From the data recorded in Table 13, it can be seen that washing the organisms did not significantly alter the results. In fact the Dubos-Davis medium may even have exerted an inhibitory effect since strains S-7 and S-9, after being washed, grew on several of the media which previously proved inadequate. This observation was not repeated, since variability may have also been a factor.

TABLE 13

Effects of Washing on the Growth of 7 Acid-fast Organisms on 9 Different Media to Indicate the Day Growth was First Observed, the Degree of Growth, Pigment and Pellicle Formation

Medium		CAFB				SAFB		TB
		S-7	S-9	S-5	M-6	M.phlei-b	M.smeg-a	H37Rv
T.....	UW	0	0	0	8++	2++++P	1++++P	0
	W	0	0	0	8+++	2++++P	1++++P	0
L.....	UW	0	0	0	7++C	1++++	1++++	18+
	W	0	0	0	8+C	1++++	1++++	18+
E-AG..	UW	41+	0	0	8++C	1++++	1++++	11+
	W	38+	0	0	7++C	1++++	1++++	12+
B-A...	UW	0	0	10+	8++C	1++++	1++++	19++
	W	0	21++C	4++	8+++C	1++++	1++++	19++
BHI...	UW	0	15+	15+	3+++	1++++P	1++++P	34+
	W	32+	15+	15++P	3+++	1++++P	1++++P	34+
E-B...	UW	0	9+	9+	3+++	1++++P	7++++P	15+
	W	19+	9+	9+	3+++	2++++P	1++++P	15+
T-Ag..	UW	0	15+C	4+	5+++C	1++++	1++++	10++
	W	18+C	21+C	4+++	5+++C	1++++	1++++	10++
D-D...	UW	4+	3++	3+	2+++	1+++	1+++	4+++
	W	4+++	3++	3+++	2+++	1+++	1+++	4+++
C-C...	UW	6++++C	4+++C	3+++C	3+++C	1++++C	1++++C	6++++
	W	6++++C	4++++C	3++++C	3++++C	1++++C	1++++C	6++++

CAFB = chromogenic acid-fast bacilli.
SAFB = saprophytic acid-fast bacilli.
TB = tubercle bacillus (M. tuberculosis var. hominis).
UW = unwashed organisms.
W = washed organisms.
C = media showing chromogenic colonies.
P = media showing pellicle formation; body of medium clear with varying degrees of growth on bottom of tube.
T = thioglycollate. L = Loeffler. E-Ag = extract agar. B-A = blood agar base. BHI = brain heart infusion. E-B = extract broth. T-Ag = tryptose agar. D-D = Dubos-Davis. C-C = Corper-Cohn.
Numbers represent the day growth was first observed.
Symbols + to ++++ represent final degree of growth after 6 weeks at 37 C.

Streptomycin Sensitivity Studies.

A. Streptomycin Sensitivity of Chromogenic Acid-fast Organisms and Tubercle Bacilli in Youmans Liquid Medium. Since some of the atypical chromogenic acid-fast bacilli have been considered potentially pathogenic for man and certain animals, and because of their possible genetic relationship to true tubercle bacilli, it appeared advisable to study their sensitivity to streptomycin. The BCG, H37Ra and H37Rv strains were included for comparison. All cultures at the completion of these experiments were checked for purity. The results presented in Table 14 show that the tubercle bacilli and all but 5 of the chromogenic strains were inhibited by less than 1 mcg. of streptomycin. Two strains were sensitive to less than 10 mcgs. of the antibiotic, and 3 strains were resistant to all the concentrations used. It is of interest to point out that W-7 was isolated from a patient who died of pulmonary tuberculosis and prior to death had received a four months' course of streptomycin. It is entirely possible that this organism acquired resistance as a result of previous therapy. It was not known if strains 6288 and M-6 had been subjected to the action of the antibiotic.

B. Effects of Prolonged Incubation on Streptomycin Sensitivity of Chromogenic Acid-fast Organisms and the Human Virulent Tubercle Bacillus in Youmans Liquid Medium. To determine the effect of prolonged incubation on streptomycin sensitivity, 6 of the cultures from the previous experiment were allowed to incubate for a total of 12 weeks. These data are presented in Table 15. Strains S-1, S-2, S-9 and H37Rv showed some degree of growth on prolonged incubation which was not manifested at the end of the second week, but M-2 did not show any evidence of growth after the twelfth week. It could not be determined from this experiment

TABLE 14

Streptomycin Sensitivity of 27 Acid-fast Organisms in Youmans Liquid Medium

Strain	Concentration of Streptomycin - Mcgs./ml. 1	10	20	Control
S-1	0	0	0	+++
S-2	++	0	0	+++
S-3	0	0	0	+++
S-4	0	0	0	+
S-5	0	0	0	++
S-6	0	0	0	++
S-7	0	0	0	+
S-8	0	0	0	++
S-9	+	0	0	+++
S-10	0	0	0	++
M-1	0	0	0	++
M-2	0	0	0	+++
M-3	0	0	0	+
M-6	++	+	+	+++
W-1	0	0	0	+
W-2	0	0	0	+
W-3	0	0	0	+
W-4	0	0	0	+
W-7	++	+	+	+++
509	0	0	0	++
786	0	0	0	+
886	0	0	0	++
6288	++	+	+	++++
98717	0	0	0	0
BCG	0	0	0	+++
H37Ra	0	0	0	++
H37Rv	0	0	0	+++

Symbols + to ++++ represent final degree of growth after 2 weeks at 37 C.

TABLE 15

The Effect of Prolonged Incubation on Streptomycin Sensitivity of 6 Acid-fast Organisms in Youmans Liquid Medium

Strain	Mcgs. S/ml.	Period of Time in Weeks								
		2	3	4	5	6	7	8	9	12
S-1	1.....	0	0	0	0	0	0	0	0	++
	10.....	0	0	0	0	0	0	0	0	0
	20.....	0	0	0	0	0	0	0	0	0
	C.....	+++	+++	+++	+++	+++	+++	+++	+++	++++
S-2	1.....	++	++	++	+++	+++	+++	+++	+++	+++
	10.....	0	0	0	0	0	0	+	+	+++
	20.....	0	0	0	0	0	0	0	0	++
	C.....	+++	+++	+++	+++	+++	+++	+++	+++	+++
S-9	1.....	+	+	++	+++	+++	+++	+++	+++	+++
	10.....	0	0	0	+	+	++	++	++	++
	20.....	0	0	0	0	0	0	0	0	0
	C.....	+++	+++	+++	+++	+++	+++	+++	+++	+++
M-2	1.....	0	0	0	0	0	0	0	0	0
	10.....	0	0	0	0	0	0	0	0	0
	20.....	0	0	0	0	0	0	0	0	0
	C.....	+++	+++	+++	+++	+++	+++	+++	+++	++++
M-6	1.....	++	++	++	++	++	++	++	++	+++
	10.....	+	+	+	+	+	+	+	+	++
	20.....	+	+	+	+	+	+	+	+	+
	C.....	+++	+++	+++	+++	+++	+++	+++	+++	+++
H37Rv	1.....	0	0	0	0	0	0	0	0	+
	10.....	0	0	0	0	0	0	0	0	0
	20.....	0	0	0	0	0	0	0	0	0
	C.....	+++	+++	+++	+++	+++	+++	+++	+++	++++

C = control.
Symbols + to ++++ represent degree of growth after each week at 37 C.

whether the streptomycin was bactericidal or merely bacteriostatic, although the appearance of growth after prolonged incubation was evidence of the latter effect.

C. Determination of Viability of Chromogenic Acid-fast Organisms and the Human Virulent Tubercle Bacillus Following Exposure to Streptomycin. To determine whether streptomycin was bacteriostatic or bactericidal, and to ascertain the extent of injury to several of the organisms after prolonged exposure (12 weeks) to the antibiotic, the following experiment was done. All the tubes containing strains M-2, M-6 and H37Rv of the previous experiment were centrifuged and the sediments washed 3 times with 0.85 per cent sodium chloride to remove the antibiotic, after which they were subcultured to tubes of Corper-Cohn and Dubos-Davis media. The data are shown in Table 16. The M-2 strain, found sensitive to less than 1 mcg. of streptomycin in the previous experiment, grew luxuriantly in both media. Development was delayed for two weeks compared with the control, but subsequent growth was rapid and heavy. The M-6 strain also showed no evidence of serious injury from the antibiotic, and, after some preliminary retardation, maximum growth was reached in both test media. The results with the H37Rv organism were of special interest, since the 20 mcg. concentration of streptomycin apparently exerted a bactericidal effect, whereas the 10 and 1 mcg. concentrations showed only a bacteriostatic influence, as evidenced by growth after washing. For some reason, yet unknown, this strain recovered more rapidly in the Dubos-Davis than in the Corper-Cohn medium. No attempt was made to induce resistance, nor is it certain whether the organism which grew on subculture after washing was identical with the parent strain or a resistant variant thereof. Additional studies will be

TABLE 16

Determination of Viability of 3 Acid-fast Organisms Following Exposure to 3 Concentrations of Streptomycin for 12 Weeks

Strain	Mcgs. S/ml.	Corper-Cohn Medium Period of Time in Weeks									Dubos-Davis Medium Period of Time in Weeks								
		1	2	3	4	5	6	7	8	9	1	2	3	4	5	6	7	8	9
H-2	1	0	0	+	++	+++	+++	+++	++++	++++	0	0	+	++	+++	+++	+++	++++	++++
	10	0	0	+	+++	+++	+++	+++	+++	+++	0	0	+	++	+++	+++	+++	+++	++++
	20	0	0	+	+	++	+++	+++	+++	+++	0	0	+	+	++	++	+++	+++	+++
	C	++	+++	+++	++++	++++	++++	++++	++++	++++	++	++	++	+++	++++	++++	++++	++++	++++
H-6	1	+	++	++	+++	+++	+++	+++	++++	++++	+	++	++	+++	+++	+++	+++	++++	++++
	10	+	++	++	+++	+++	+++	+++	+++	++++	+	++	++	+++	+++	+++	+++	++++	++++
	20	+	++	++	+++	+++	+++	+++	+++	++++	+	++	++	+++	+++	+++	+++	++++	++++
	C	+	+++	+++	++++	++++	++++	++++	++++	++++	++	+++	+++	++++	++++	++++	++++	++++	++++
H37Rv	1	0	0	0	0	0	0	0	0	+	0	0	++	+++	+++	++++	++++	++++	++++
	10	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	+	++	+++
	20	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	C	++	+++	++++	++++	++++	++++	++++	++++	++++	++	+++	++++	++++	++++	++++	++++	++++	++++

C = control-medium without Streptomycin.
Symbols + to ++++ represent degree of growth after each week at 37 C.

required to establish this fact. In any case, it is clear that prolonged contact with streptomycin in concentrations which are thought to be bactericidal may actually be only bacteriostatic.

Animal Studies.

A. Preliminary Experiments. The preliminary animal experiments were designed to screen the pathogenic potentialities of the various chromogenic acid-fast bacilli in guinea pigs and to determine whether any strains were capable of sensitizing the animals to mammalian tuberculin. Twenty-six of these strains were selected for study and, for controls, 3 saprophytic strains (M. smegmatis-b, M. phlei-a and M. butyricum) and one human virulent strain (H37Rv) were also included. It was arbitrarily decided to use an intraperitoneal inoculation of 25 milligrams and to sacrifice surviving animals after 11 weeks, at which time all the animals were tuberculin tested with second strength PPD. Rectal temperatures were also taken on all animals which showed weight loss. A summary of the pathologic groups and the record of weights and tuberculin reactions are presented in Table 17. The results of direct smears of the lungs, liver, spleen, kidneys and lesions, and cultures of all these tissues, are summarized in Table 18. The record of rectal temperatures is recorded in Table 19.

1. Pathogenicity. In regard to pathogenicity the strains could be grouped as follows:

Group I. Strains which produce death with macroscopic lesions.

Group II. Strains which do not produce death, but produce macroscopic lesions.

Group III. Strains which do not produce death or macroscopic lesions.

Seven animals infected with the chromogenic organisms expired in 14 to 31

TABLE 17

Summary of Pathologic Group, Record of Weights and PPD Tuberculin Reactions in Guinea Pigs Infected with 25 Milligram Intraperitoneal Doses of 30 Acid-fast Organisms

	Strain	DE	WPI	Weights in Grams - Weeks After Infection											P
				1	2	3	4	5	6	7	8	9	10	11	
Group I	S-3.....	14	279	298	260										
	M-6.....	15	283	229	232	207									
	S-9.....	23	256	247	281	250	223								
	W-4.....	23	259	292	285	248	221								
	886.....	23	315	300	332	351	321								
	S-2.....	26	298	302	314	316	285								
	S-1.....	31	282	272	295	270	263	270							
Group II	6288.....		312	260	238	245	253	251	237	316	315	324	329	316	E[1]
	S-7.....		333	303	293	324	372	408	470	489	482	528	550	517	+
	M-1.....		349	280	248	243	248	280	303	370	374	415	441	446	++
	S-8.....		280	258	274	268	275	301	314	356	378	401	431	451	++
	M-2.....		289	343	366	360	423	464	549	595	609	665	719	736	-
	M-3.....		342	353	380	359	366	392	494	563	566	600	646	652	++
	509.....		276	252	260	253	256	257	253	321	338	367	412	407	++
	W-7.....		272	226	206	244	233	272	297	349	361	360	380	395	++
	S-4V.....		231	265	285	320	360	407	438	450	490	500	520	537	++
	Parks.....		309	270	279	315	367	413	432	515	514	529	521	535	++
	S-4.....		314	329	318	350	355	404	486	557	541	552	608	629	+
	S-6.....		259	295	332	334	349	372	424	477	476	522	571	598	-

TABLE 17 Continued

	Strain	DE	WPI	Weights in Grams - Weeks After Infection 1	2	3	4	5	6	7	8	9	10	11	P
Group III	S-5.....		276	263	270	291	288	321	401	411	395	436	459	467	-
	S-10.....		334	372	397	416	435	472	518	586	579	605	613	622	+
	W-1.....		296	258	267	283	293	331	380	431	477	505	578	588	+
	W-2.....		275	322	365	393	425	453	552	574	575	626	670	725	-
	W-3.....		287	310	329	317	290	322	387	395	402	449	497	521	+
	786.....		324	349	324	403	419	484	537	601	610	580	643	670	+
	98717.....		334	338	388	384	410	475	496	519	554	566	666	702	-
Controls	M. smeg.-b		233	219	233	260	277	322	327	373	351	301	295	315	-
	M. phlei-a		341	289	325	362	403	434	436	511	542	590	630	608	-
	M. butyr..		367	450	477	531	572	595	570	651	700	695	736	800	-
	H37Rv.....	19	272	267	239	220									

Group I = strains which produce death with macroscopic lesions. Group II = strains which do not produce death but produce macroscopic lesions. Group III = strains which do not produce death or macroscopic lesions.

Controls = M. smeg.-b = M. smegmatis-b. M. butyr. = M. butyricum.

DE = number of days after infection animals expired. WPI = weight prior to infection. ___ = loss in weight. P = PPD tuberculin reaction at completion of experiment. E^1 = expired several hours after test.

Symbols + to ++ represent the degree of tuberculin reaction.

TABLE 18

Summary of Direct Smears of Organs and Lesions, and Cultures from Mixed Specimens of These Tissues from Guinea Pigs Infected with 25 Milligram Intraperitoneal Doses of 30 Acid-fast Organisms

	Strain	DE	Lungs	Liver	Spleen	Kidneys	Lesions	Culture
Group I	S-3.....	14	0	+	0	0	+	14
	M-6.....	15	+	++	++	+	+	15
	S-9.....	23	0	0	0	0	0	0
	W-4.....	23	0	+	+	0	0	0
	686.....	23	+	+	+	0	++	19
	S-2.....	26	0	+	+	0	++	18
	S-1.....	31	+	+	+	0	+	0
Group II	6288.....		++	++++	++	0	+++	0
	S-7.....		0	0	+	0	++	31
	M-1.....		0	+	+	0	+	21
	S-8.....		0	0	0	0	++	0
	M-2.....		0	0	0	0	0	0
	M-3.....		0	0	0	0	+	20
	509.....		0	0	0	0	0	0
	W-7.....		0	0	0	0	0	0
	S-4V.....		0	0	0	0	0	0
	Parks.....		0	0	0	0	+	0
	S-4.....		0	0	0	0	0	0
	S-6.....		0	0	0	0	0	0

TABLE 18 Continued

	Strain	DE	Lungs	Liver	Spleen	Kidneys	Lesions	Culture
Group III	S-5.....		0	0	0	0	NF	0
	S-10.....		0	0	0	0	NF	0
	W-1.....		0	0	0	0	NF	0
	W-2.....		0	0	0	0	NF	0
	W-3.....		0	0	0	0	NF	0
	786.....		0	0	0	0	NF	0
	98717.....		0	0	0	0	NF	0
Controls	M. smeg.-b...		0	0	0	0	NF	0
	M. phlei-a...		0	0	0	0	NF	0
	M. butyr.....		0	0	0	0	NF	0
	H37Rv.....	19	++	++	++	+	++	16

Group I = strains which produce death with macroscopic lesions. Group II = strains which do not produce death, but produce macroscopic lesions. Group III = strains which do not produce death or macroscopic lesions.

Controls = M. smeg.-b = M. smegmatis-b. M. butyr. = M. butryicum.

DE = number of days after infection animals expired. NF = no lesions found.

Numbers in culture column represent the day growth was first observed.

Symbols + to ++++ represent the degree of acid-fast bacilli found.

TABLE 19

Summary of Rectal Temperatures in Guinea Pigs Infected with 25 Milligram Intraperitoneal Doses of 30 Acid-fast Organisms

	Strain	DE	TI	Temperature in Degrees F. - Weeks After Infection 1	2	3	4	5	6	7	8	9	10	11
Group I	S-3.....	14	102	-	93	-	-	-	-	-	-	-	-	-
	M-6.....	15	103	104	-	-	-	-	-	-	-	-	-	-
	S-9.....	23	103	104	-	102	-	-	-	-	-	-	-	-
	W-4.....	23	104	-	103	100	-	-	-	-	-	-	-	-
	886.....	23	103	104	-	-	-	-	-	-	-	-	-	-
	S-2.....	26	103	-	-	-	-	-	-	-	-	-	-	-
	S-1.....	31	104	105	-	101	101	-	-	-	-	-	-	-
Group II	6288.....	-	103	105	103	-	-	102	99	-	100	-	-	101
	S-7.....	-	103	104	104	-	-	-	-	-	102	-	-	102
	M-1.....	-	103	105	106	103	-	-	-	-	-	-	-	-
	S-8.....	-	104	104	-	103	-	-	-	-	-	-	-	-
	M-2.....	-	102	-	-	102	-	-	-	-	-	-	-	-
	M-3.....	-	103	-	-	101	-	-	-	-	-	-	-	-
	509.....	-	103	105	-	104	-	-	101	-	-	-	-	-
	W-7.....	-	103	105	103	-	102	-	-	-	-	101	-	-
	S-4V.....	-	103	-	-	-	-	-	-	-	-	-	-	-
	Parks.....	-	104	104	-	-	-	-	-	-	102	-	102	-
	S-4.....	-	102	-	103	-	-	-	-	-	102	-	-	-
	S-6.....	-	103	-	-	-	-	-	-	-	102	-	-	-

TABLE 19 Continued

	Strain	DE	TI	Temperature in Degrees F. - Weeks After Infection 1	2	3	4	5	6	7	8	9	10	11
Group III	S-5.....	-	103	104	-	-	102	-	-	-	102	-	-	-
	S-10.....	-	103	-	-	-	-	-	-	-	102	-	-	-
	W-1.....	-	102	105	-	-	-	-	-	-	-	-	-	-
	W-2.....	-	102	-	-	-	-	-	-	-	-	-	-	-
	W-3.....	-	102	-	-	103	103	-	-	-	-	-	-	-
	786.....	-	103	-	102	-	-	-	-	-	-	101	-	-
	98717.....	-	103	-	-	102	-	-	-	-	-	-	-	-
Controls	M.smeg.-b.	-	104	104	-	-	-	-	-	-	102	102	101	-
	M.phlei-a.	-	103	104	-	-	-	-	-	-	-	-	-	-
	M.butyr...	-	102	-	-	-	-	-	101	-	-	101	-	-
	H37Rv.....	19	103	106	104	-	-	-	-	-	-	-	-	-

Group I = strains which produce death with macroscopic lesions. Group II = strains which do not produce death, but produce macroscopic lesions. Group III = strains which do not produce death or macroscopic lesions.

Controls = M. smeg.-b = M. smegmatis-b. M. butyr. = M. butyricum.

DE = number of days after infection animals expired. TI = rectal temperatures prior to infection.

Temperatures were only taken on animals showing weight loss. The normal rectal temperature in guinea pigs, based upon readings of 30 male and 10 female animals, averaged 103 F.

days. The animal inoculated with the H37Rv control strain expired in 19 days. Details of the pathologic findings are discussed in the next section.

a. Correlation Between Extent of Lesions, Expiration and Lesion Production Time. The strains in Group I were arranged in order of expiration time, from those producing death in the shortest to the longest period. This was done to determine whether there was any correlation between the extent of the macroscopic lesions and the expiration time, or between the extent of the lesions and the time required for their production. No such correlation was found. Some animals with few lesions died as early or later than those with many lesions.

b. Correlation Between Extent of Lesions and PPD Tuberculin Sensitivity. The strains in Group II were arranged in order of macroscopic lesion production, from those producing the largest to the least amount. This was done to see if there was any correlation between the extent of the lesions and the degree of PPD tuberculin sensitivity. From the results in Table 17 it can be seen that little if any correlation was evident, for the animals with the largest number of lesions did not react more strongly to tuberculin than some of the animals with fewer lesions. However, when the results of the Group II animals were compared with those of Group III there appeared to be some correlation, since the degree of sensitivity in the latter group was less than in the former. It would seem that as long as some lesions are present, the degree of sensitivity is not necessarily proportional to the number, but in animals with minimal lesions (detected only microscopically), the degree of allergy is less. In the subsequent experiments on skin allergy it will be seen how often variability is manifested, and because of this it is difficult

to evaluate allergic reactions. The animals infected with the saprophytic strains were tuberculin negative. These findings suggest an immunologic similarity between some of the chromogenic organisms and the mammalian tubercle bacilli, each possibly possessing related antigens, either group or specific. Under the conditions of these experiments the saprophytic strains differed in that they did not manifest this relationship.

c. Results of Direct Smears and Cultures of Tissues. It will be noted in Table 18 that organisms could be frequently demonstrated in the tissues on both smear and culture, particularly in specific lesions and also in the spleen and liver. Of special interest is the fact that these were found most often in the Group I aminals, to a less extent in Group II and not at all in Group III. Organisms were also recovered from the tissues on both smear and culture in the control animal infected with the H37Rv strain.

2. Weight Changes. It will be noted that loss in weight in over 50 per cent of the animals occurred early, during the first week after infection. All animals with the exception of one, in Group I, lost weight prior to death. In the other groups weight loss, in general, occurred early, but in a number of instances the weight loss was irregular or was observed near the end of the experiment. Weight change did not appear to be an entirely reliable measure of an active pathologic process.

3. Rectal Temperature Changes. Rectal temperatures[1] of most of the animals were highest in the first week of infection, after which time they were, in general, somewhat lower than normal. The lowest temperature

[1]The normal rectal temperature in guinea pigs, based upon readings of 30 male and 10 female animals, averaged 103 F.

found was 93 F. in the guinea pig infected with strain S-3, and the highest was 106 F. in the animals infected with strains M-1 and H37Rv. Temperature change also did not prove to be an index of the severity of infection in guinea pigs.

4. Pathologicoanatomical Characteristics of Group Strains.

a. Findings in Animals Infected with Group I Strains. After the intraperitoneal inoculation of a 25 mg. dose of the organisms in this group, the following changes have been observed:

At the site of inoculation, a small, nodular, indurated subcutaneous swelling formed, which became palpable within 1 week. This lesion increased in size, and underwent liquefaction and abscess formation. The process reached its height in approximately 2 to 3 weeks, after which time the lesion slowly regressed and began to heal (Fig. 1). The regional lymph nodes were often palpable. Some were greatly enlarged and underwent suppuration. Although this response has been observed in intraperitoneal inoculations, it was chiefly characteristic of those made subcutaneously.

The abdomens of the animals were occasionally distended and contained bloody serosanguinous ascitic fluid. The pleural cavity was similarly involved. There was evidence of peritonitis, and the viscera were invariably firmly adherent to one another and separated with difficulty. A seropurulent exudate was often present especially between the loops of the bowel.

Throughout the peritoneal cavity were varying numbers of yellowish-white, firmly encapsulated nodules measuring from 0.5 mm. to 3 cm. in diameter. These were smooth or irregular and varied in texture from very soft to very hard. On cut section the softer nodules were composed

Figure 1

Local abscess at the site of injection in a guinea pig 2 weeks after subcutaneous infection with a 1 mg. dose of a chromogenic acid-fast organism (strain M-6). Several of the regional lymph nodes were palpable. The lesion had already opened and was partly drained. Healing is occurring with scar formation.

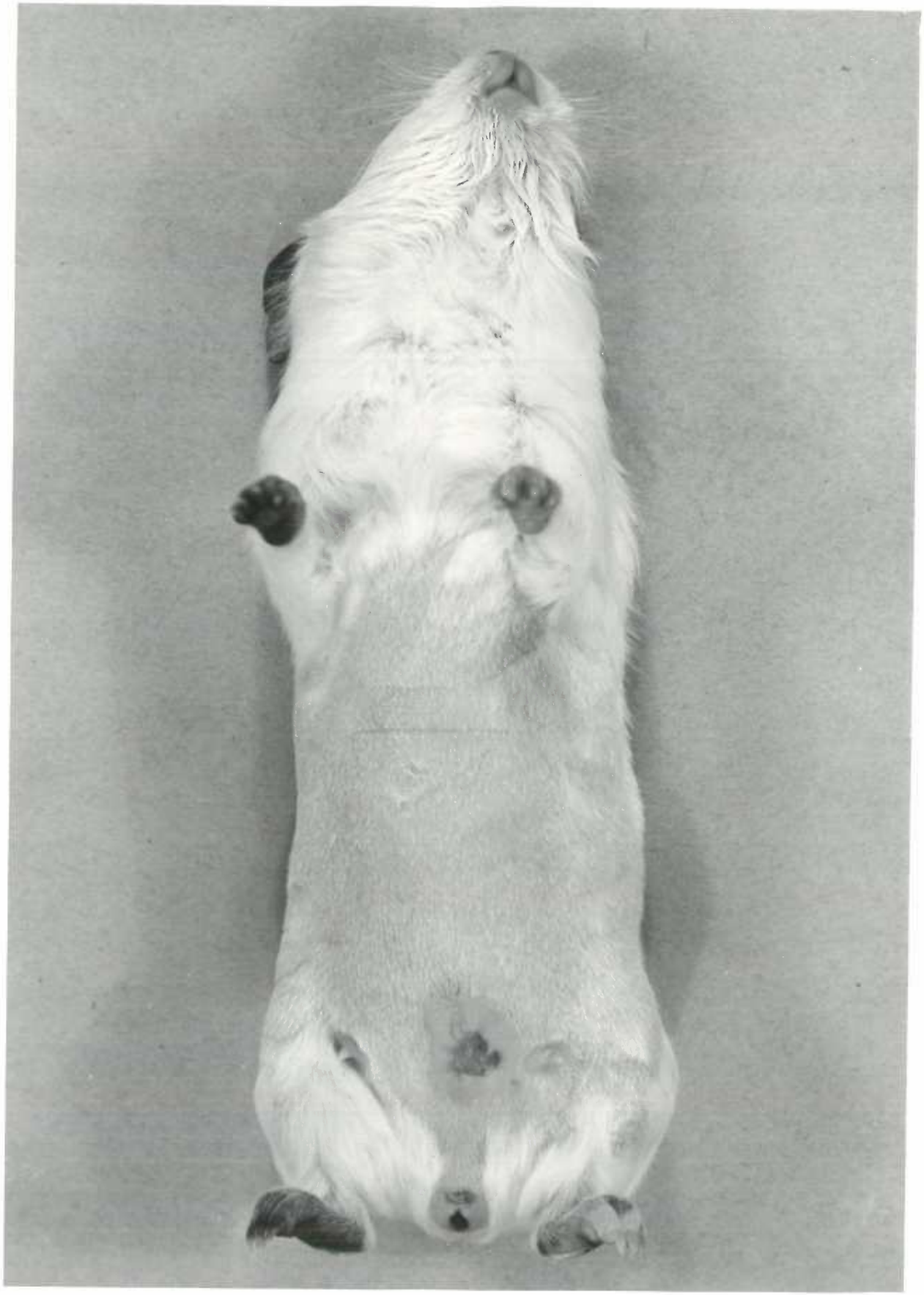


Fig. 1

of thin fibrous capsules and contained fluid pus. Other nodules revealed a thicker somewhat caseous exudate which was surrounded by a dense fibrous capsule. Still others were composed almost entirely of a spongy, fibrous tissue, and little or no exudate was seen. The nodules were either attached firmly to the peritoneal wall, or else occurred on, between, or in the various organs (Figs. 2 and 3).

The omentum was always rolled up into a sausage-like mass as a result of the inflammatory process. At times sections of the bowel showed unusual contraction, and portions were firmly adherent to each other and to the other organs by the adhesions and nodules previously described. Occasionally small hemorrhagic areas were seen.

Some of the livers presented small or large areas of light brown to yellowish-brown discoloration, but were otherwise normal in appearance. The changes were mainly limited to the capsular surface where one encountered varying numbers of nodules and adhesions to the adjacent viscera and peritoneum. Occasionally, within the parenchyma, there were seen a few small scattered yellowish-white nodules, approximately 1.0 to 5.0 mm. in diameter and similar to those already described.

The spleen was either normal in size or showed varying degrees of enlargement. It was invariably adherent to the stomach, kidney and peritoneum, often so much so that it was only removed with great difficulty. As in the case of the liver, the changes were mainly limited to the capsule where one found nodules and adhesions. Occasionally from one to several marked hemorrhagic areas were seen, and when this was found the organ was soft and friable. Rarely, as in the liver, one encountered from one to several small yellowish-white parenchymal nodules.

The lungs were almost constantly intensely hemorrhagic and markedly

Figure 2

Multiple abscess formation and other visceral involvement in a guinea pig 11 weeks after intraperitoneal infection with a 25 mg. dose of a chromogenic acid-fast organism (strain 6288). Note the extensive adherence of the viscera by fibrous adhesions.

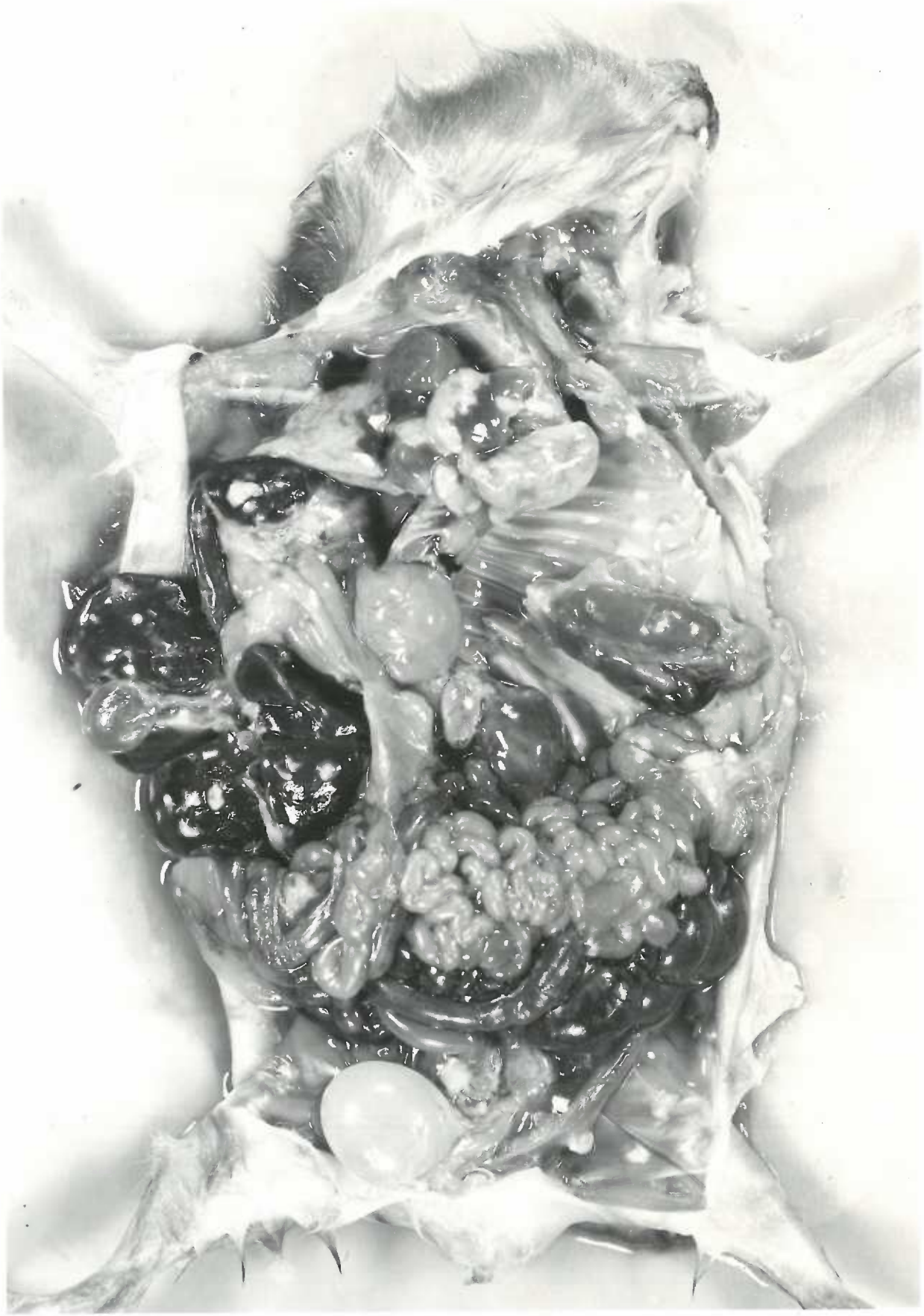


Fig. 2

Figure 3

Tissues removed from animal shown in Figure 2 with multiple abscess formation and other visceral involvement in a guinea pig 11 weeks after intraperitoneal infection with a 25 mg. dose of a chromogenic acid-fast organism (strain 6288).

a. Liver with large multiple abscesses, which are chiefly extraparenchymal. Some abscesses can be seen beneath the capsule and a few within the liver.

b. Section of entire abdominal wall showing multiple abscesses some of which are under the peritoneum and deeply imbedded in the muscle tissue. One portion is adherent to the liver as a result of the peritonitis.

c. Lobes of lung separated to show the numerous grayish-white plaques which suggest areas of scar tissue.

d. Large extraparenchymal abscess on under surface of right kidney.

e. Spleen and left kidney firmly adherent by thick fibrous adhesions. Note the enlargement of the spleen and the extent of the lesions.

Fig. 3

edematous, but this was probably chiefly artifact, attributable to the ether inhalation used in sacrificing the animals. Quite often one found varying numbers of small grayish-white plaques near the peripheral boundaries. Rarely also, as in the liver and spleen, one or more small yellowish-white parenchymal nodules were noted. In animals showing evidence of hemorrhagic effects, the lungs were unusually pale.

Many of the lymph nodes were enlarged and some showed suppuration. The superficial nodes at the site of the inoculation were often affected. The deep inguinals, the lumbar, the mesenteric and portal nodes showed varying degrees of involvement. Enlargement and suppuration of the pulmonary nodes were not observed.

b. Findings in Animals Infected with Group II Strains. The findings in this group were essentially the same as those in Group I with some of the following exceptions. Adhesions were more fibrous in character, and abdominal swelling with bloody ascites was found in only one animal. Liver discoloration and intestinal involvement was less prominent, and exudates were purulent in character. Numerous nodules in all stages were seen but consisted chiefly of the latter two types described in the Group I pigs. The most severe reactions were produced by strain 6288, outstanding of which was the massive nodular formation throughout the entire viscera and the peritoneal wall of the infected animal (Fig. 2). Of special interest is the fact that this pig expired several hours after being tuberculin tested. This suggested a possible general systemic reaction similar to the type of death produced in tuberculous guinea pigs with large amounts of tuberculin several weeks following infection. Since this experiment was not repeated, no definite conclusions can be drawn regarding this observation.

As already mentioned, organisms could be recovered from the tissues on both smear and culture, but to a lesser extent than the strains of Group I.

c. Findings in Animals Infected with Group III Strains. No macroscopic lesions were found in this group of animals. Of special interest is the fact that no organisms could be recovered from the tissues either on smear or culture.

5. Histological Characteristics of Group Strains. Histologic sections were done to determine if the lesions in any way resembled true tuberculous reactions. The results in general confirmed the gross findings. In the earlier stages of infection as seen in the Group I animals, the lesions consisted of heterogeneous granulomatous tissue in which epithelioid cells, macrophages and atypical multinucleated giant cells were irregularly distributed together with a few granulocytes and lymphocytes. Abscess formation was not yet evident.

In the more advanced stages, as seen in the Group II pigs, most of the lesions were characterized by areas of central necrosis and abscess, surrounded by polymorphous granulation tissue, often with unusual degrees of hyperemia, a feature which is not distinctive of the true tuberculous granulomas. Most of these abscesses originated on the capsular surface of the organ rather than within the parenchyma. Other lesions showed evidence of the later stages of healing in which the granulation tissue was being transformed into connective tissue, chiefly hyaline and fibrous in character.

The kidneys showed varying degrees of hyperemia, but this may have been an artifact manifestation resulting from ether inhalation used in sacrificing the animals. A few organisms were recovered from this organ

in several instances, but lesions were never found.

A few small chronic-type interstitial granulomata were also found in one section of the macroscopically normal lungs of the chromogenic-infected animals of Group III. Nothing noteworthy was present in the other animals of this group.

In general the lesions may be described as benign suppurative inflammations which develop into non-specific granulomata. They exhibited unusual cellular polymorphism, and the foreign-body type of reaction was often mixed with a subacute process resulting in subserosal abscess formation. A few typical advanced lesions are shown in Figure 4.

6. Summary. The following summary is based on the findings of Dr. Vinton Smeeden of the Department of Pathology.

a. Findings in Animals Infected with Group I Strains.

(1). Fibrinous peritonitis.

(2). Serosanguinous ascites.

(3). Interstitial pneumonitis.

(4). Granulomatous inflammation of liver.

(5). Granulomatous inflammation of spleen.

(6). Subcutaneous abscess at site of injection.

(7). Hyperemia and edema of lungs (artifact?).

(8). Passive hyperemia of viscera (artifact?).

b. Findings in Animals Infected with Group II Strains.

(1). Fibrous peritonitis with focal abscess formation.

(2). Serosanguinous ascites.

(3). Interstitial inflammation of lung.

(4). Interstitial and alveolar granuloma of lung.

(5). Perihepatic abscess of liver.

Figure 4

Characteristic histopathology in guinea pigs 11 weeks after infection with 25 mg. intraperitoneal doses of chromogenic acid-fast bacilli.

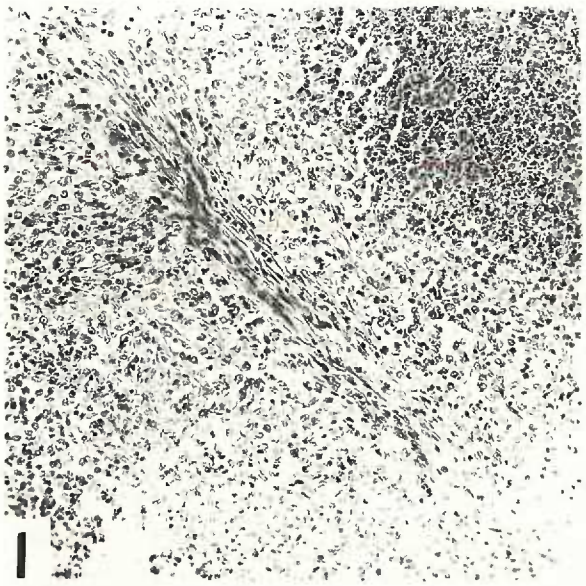
1. Perihepatic abscess of liver. This section shows an area of necrosis and abscess formation, the cellular composition of which is that of an acute inflammatory exudate. Immediately adjacent to the abscess and between it and the capsule is the typical accumulation and zone of large pale epithelioid cells of the phagocytic type. Fat stains of these reveal the presence of finely divided fat. This is in accord with their phagocytic activity and fine vacuolization of the cytoplasm.

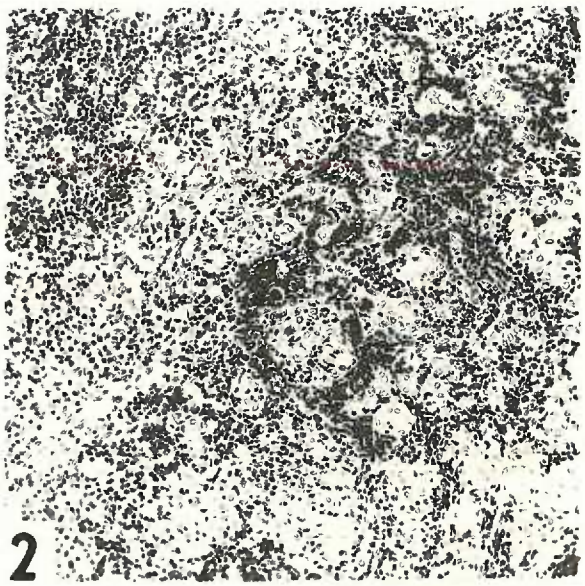
2. Granulomatous inflammation of spleen. This section shows the diffuse epithelioid cell accumulation similar to that about the abscess on the liver. Here the proliferation is in the sinusoids and a variable and usually fairly marked number of multinucleated cells are seen. Morphologically these are composed of undivided or fused cells of the same type as the epithelioid macrophages, but more diffusely and overwhelmingly distributed throughout the pulp.

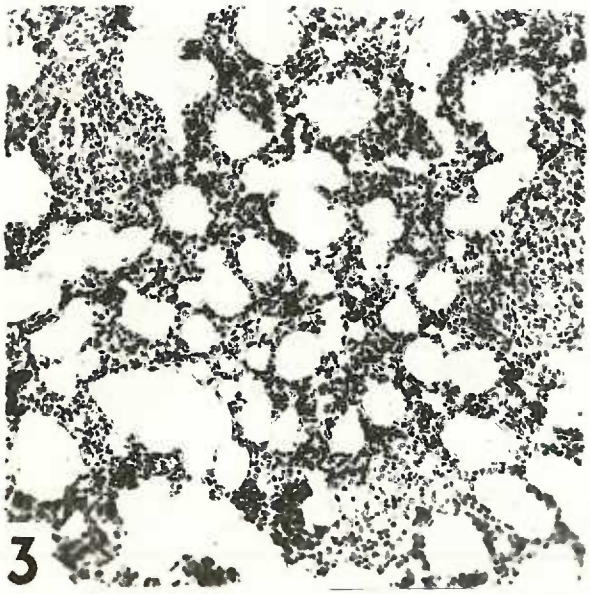
3. Interstitial inflammation of lung. This section shows focal areas of interstitial nodule formation with a whorled-like arrangement of cells that are spindle in shape and appear to be of fibroblastic origin. A few lymphocytes are present, but no epithelioid, phagocytic or multinucleated giant cells.

4. Interstitial and alveolar granuloma of lung. This section shows an area of interstitial fibrosis with some compression and atelectasis which may be artifact. In this area there is also a minimal alveolar infiltration of inflammatory cells, predominately lymphocytes.

5. Lymphocytic infiltration of liver triads. This section shows an accumulation of lymphocytes in the fibrous connective tissue at the edges of the lobules. The reaction consists entirely of a lymphocytic infiltration. The degree of involvement is roughly in proportion to the degree of abscess formation elsewhere. Again epithelioid cells and giant cells of the multinucleated type are not present.







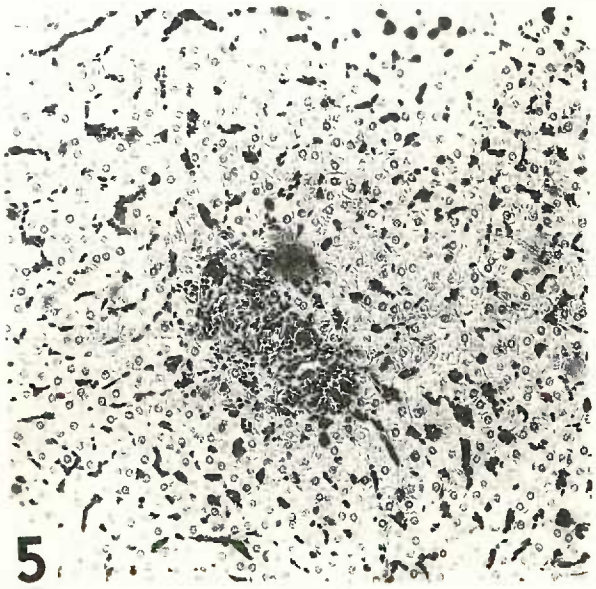


Fig. 4

(6). Lymphocytic infiltration of liver triads.

(7). Granulomatous inflammation of spleen.

(8). Subcutaneous abscess at site of injection.

(9). Hyperemia and edema of lungs (artifact?).

(10). Passive hyperemia of viscera (artifact?).

c. Findings in Animals Infected with Group III Strains.

(1). Interstitial granuloma of lung.

(2). Passive hyperemia of viscera (artifact?).

Although massive inocula, and in some instances smaller doses, of the chromogenic acid-fast bacilli may produce progressive and fatal disease in animals from whom the organisms can be recovered, the findings indicate that the pathology is not identical with tuberculosis.

On the basis of both the gross and microscopic studies, the lesions produced by these organisms must be classed as tuberculoid in character. They differ from true guinea pig tuberculosis in the following ways:

1. The involvement is primarily serosal rather than parenchymal.

2. In the earlier stages of infection the cellular response consists of heterogeneous granulomatous tissue in which epithelioid cells, macrophages and atypical multinucleated giant cells are predominant but typical tubercles are absent.

3. In the more advanced stages many of the lesions are characterized by areas of central necrosis which undergo abscess formation. These are enclosed by polymorphous granulation tissue, often with unusual degrees of hyperemia, a feature which is not distinctive of tuberculosis.

4. The majority of strains do not produce progressive disease; instead healing is manifested as polymorphous granulations which proceed to scar formation.

B. Effects of Bacterial Dosage on Pathogenicity and PPD Tuberculin Sensitivity. The following experiment was designed to determine the effect of bacterial dosage on pathogenicity and PPD tuberculin sensitivity. It was divided into two parts, I and II. The purpose of Part I was to determine the effects in guinea pigs of varying doses of two chromogenic strains on pathogenicity and tuberculin sensitivity. The purpose of Part II was to determine the effects in guinea pigs of a single dose of 19 chromogenic strains on pathogenicity and tuberculin sensitivity.

1. Part I. Strains M-6 and 886 of Group I were selected for study, and doses of 10.0, 1.0, 0.1, and 0.01 mgs. were inoculated intraperitoneally into each of 2 guinea pigs. For a control, the same 4 dosages of the H37Rv strain were inoculated. It will be recalled that in the previous experiment an intraperitoneal dose of 25 mgs. per guinea pig was used. The animals were weighed and PPD tuberculin tested weekly for 8 weeks after which time they were sacrificed, autopsied, and smears and cultures made of the tissues in the manner described under methods. Tissues were not prepared for histologic examination and no rectal temperatures were taken. The results of this experiment are presented in Tables 20 and 21.

a. Results of Part I.

(1). Weight Changes. It will be noted that weight loss in this experiment occurred in very few cases and, with the exception of two instances, loss of weight was manifested during the first 3 weeks, after which time all the animals gained. Weight loss was more evident in the control group but even here the results were not always consistent.

(2). PPD Tuberculin Sensitivity Reactions. Sensitivity to PPD was first manifested at the end of the third week in 2 animals

TABLE 20

Record of Weights and PPD Tuberculin Reactions on Guinea Pigs Infected with Varying Intraperitoneal Doses of 3 Acid-fast Organisms

	Dosage in Mgs.	Pig No.	WPI	PI	Weights and Tuberculin Reactions - Weeks After Infection													
					1		2		3		4		5		6		7		8	
					W	P	W	P	W	P	W	P	W	P	W	P	W	P	W	P
Strain M-6	10.0	1	203	-	246	-	280	-	321	+	357	++	384	++	432	++	444	-	463	-
		2	279	-	290	-	333	-	377	-	428	-	442	-	490	-	530	+	560	-
	1.0	1	249	-	250	-	266	-	301	-	340	-	320	E^1						
		2	320	-	308	-	291	-	280	-	308	-	356	+	401	+	438	-	463	-
	0.1	1	367	-	342	-	354	-	374	-	412	-	492	-	546	-	608	-	654	-
		2	220	-	229	-	252	-	286	+	320	+	348	+	376	++	419	-	448	-
	0.01	1	275	-	310	-	349	-	391	-	435	-	487	-	542	-	530	-	580	-
		2	272	-	318	-	358	-	389	-	445	-	480	-	513	-	558	-	569	-
Strain 886	10.0	1	286	-	271	-	280	-	342	-	373	-	373	-	437	-	463	-	480	-
		2	230	-	227	-	229	-	246	-	286	-	310	-	345	-	370	+	388	-
	1.0	1	283	-	305	-	338	-	338	-	399	-	448	-	478	-	511	-	530	-
		2	216	-	248	-	285	-	308	-	342	-	383	-	410	-	440	-	460	-
	0.1	1	299	-	326	-	370	-	416	-	452	-	496	-	560	-	570	-	609	-
		2	355	-	378	-	425	-	477	-	503	-	545	-	570	-	602	-	620	-
	0.01	1	355	-	373	-	423	-	470	-	509	-	545	-	583	-	601	-	625	-
		2	256	-	286	-	309	-	355	-	398	-	434	-	453	-	463	-	495	-
Strain H37Rv	10.0	1	366	-	347	-	358	-	328	-	291	-	240	E^2						
	1.0	2	361	-	355	-	359	-	298	-	274	-	219	E^3						
	0.1	3	280	-	325	-	356	-	378	-	401	-	385	-	422	+	440	+++	460	+
	0.01	4	250	-	291	-	319	-	346	-	365	+++	343	+++	386	++++	424	+++	443	+

WPI = weight prior to infection. PI = PPD tuberculin reaction prior to infection. W = weight in grams. ___ = loss in weight. P = PPD tuberculin reactions at weekly intervals. E = expired. E^1 = 33days. $E^{2,3}$ = 32 days. Symbols + to ++++ represent the degree of tuberculin reaction.

TABLE 21

Summary of Direct Smears of Organs and Lesions, and Cultures from Mixed Specimens of These Tissues from Guinea Pigs Infected with Varying Intraperitoneal Doses of 3 Acid-fast Organisms

	Dosage in Mgs.	Pig No.	Lungs	Liver	Spleen	Kidneys	Lesions	Culture
Strain M-6	10.0	1.....	0	0	0	0	0	0
		2.....	0	0	0	0	++	0
	1.0	1.....	0	0	0	0	+++	0
		2.....	0	0	0	0	++	0
	0.1	1.....	0	0	0	0	NF	0
		2.....	0	0	0	0	NF	0
	0.01	1.....	0	0	0	0	NF	0
		2.....	0	0	0	0	NF	0
Strain 886	10.0	1.....	0	0	0	0	0	0
		2.....	0	0	0	0	++	0
	1.0	1.....	0	0	0	0	NF	0
		2.....	0	0	0	0	0	0
	0.1	1.....	0	0	0	0	NF	0
		2.....	0	0	0	0	NF	0
	0.01	1.....	0	0	0	0	0	0
		2.....	0	0	0	0	NF	0
Strain H37Rv	10.0	1.....	++	+	+	0	++	17
	1.0	2.....	0	0	0	0	0	0
	0.1	3.....	0	0	0	0	++	15
	0.01	4.....	0	0	0	0	++	10

NF = no lesions found.
Numbers in culture column represent the day growth was first observed.
Symbols + to +++ represent the degree of acid-fast bacilli found.

infected with 10.0 and 0.1 mg. doses of chromogenic strain M-6. This was interesting in comparison with the control strain where no reaction was elicited in the same period of time. At the end of the fourth week the same results were obtained and in addition one of the H37Rv control animals infected with the 0.01 mg. dose gave a +++ reaction. At the end of the fifth, sixth and seventh weeks 4 additional animals, including 1 control, showed sensitivity, and by the end of the eighth week all chromogenic infected animals had lost sensitivity, but the 2 control animals still maintained slight positive reactions. Of interest in this experiment is the fact that the animals receiving the largest infective doses were often, but not necessarily, the ones to react first to PPD or to manifest the more marked reactions or to maintain allergy for the longest period of time. In the previous experiment however, where 25 mg. inocula were used, allergy was still detected at the end of the eleventh week with slight to moderate degrees of positivity. This observation suggests that the amount of the inoculum and the ability to sustain progressive infection play a role in the maintenance of allergy.

(3). Pathogenicity. With smaller doses of organisms (0.01 to 10.0 mg.) the results were much less striking than those in the previous experiment where 25 mg. doses proved lethal. Only one animal expired, and surprisingly, from a 1.0 mg. inoculum of strain M-6. This is, no doubt, a result of individual variation. The more pronounced macroscopic lesions were confined entirely to the animals infected with the 10.0 and 1.0 mg. doses, and in general, were similar to those observed in the previous experiment. The number and size of the nodular lesions and abscesses were less, and the other serosal reactions were also of lesser intensity. Some of the animals infected with the

1.0 mg. doses showed reactions equivalent to those infected with the 10.0 mg. doses. The animals infected with the 0.1 and 0.01 mg. doses showed nothing more than some omental involvement, resulting from the inflammatory reaction of the organisms. Only one tiny nodular lesion was found on the surface of the liver of the animal infected with the 0.01 mg. dose of strain 886. Of special interest is the fact that no organisms were recovered from any of the organs either on direct smear or culture regardless of dosage. Organisms were found in the lesions of four animals, but these stained poorly and showed evidence of autolytic change. The control H37Rv strain, by comparison, yielded organisms on direct smears made from the organs and lesions of the animal infected with the 10.0 mg. inoculum, and from only the lesions of the animals infected with the 0.1 and 0.01 mg. inocula. Positive cultures were obtained from 3 of the control pigs, in which typical progressive tuberculosis was evident macroscopically. Of interest also is the fact that the animal infected with the 0.1 mg. dose produced practically no macroscopic lesions, whereas the animal infected with the 0.01 mg. dose produced massive infection involving all the organs. This again demonstrates pathologic variability.

2. Part II. Nineteen chromogenic strains of Groups II and III were selected for study, and doses of 1.0 mg. each were inoculated intraperitoneally into single guinea pigs. For controls the same dosage of each of 3 saprophytic strains (M. smegmatis-b, M. phlei-a, and M. butyricum) and the H37Rv strain were also inoculated into single pigs. The animals were weighed and PPD tuberculin tested weekly for 7 weeks, after which time they were autopsied and smears and cultures made of the tissues in the manner described under methods. Tissues were not prepared

for histologic examination and no rectal temperatures were taken. The results of this experiment are presented in Tables 22 and 23.

a. Results of Part II.

(1). Weight Changes. Again, as in Part I, weight loss occurred more frequently early, and in only a few of the animals. No weight loss was observed in the saprophytic control animals, but it did occur consistently in the H37Rv control animal.

(2). PPD Tuberculin Sensitivity Reactions. Tuberculin sensitivity to PPD in this experiment was first manifested at the end of the second week in 8 of the animals infected with the chromogenic strains, and then with the exception of 1 animal no further reactions could be elicited at any time during the remainder of the experiment. In the case of the latter animal sensitivity could not be detected at the end of both the third and fourth weeks, but was detected at the end of the fifth week, and was lost again at the end of the sixth week. The reactions were only slightly positive in every case except one (509). This indicates further that animals infected with small doses of chromogenic organisms exhibit less marked and more transient sensitivity to mammalian tuberculin than do animals infected with larger doses. No sensitivity could be detected in either the saprophytic or H37Rv control animals. The reactions at the end of the second week in 2 of the animals infected with strains M-3 and 509 and 1 animal infected with control strain M. butyricum are shown in Figure 5.

(3). Pathogenicity. With the 1.0 mg. dose of organisms the results were even less striking than either of those in the two previous experiments. Besides varying degrees of omental involvement, resulting from the inflammatory reaction of the organisms, and a few

TABLE 22

Record of Weights and PPD Tuberculin Reactions on Guinea Pigs Infected with 1 Milligram Intraperitoneal Doses of 23 Acid-fast Organisms

				Weights and Tuberculin Reactions - Weeks After Infection													
				1		2		3		4		5		6		7	
	Strain	WPI	PI	W	P	W	P	W	P	W	P	W	P	W	P	W	P
Group II	6288.....	262	-	265	-	278	-	302	-	334	-	361	-	394	-	411	-
	S-7.....	343	-	371	-	415	+	449	-	488	-	529	+	537	-	558	-
	M-1.....	250	-	298	-	337	-	389	-	398	-	434	-	449	-	460	-
	S-8.....	250	-	289	-	340	-	389	-	445	-	493	-	567	-	583	-
	M-2.....	332	-	379	-	415	-	456	-	498	-	486	-	520	-	560	-
	M-3.....	387	-	392	-	431	+	445	-	477	-	500	-	525	-	550	-
	509.....	266	-	316	-	339	++	373	-	403	-	432	-	472	-	514	-
	W-7.....	290	-	281	-	298	-	345	-	391	-	443	-	465	-	483	-
	S-4V.....	262	-	306	-	329	-	346	-	319	-	402	-	437	-	482	-
	Parks....	260	-	251	-	298	-	352	-	350	-	432	-	481	-	518	-
	S-4.....	294	-	296	-	329	-	368	-	371	-	419	-	370	-	380	-
	S-6.....	333	-	359	-	384	+	413	-	429	-	456	-	482	-	513	-
Group III	S-5.....	340	-	333	-	369	+	416	-	427	-	465	-	467	-	484	-
	S-10.....	253	-	274	-	304	-	345	-	375	-	405	-	445	-	462	-
	W-1.....	257	-	299	-	337	+	348	-	369	-	411	-	441	-	471	-
	W-2.....	333	-	348	-	373	-	374	-	411	-	457	-	493	-	501	-
	W-3.....	312	-	344	-	395	+	427	-	440	-	482	-	539	-	591	-
	786.....	282	-	286	-	300	+	313	-	321	-	334	-	358	-	377	-
	98717....	290	-	317	-	350	-	383	-	351	-	420	-	463	-	483	-
Controls	M.smeg.-b	264	-	278	-	309	-	369	-	416	-	432	-	507	-	595	-
	M.phlei-a	325	-	331	-	362	-	405	-	456	-	479	-	593	-	638	-
	M.butyr..	250	-	258	-	266	-	297	-	334	-	337	-	373	-	388	-
	H37Rv....	258	-	242	-	221	-	180	-	153	E^1						

Controls = M. smeg.-b = M. smegmatis-b. M. butyr. = M. butyricum.
WPI = weight prior to infection. PI = PPD tuberculin reaction prior to infection. W = weight in grams.
___ = loss in weight. P = PPD tuberculin reactions at weekly intervals. E^1 = expired in 27 days.
Symbols + to ++ represent the degree of tuberculin reaction.

TABLE 23

Summary of Direct Smears of Organs and Lesions, and Cultures from Mixed Specimens of These Tissues from Guinea Pigs Infected with 1 Milligram Intraperitoneal Doses of 23 Acid-fast Organisms

	Strain	Lungs	Liver	Spleen	Kidneys	Lesions	Culture
Group II	6288	0	0	0	0	0	0
	S-7	0	0	0	0	0	0
	M-1	0	0	0	0	0	0
	S-8	0	0	0	0	0	0
	M-2	0	0	0	0	0	0
	M-3	0	0	0	0	NF	0
	509	0	0	0	0	NF	0
	W-7	0	0	0	0	NF	0
	S-4V	0	0	0	0	NF	0
	Parks	0	0	0	0	NF	0
	S-4	0	0	0	0	NF	0
	S-6	0	0	0	0	0	0
Group III	S-5	0	0	0	0	NF	0
	S-10	0	0	0	0	NF	0
	W-1	0	0	0	0	NF	0
	W-2	0	0	0	0	NF	0
	W-3	0	0	0	0	NF	0
	786	0	0	0	0	NF	0
	98717	0	0	0	0	NF	0
Controls	M. smegmatis-b	0	0	0	0	NF	0
	M. phlei-a	0	0	0	0	NF	0
	M. butyricum	0	0	0	0	NF	0
	H37Rv	++	+	++	0	+++	14

NF = no lesions found.
Number in culture column represents the day growth was first observed.
Symbols + to +++ represents the degree of acid-fast bacilli found.

Figure 5

Tuberculin reactions to second strength PPD in guinea pigs at the end of the second week of infection following intraperitoneal inoculation with 1 mg. doses of 2 chromogens and 1 saprophytic acid-fast organism.

The animal infected with strain 509 gave a ++ reaction with slight beginning necrosis, while the animal infected with strain M-3 gave a + reaction. The animal inoculated with the saprophytic control strain M. butyricum gave no reaction. The circles indicate the extent of the edema and hyperemia.

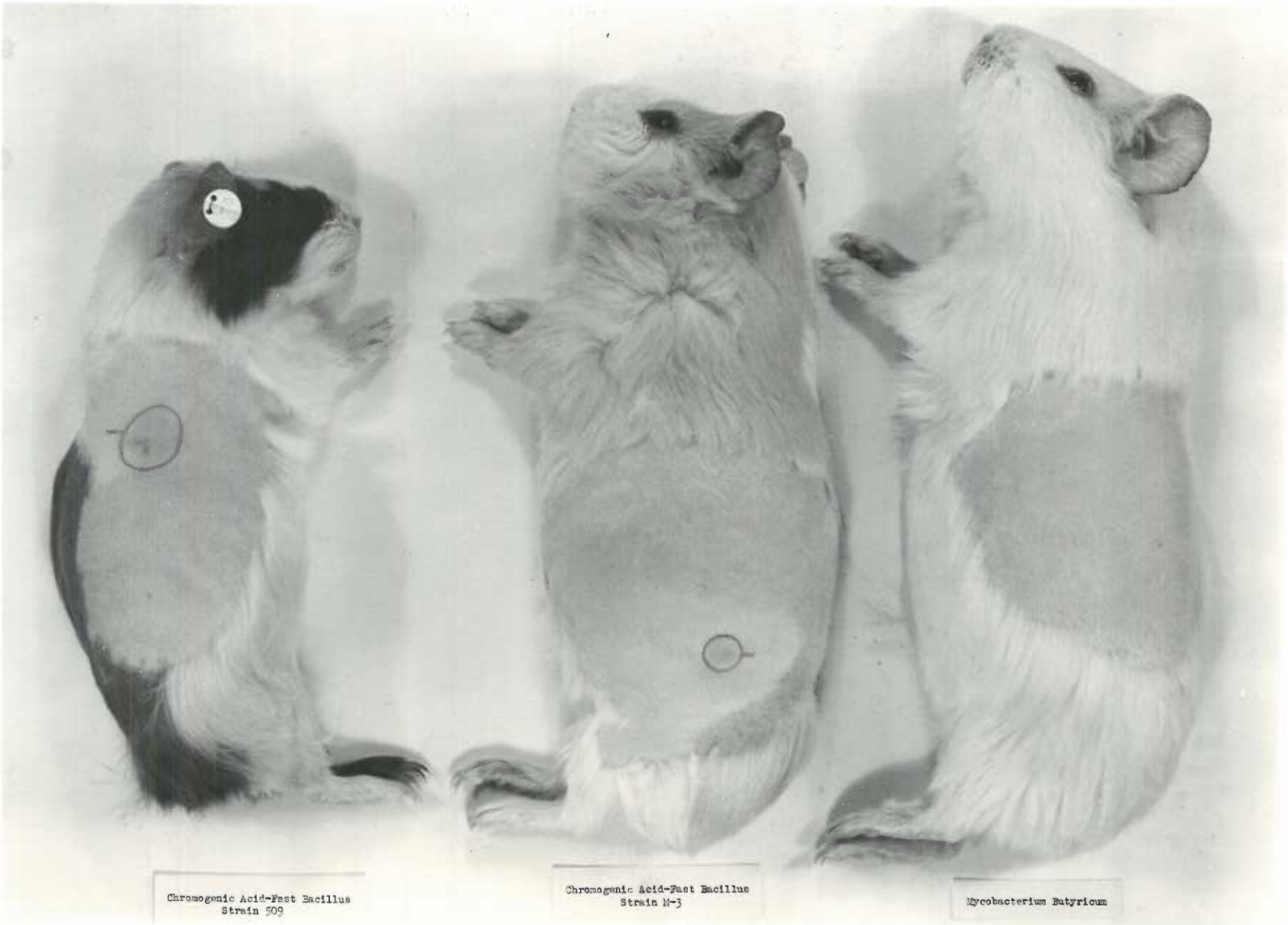

Chromogenic Acid-Fast Bacillus
Strain 509
Chromogenic Acid-Fast Bacillus
Strain M-3
Mycobacterium Butyricum

Fig. 5

nodular lesions no other noteworthy gross changes were found. Here too, as in Part I, no organisms were recovered from any of the tissues, either on direct smear or culture. No macroscopic lesions were found in any of the saprophytic control animals, nor were any organisms recovered from their tissues. Organisms were recovered on both smear and culture from all the tissues, except the kidneys, of the H37Rv control animal, in which typical progressive tuberculosis was evident macroscopically. The findings in this control pig were just the opposite of the comparable control in Part I where no organisms were found in any of the tissues either on direct smear or culture, which again demonstrates pathologic variability.

C. Effects of Route of Inoculation on Pathogenicity and PPD Tuberculin Sensitivity. In view of the fact that all previous inoculations were made intraperitoneally, it seemed desirable to study the differences in pathogenicity and tuberculin sensitivity by varying the route of inoculation. For this experiment strains M-6 and 886 were selected for study, and doses of 1.0 mg. were inoculated subcutaneously into the right inguinal region of each of 2 guinea pigs. For a control the same dosage of the H37Rv strain was inoculated into one guinea pig. The animals were weighed and tuberculin tested weekly for 8 weeks, after which time they were autopsied, and smears and cultures made of the tissues in the manner described under methods. Tissues were not prepared for histologic examination, and no rectal temperatures were taken. The data recorded in Tables 24 and 25 indicate results comparable with those of the intraperitoneal route. The important differences noted were the almost complete absence of weight loss, the recovery in only two instances on direct smear of organisms from the tissues of both animals

TABLE 24

Record of Weights and PPD Tuberculin Reactions on Guinea Pigs Infected with 1 Milligram Subcutaneous Doses of 3 Acid-fast Organisms

Strain	Pig No.	WPI	PI	Weights and Tuberculin Reactions - Weeks After Infection															
				1		2		3		4		5		6		7		8	
				W	P	W	P	W	P	W	P	W	P	W	P	W	P	W	P
M-6	1	323	-	365	-	406	-	441	-	480	+	528	+	541	+	575	+	598	-
	2	226	-	262	-	301	-	331	-	362	-	385	-	418	+	468	-	487	-
886	1	378	-	422	-	469	-	520	-	598	-	635	+	644	+	678	+	689	-
	2	320	-	348	-	400	-	450	-	502	-	544	-	599	-	578	-	586	-
H37Rv	1	297	-	348	-	374	-	404	-	421	++	450	++	422	+++	422	++++	404	+

WPI = weight prior to infection. W = weight in grams. ___ = loss in weight. PI = PPD tuberculin reaction prior to infection. P = PPD tuberculin reactions at weekly intervals.
Symbols + to ++++ represent the degree of tuberculin reaction.

TABLE 25

Summary of Direct Smears of Organs and Lesions, and Cultures from Mixed Specimens of These Tissues from Guinea Pigs Infected with 1 Milligram Subcutaneous Doses of 3 Acid-fast Organisms

Strain	Pig No.	Lungs	Liver	Spleen	Kidneys	Lesions	Culture
M-6	1	0	0	0	0	NF	0
	2	0	0	0	0	++	0
886	1	0	+	0	0	NF	0
	2	0	0	0	0	NF	0
H37Rv	1	+	+	+	0	++	21

NF = no lesions found.
Number in culture column represents the day growth was first observed.
Symbols + to ++ represent the degree of acid-fast bacilli found.

infected with the chromogenic strains, and the manifestation of more persistent, but not more marked, tuberculin sensitivity than that noted in all previous experiments with the same infective dose. Whether this is due to the characteristics of the organisms, the route of inoculation, or to individual animal variation cannot be concluded since the experiment was not repeated. In any case, a large series of animals would have to be studied to determine this. Pathologic results were essentially the same as those obtained in the previous experiment. In the animals infected with strains M-6 and H37Rv, large local abscesses formed at the site of inoculation.

D. Range of Pathogenicity. These experiments were undertaken to learn something about the possible range of pathogenicity of the chromogenic acid-fast bacilli. The purpose was merely a preliminary attempt to screen the pathogenic potentialities of these organisms in white Swiss mice and rabbits with an arbitrarily selected standard wet-weight dose. Strains M-6 and 886, previously classified for pathogenicity in Group I, were selected for study. The H37Rv strain was included for a control. A 1.0 mg. dose of each organism was inoculated intraperitoneally into each of 6 mice and intravenously into each of 2 rabbits. The animals were weighed weekly. Three mice, each infected with a different strain, were sacrificed at the end of 25 days to determine the nature of possible gross pathology. The remaining mice were sacrificed at the end of the ninth week, and the rabbits at the end of the eighth week, at which time they were autopsied and smears and cultures made of the tissues in the manner described under methods. Tissues were prepared for histologic examination. No rectal temperatures were taken. These data are presented in Tables 26 and 27.

TABLE 26

Record of Weights in White Swiss Mice and Rabbits Infected with 1 Milligram Intraperitoneal and Intravenous Doses of 3 Acid-fast Organisms

Strain	Animal	No.	RI	WPI	Weights - Weeks After Infection 1	2	3	4	5	6	7	8	9
M-6	Mouse	1	I-P	13	16	13	15	13	12	10	7^{E1}		
		2		14	16	12	12	13	15	14	15	17	20
		3		16	17	13	12	$12S^{1}$					
		4		11	13	11	12	13	14	13	13	16	18
		5		12	15	13	13	12	12	11	12	14	16
		6		13	13	12	14	15	16	15	16	17	19
886	Mouse	1	I-P	14	12	13	14	15	15	15	11	18	20
		2		11	12	13	13	14	15	14	14	16	19
		3		16	15	15	16	$16S^{2}$					
		4		13	12	12	14	14	14	13	14	16	17
		5		14	14	13	15	15	17	17	17	18	20
		6		11	12	10	8	7^{E2}					
H37Rv	Mouse	1	I-P	15	16	15	15	14	14	13	13	15	16
		2		14	17	15	15	15	14	14	13	13	15
		3		12	14	14	14	15	13	13	12	12	13
		4		14	14	14	13	$13S^{3}$					
		5		17	17	17	17	17	16	15	15	17	20
		6		13	12	10	9	7^{E3}					
M-6	Rabbit	1	I-V	1440	1680	1720	1720	1780	1720	1920	2000	2000	
		2		1640	1760	1800	1800	1800	1795	2020	2200	2300	
886	Rabbit	1	I-V	1660	1740	1745	1760	1860	1800	1560	1495^{E4}		
		2		2140	2340	2590	2680	2940	3340	3340	3600	3760	
H37Rv	Rabbit	1	I-V	2140	2300	2260	2340	2500	2720	2940	3120	3300	
		2		2238	2060	1640	1800	2080	2160	2100^{E5}			

RI = route of inoculation. I-P = intraperitoneal. I-V = intravenous. WPI = weight prior to infection
W = weight in grams. ___ = loss in weight. E = expired. E^{1} = 45 days. E^{2} = 23 days. E^{3} = 24 days.
E^{4} = 45 days. E^{5} = 41 days. S = sacrificed. $S^{1,2,3,}$ = 25 days.

TABLE 27

Summary of Direct Smears of Organs and Lesions, and Cultures from Mixed Specimens of these Tissues from White Swiss Mice and Rabbits Infected with 1 Milligram Intraperitoneal and Intravenous Doses of 3 Acid-fast Organisms

Strain	Animal	No.	RI	DS	DE	Lungs	Liver	Spleen	Kidneys	Lesions	Culture
M-6	Mouse	1	I-P		45	+	++	+	0	+++	21
		2		63		+	+	+	0	+++	15
		3		25		+	+	++	0	+++	17
		4		63		+	++	+	0	++++	14
		5		63		+	+	+	0	+++	20
		6		63		+	+	+	0	++++	15
886	Mouse	1	I-P	63		0	0	0	0	NF	0
		2		63		0	0	0	0	NF	0
		3		25		+	+	+	0	+++	19
		4		63		0	0	0	0	NF	0
		5		63		0	0	0	0	NF	0
		6			23	+	+	+	0	++	23
H37Rv	Mouse	1	I-P	63		++	+++	+	+	++++	14
		2		63		++	+	++	+	++++	17
		3		63		++	+	+	+	++++	23
		4		25		+	+	++	0	+++	27
		5		63		+	+	+	0	+++	28
		6			24	+++	++	+++	0	+++	13
M-6	Rabbit	1	I-V	56		0	0	0	0	+	0
		2		56		0	0	0	0	+	0
886	Rabbit	1	I-V		45	0	0	0	0	0	0
		2		56		0	0	0	0	+	0
H37Rv	Rabbit	1	I-V	56		++	+	+	0	++	0
		2			41	++	+	+	0	++	13

RI = route of inoculation. I-P = intraperitoneal. I-V = intravenous. DS = number of days after infection that animals were sacrificed. DE = number of days after infection that animals expired. NF = no lesions found.

Numbers in culture column represent the day growth was first observed.

Symbols + to ++++ represent the degree of acid-fast bacilli found.

1. Findings in Mice.

a. Weight Changes. Weight changes occurred most frequently in the mice infected with strain M-6, but in general these were not especially noteworthy. Weight loss occurred in all these animals at the end of the second week of infection, after which time it was irregular, and none occurred after the seventh week. Mouse No. 1 lost weight consistently after the fourth week and up to the time it expired. Weight loss in the mice infected with strain 886 and control strain H37Rv was essentially the same as that in the animals infected with strain M-6.

b. Pathogenicity. One of the animals infected with strain M-6 expired in 45 days and one infected with strain 886 expired in 23 days. The lesions in mice were similar to those found in guinea pigs infected with the same dose of organisms. Macroscopic lesions were found in all the animals infected with strain M-6, and in 2 of the animals infected with strain 886. The lesions consisted almost entirely of varying numbers of the yellowish-white nodules described in the preliminary experiments. Several of the mice showed unusually large spleens. Visceral adhesions such as those described in the guinea pigs were almost entirely lacking in these animals. Histologically the only finding of significance was pulmonary, hepatic and splenic granulomata similar to those observed in both the Group I and II guinea pigs. Acid-fast bacilli were recovered on both smear and culture from the various tissues of all the mice infected with strain M-6 and from only 2 of the mice infected with strain 886. In the case of the animals infected with the latter strain, organisms were isolated from the animal that expired on the twenty-third day and from the one which was sacrificed on the

twenty-fifth day of infection which, in these cases at least, indicates that isolation can only be made in the earlier phases of infection. Only one mouse infected with the H37Rv control strain expired in 24 days, and organisms were also isolated on both smear and culture from the tissues of all the animals. Of special interest is the fact that typical progressive tuberculosis could be produced in the lungs, spleen and liver by intraperitoneal inoculations.

2. Findings in Rabbits.

a. Weight Changes. Weight changes occurred occasionally in the infected rabbits, but most of these were not significant. Rabbit No. 1, infected with strain 886, began to lose weight at the end of the fifth week and continued to do so until it expired. The greatest weight loss occurred at the end of the second week in rabbit No. 2 infected with the control H37Rv strain.

b. Pathogenicity. The results in rabbits were the least striking of any of the animal experiments. One animal infected with strain 886 expired on the forty-fifth day, but this was probably the result of septic infection acquired after a severe fight with another animal in the same cage. This assumption was based upon the fact that no acid-fast organisms could be recovered either on smear or culture, and one large peritoneal abscess, different from any of those produced by the chromogenic strains previously, was found to contain massive numbers of staphylococci. Varying numbers of small yellowish-white nodules measuring from 0.5 to 2 cm in diameter, and similar in composition to those described in the other animals were found in the livers of the other three rabbits. The infecting organisms could be recovered from these lesions on smear but not on culture. No bacilli were

recovered from any of the other tissues. Histologically the lesions consisted of granulomata also of the type described in both the Group I and II guinea pigs. No other noteworthy changes were found. In the rabbits infected with the H37Rv control strain, typical tuberculosis characteristic of these animals was found. One of the rabbits expired in 41 days, and tubercle bacilli were recovered on smear from all of the tissues except the kidney. The cultures were positive. The other animal survived until it was sacrificed. Tubercle bacilli were also recovered on smear from all the tissues except the kidney, but the cultures were negative.

E. Specificity of Tuberculins. Since relatively few of the animals infected with chromogenic acid-fast bacilli developed PPD sensitivity, despite the appearance of large numbers of lesions, it seemed desirable to test the guinea pigs against their homologous tuberculins and some heterologous ones as well. For this purpose a number of these were prepared as described in the section on methods. In addition the same animals were tested with the bacterial sediments from which the tuberculins had been extracted. For purposes of clarity the following terminology has been adopted: PPD (purified protein derivative), H37Rv (human) and CAF (chromogenic acid-fast) tuberculins, either homologous or heterologous and CAF bacilli (chromogenic acid-fast), either homologous or heterologous.

1. Effects of Homologous Tuberculins and Bacterial Suspensions on Guinea Pigs Infected with Chromogenic Acid-fast Organisms and the Human Virulent Tubercle Bacillus. This experiment was done to determine the effects of CAF and H37Rv homologous tuberculins and bacterial suspensions on PPD tuberculin negative and positive guinea pigs infected

with chromogenic acid-fast organisms and the H37Rv strain of the human tubercle bacillus. These preparations were tested weekly for 6 weeks in 17 of the guinea pigs which had been infected with 1.0 mg. intraperitoneal doses (See Table 22). The saline-phenol solution was used as a control. From the data presented in Table 28 it can be seen that sensitivity to one bacterial suspension was first manifested at the end of the first week in the animal infected with the M-3 chromogenic strain. Sensitivity to the CAF bacterial suspensions was increased with time, and by the end of the fifth week all the animals, with the exception of W-1 reacted. The CAF homologous tuberculins were next in order of activity and paralleled the bacterial suspensions by the fifth week. Despite these allergic manifestations, the PPD tuberculin elicited fewer positive reactions than either of the homologous test agents. By contrast the greatest number of reactions occurred with PPD after the second week of infection. It should also be noted that the H37Rv control pig remained completely anergic and expired at the end of the fourth week.

Skin sensitivity was variable throughout the entire experiment. One of the animals lost sensitivity completely, others lost and then regained it, while still others at one time showed more marked reactions to the homologous tuberculins and less to the bacterial suspensions, and at other times the reverse effects were noted. The skin reactions at the end of the fifth week in 2 animals infected with strains S-4 and S-7 are illustrated in Figure 6.

2. Effects of Homologous Tuberculins on Guinea Pigs Infected with Chromogenic Acid-fast Organisms. Since CAF homologous tuberculins were tested on animals infected with a small inoculum of organisms

TABLE 28

Effects of Homologous Tuberculins and Bacterial Suspensions on PPD Negative and Positive Guinea Pigs Infected with 1 Milligram Intraperitoneal Doses of 17 Acid-fast Organisms

	Infection with Strain	PI	Tuberculin Reactions - Weeks After Infection																					
			1				2				3				4				5				6			
			P	C	T	BS	P	C	T	BS	P	C	T	BS	P	C	T	BS	P	C	T	BS	P	C	T	BS
Group II	6288	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	+	+	-	-	+	+	-	-	+	++
	S-7	-	-	-	-	-	+	-	+	+	-	-	+	+	-	-	++	+	+	-	+++	+++	-	-	+	+
	M-1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	-	+	+
	S-8	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	+	+	-	-	+	+
	M-2	-	-	-	-	-	-	-	-	+	-	-	+	+	-	-	-	+	-	-	+	+	-	-	+	++
	M-3	-	-	-	-	+	+	-	+	+	-	-	-	+	-	-	-	+	-	-	+	+	-	-	+	++
	509	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	+	+	-	-	+	+	-	-	+	+
	S-4	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	++	+++	-	-	++	+
	S-6	-	-	-	-	-	+	-	+	+	-	-	+	+	-	-	+	+	-	-	+	+	-	-	+	+
Group III	S-5	-	-	-	-	-	+	-	-	+	-	-	+	+	-	-	+	+	-	-	+	+	-	-	+	+
	S-10	-	-	-	-	-	-	-	+	+	-	-	+	+	-	-	-	+	-	-	+	+	-	-	+	+
	W-1	-	-	-	-	-	+	-	+	+	-	-	++	+	-	-	-	-	-	-	-	-	-	-	-	-
	W-2	-	-	-	-	-	-	-	-	+	-	-	+	+	-	-	+	+	-	-	+	+	-	-	+	+
	W-3	-	-	-	-	-	+	-	+	+	-	-	+	++	-	-	+	+	-	-	+	+	-	-	+	+
	786	-	-	-	-	-	+	-	+	+	-	-	-	+	-	-	+	+	-	-	+	+	-	-	+	+
	98717	-	-	-	-	-	-	-	-	+	-	-	-	+	-	-	+	+	-	-	+	+	-	-	+	+
	H37Rv	-	-	-	-	-	-	-	-	-	-	-	-	-	E^1											

PI = PPD tuberculin reaction prior to infection. P = PPD tuberculin reactions at weekly intervals. C = saline-phenol control. T = CAF and H37Rv homologous tuberculins. BS = CAF and H37Rv homologous bacterial suspensions. E^1 = expired on 27th day.

Symbols + to +++ represent the degree of tuberculin reaction.

Figure 6

Reactions to homologous tuberculins and bacterial suspensions in guinea pigs at the end of the fifth week of infection following intraperitoneal inoculation of 1 mg. doses of 2 chromogenic acid-fast organisms.

I. Animal infected with strains S-7.

a = saline-phenol control.

b = CAF homologous tuberculin; +++ reaction.

c = CAF homologous bacterial suspension; +++ reaction with slight beginning necrosis.

II. Animal infected with strain S-4.

a = saline-phenol control.

b = CAF homologous tuberculin; ++ reaction.

c = CAF homologous bacterial suspension; +++ reaction with slight beginning necrosis.

The circles indicate the extent of edema and hyperemia.

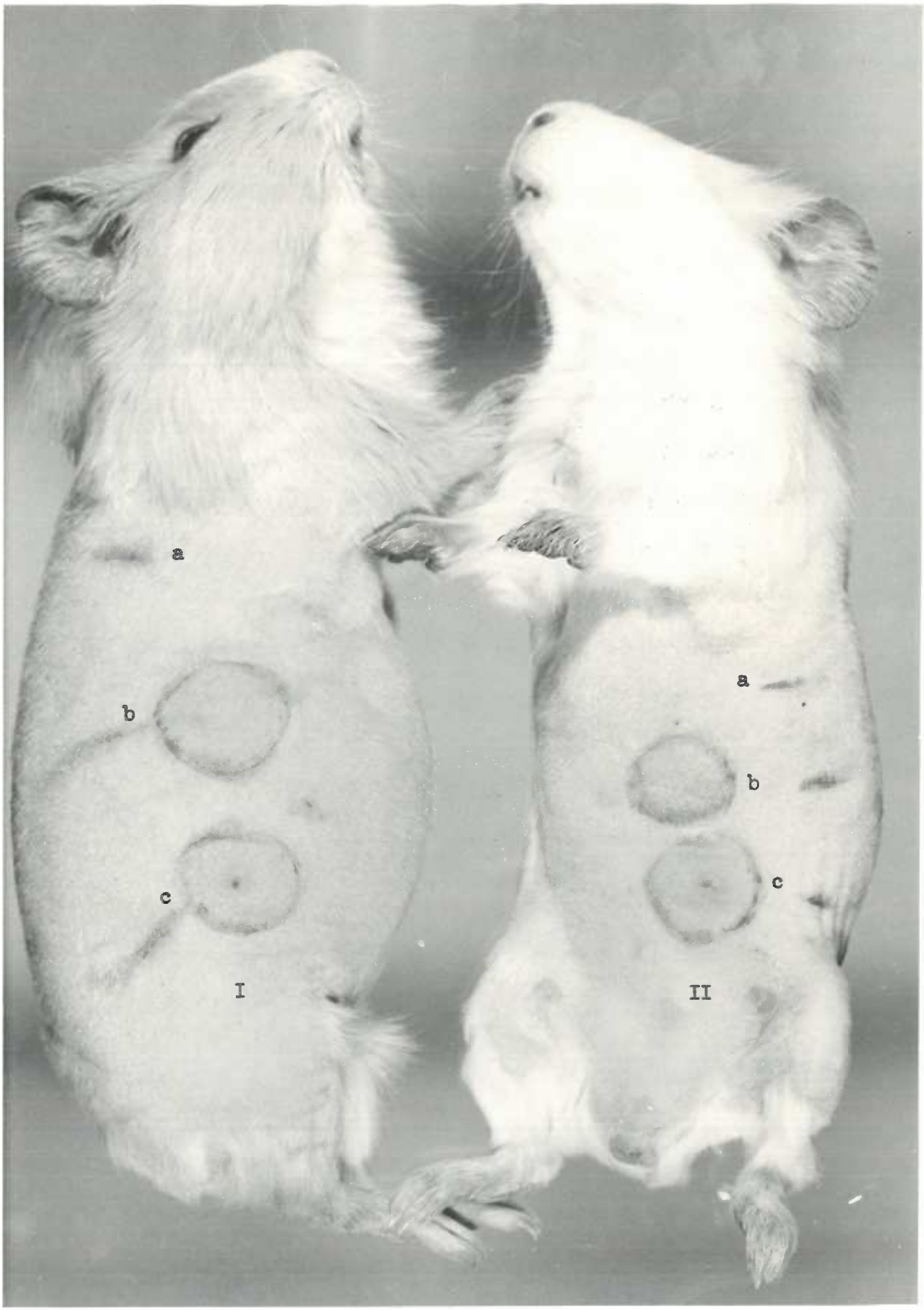


Fig. 6

(1.0 mg.) it seemed desirable to study the effects of these tuberculins on both PPD tuberculin negative and positive animals infected with a larger dose. Two guinea pigs which had been inoculated with 10.0 mg. intraperitoneal doses of strains W-6 and 886 (see Table 20) were tested at the end of the sixth and seventh weeks of infection. The saline-phenol solution was used as a control. From the results recorded in Table 29 it can be seen that both animals reacted strongly to their homologous tuberculins at the end of the sixth week, and slightly less so at the end of the seventh week. The reactions with PPD were irregular and less intense than the homologous tuberculins.

3. Effects of Homologous and Heterologous Tuberculins on Guinea Pigs Infected with the Human Virulent Tubercle Bacillus. The following experiment was done to determine the effects of the H37Rv homologous and CAF heterologous tuberculins on PPD tuberculin negative and positive guinea pigs infected with the H37Rv strain of the human tubercle bacillus. Two guinea pigs were tested at the end of the fourth week of infection (see Table 20).

The first pig had been inoculated intraperitoneally with a 0.1 mg. dose of the organism and was tuberculin negative to second strength PPD. The second animal had received a 0.01 mg. dose of the organism intraperitoneally and was PPD positive with a +++ reaction. These animals were tested with the H37Rv and 17 CAF tuberculins prepared previously. The saline-phenol solution was used as a control. Final readings were made after 48 hours. The findings recorded in Table 30 show that on the PPD tuberculin negative pig only 4 of the CAF tuberculins elicited reactions, whereas on the tuberculin positive pig all but 2 of the CAF tuberculins produced reactions. Of interest is the fact that the

TABLE 29

Effects of Homologous Tuberculins on PPD Tuberculin Negative and Positive Guinea Pigs After 6 and 7 Weeks of Infection with 10 Milligram Intraperitoneal Doses of 2 Acid-fast Organisms

Infection with Strain	Tuberculin Reactions - Weeks After Infection						
	PI	6			7		
		P	C	T	P	C	T
M-6	-	++	-	++++	-	-	+++
886	-	-	-	+++	+	-	++

PI = PPD tuberculin reaction prior to infection.
P = PPD tuberculin reactions after infection.
C = saline-phenol control.
T = CAF homologous tuberculin.
Symbols + to ++++ represent the degree of tuberculin reaction.

TABLE 30

Effects of Homologous and Heterologous Tuberculins on PPD Tuberculin Negative and Positive Guinea Pigs After 4 Weeks of Infection with 0.1 and 0.01 Milligram Intraperitoneal Doses of the Human Virulent Tubercle Bacillus

	Tuberculin from Strain	PPD Negative Guinea Pig	PPD Positive Guinea Pig
Group II	6288.....	-	-
	S-7.....	-	+
	M-1.....	-	++
	S-8.....	-	+
	M-2.....	-	++
	M-3.....	-	++
	509.....	+	+
	S-4.....	-	+
	S-6.....	-	+
Group III	S-5.....	-	++
	S-10.....	-	+
	W-1.....	+	+
	W-2.....	-	+
	W-3.....	+	+
	786.....	+	-
	98717.....	-	+
	H37Rv.....	-	+
	C.....	-	-

Tuberculin negative pig inoculated with 0.1 mg. of the H37Rv strain.
Tuberculin positive pig inoculated with 0.01 mg. of the H37Rv strain.
C = saline-phenol control.
Symbols + to ++ represent the degree of tuberculin reaction.

homologous H37Rv tuberculin failed to produce a reaction in the first pig, whereas 4 of the heterologous CAF tuberculins did so. This indicates further the variability of skin allergy in animals infected with acid-fast organisms.

4. Effects of Homologous and Heterologous Tuberculins on Guinea Pigs Infected with Chromogenic Acid-fast Organisms and the Human Virulent Tubercle Bacillus. This experiment was done to determine the effects of the H37Rv homologous and heterologous tuberculin against PPD and CAF tuberculin negative and positive guinea pigs infected with chromogenic acid-fast organisms and the H37Rv strain of the human tubercle bacillus. Seventeen guinea pigs which had been inoculated with 1.0 mg. intraperitoneal doses of the organisms (see Table 22) were tested with the H37Rv tuberculin at the end of the third week of infection. The saline-phenol solution was used as a control. Final readings were made after 48 hours. The results recorded in Table 31 show that none of the animals infected with chromogenic organisms responded to the H37Rv or PPD tuberculin at this stage of the infection but 8 of them did react to their homologous tuberculin. The animal infected with the H37Rv strain reacted slightly to its homologous tuberculin but not to the others. It would have been interesting to test these animals at other intervals to see whether the same or different results could be obtained. In view of the fact that chromogenic acid-fast bacilli can sensitize animals to PPD, they should be expected to do so to other mammalian tuberculins, and possibly even to unrelated ones.

5. Effects of Homologous and Heterologous Tuberculins (Cross Sensitization) on Guinea Pigs Infected with Chromogenic Acid-Fast

TABLE 31

Effects of Homologous and Heterologous Tuberculins on PPD and CAF Tuberculin Negative and Positive Guinea Pigs After 3 Weeks of Infection with 1 Milligram Intraperitoneal Doses of 17 Acid-fast Organisms

	Infection with Strain	CAF Tuberculin Reaction	PPD Tuberculin Reaction	H37Rv Tuberculin Reaction	C
Group II	6288.....	-	-	-	-
	S-7.....	+	-	-	-
	M-1.....	-	-	-	-
	S-8.....	-	-	-	-
	M-2.....	+	-	-	-
	M-3.....	-	-	-	-
	509.....	-	-	-	-
	S-4.....	-	-	-	-
	S-6.....	+	-	-	-
Group III	S-5.....	+	-	-	-
	S-10.....	+	-	-	-
	W-1.....	++	-	-	-
	W-2.....	+	-	-	-
	W-3.....	+	-	-	-
	786.....	-	-	-	-
	98717.....	-	-	-	-
	H37Rv.....	-	-	+	-

C = saline-phenol control.

Symbols + to ++ represent the degree of tuberculin reaction.

Organisms and the Human Virulent Tubercle Bacillus. This experiment was done to determine the effects of H37Rv and CAF homologous and heterologous tuberculins on guinea pigs infected with chromogenic acid-fast organisms and the H37Rv strain of the human tubercle bacillus, and to study further some of the effects of cross sensitization. Twelve guinea pigs which had been inoculated with 1.0 mg. intraperitoneal doses of the organisms (see Table 22) were cross tested with 12 tuberculins at the end of the seventh week of infection. The saline-phenol solution was used as a control. Final readings were made after 48 hours. From the data presented in Table 32 it can be seen that most of these strains are not only capable of producing reactions to their homologous tuberculins, but to many of the heterologous ones as well. Cross sensitization is definitely evident. Two animals infected with strains S-5 and 886 were anergic and failed to respond to any of the tuberculins. Of special interest is the fact that reactions to the homologous tuberculins in three instances were less marked than the reactions to the heterologous ones; however, the other animals reacted as strongly, and in two instances more markedly to the former than to the latter tuberculins. The animal infected with the H37Rv strain showed the greatest degree of reaction against the homologous tuberculin. Again, as in the previous experiments, it would have been interesting to test these preparations at other intervals to see whether the same or different results could be obtained. On the basis of previous experience, it is assumed that more positive as well as more marked reactions could be expected if the animals had been tested in the earlier phases of infection.

6. Effects of Homologous and Heterologous Tuberculins on White Swiss Mice and Rabbits Infected with Chromogenic Acid-fast Organisms and

TABLE 32

Effects of Homologous and Heterologous Tuberculins (Cross Sensitization) on PPD and CAF Tuberculin Negative and Positive Guinea Pigs After 7 Weeks of Infection with 1 Milligram Intraperitoneal Doses of 12 Acid-fast Organisms

Infection with Strain	Tuberculins													
	P	S-5	S-7	M-2	M-6	W-2	W-3	509	786	886	6288	98717	H37Rv	C
S-5	-	(-)	-	-	-	-	-	-	-	-	-	-	-	-
S-7	-	+	(++)	-	+	+	+	+	+	+	+	++	+	-
M-2	-	+	+	(+)	-	++	+	+	+	++	-	+	+	-
M-6	-	-	-	-	(++)	++	++	+	+	++	++	+	+	-
W-2	-	+	+	+	+	(+)	+	-	+	-	++	+	+	-
W-3	-	-	+	+	-	++	(++)	+	++	+	-	++	+	-
509	-	+	+	+	+	+	+	(+)	+	+	-	+	+	-
786	-	+	+	+	-	+	+	+	(++)	+	+	+	+	-
886	-	-	-	-	-	-	-	-	-	(-)	-	-	-	-
6288	-	+	-	-	+	+	+	+	+	+	(+)	+	++	-
98717	-	+	+	+	-	+	++	+	+	+	-	(++)	+	-
H37Rv	+	+	+	+	-	+	-	-	+	-	-	+	(++++)	-

P = PPD tuberculin reaction at the end of the seventh week of infection. C= saline-phenol control.
The results of homologous tuberculins are indicated in parenthesis.
Symbols + to ++++ represent the degree of tuberculin reaction.

the Human Virulent Tubercle Bacillus. The last experiment was done to determine whether the mice or rabbits infected with the organisms in one of the previous experiments (see Table 26) would manifest any allergic skin reactions to either PPD, H37Rv and CAF homologous or heterologous tuberculins as late as 8 weeks after being infected. From the results recorded in Table 33 it will be noted that both rabbits infected with strain M-6 gave slight reactions to the homologous tuberculin, but failed to respond to any of the other tuberculins. The other animals were negative to all the tuberculin preparations. The negative reactions in mice were anticipated since these animals are well known for their anergy to mammalian tuberculin.

TABLE 33

Effects of Homologous and Heterologous Tuberculins on White Swiss Mice and Rabbits After 8 Weeks of Infection with 1 Milligram Intraperitoneal and Intravenous Doses of 3 Acid-fast Organisms

Infection with Strain	Animal	No.	R-I	Tuberculin M-6	886	H37Rv	PPD	C
M-6	Mouse	2	I-P	-	-	-	-	-
		4		-	-	-	-	-
		5		-	-	-	-	-
		6		-	-	-	-	-
886	Mouse	1	I-P	-	-	-	-	-
		2		-	-	-	-	-
		4		-	-	-	-	-
		5		-	-	-	-	-
H37Rv	Mouse	1	I-P	-	-	-	-	-
		2		-	-	-	-	-
		3		-	-	-	-	-
		5		-	-	-	-	-
M-6	Rabbit	1	I-V	+	-	-	-	-
		2		+	-	-	-	-
886	Rabbit	2	I-V	-	-	-	-	-
H37Rv	Rabbit	1	I-V	-	-	-	-	-

R-I = route of inoculation. I-P = intraperitoneal.
I-V = intravenous. C = saline-phenol control.
Symbol + represents the degree of tuberculin reaction.

DISCUSSION

The isolation of an acid-fast bacillus from tissues, regardless of its source, should be carefully evaluated, so as to avoid the pitfall of ascribing etiologic significance to an organism which actually has nothing to do with the pathologic process. This must of necessity include a consideration of the frequency of isolation of the same organism, an appreciation of the ubiquity of the saprophytic mycobacteria and the possible sources of error in cultural and animal work. The problem of classifying the saprophytic acid-fast organisms and strains other than true tubercle bacilli is complicated by the great heterogeneity which exists among these groups regarding individual and colony morphology, staining reaction, chemical composition, metabolic activity, antigenic structure, serologic specificity and pathogenicity. Further, since it has been difficult to determine which of these species characteristics are stable, and which are variable, depending upon environmental influences, any of the above criteria which may be used in an attempt at classification would be questionable unless the experimental conditions were rigidly controlled.

Some of the atypical chromogenic bacilli studied in this investigation have shown characteristics which might possibly prove of value in the differentiation of these organisms from other mycobacteria, particularly the saprophytic group. No attempt has been made to indicate methods of typing the mammalian tubercle bacilli since adequate differential schemes are presented in many of the standard texts. The purpose here is merely a general comparison of the principal characteristics of the chromogenic strains, based upon the findings in this study, with the mammalian and saprophytic mycobacteria, since these are the

types most commonly isolated from human sources. Then too, it was felt desirable to present something in the way of a working scheme, which although far from complete, might serve as a basis for further studies along similar lines.

A. Major Differential Characteristics of Some of the Mycobacteriaceae.

1. Genus Mycobacterium.

a. The mammalian tubercle bacilli.

(1). Type species.

M. tuberculosis var. hominis.

M. tuberculosis var. bovis.

(a). On Corper-Cohn or similar glycerine-egg media the human strains show eugonic development. Colonies are typically rough, warty, coarsely granular, friable and creamy to grayish-white in color. Bovine strains give similar, but somewhat dysgonic growth. Glycerine is stimulatory to the former, but not to the latter strains.

(b). Development on standard tuberculosis media is slow at 37 C.

(c). Growth on non-standard media is slow, selective and variable.

(d). In the liquid media used in this study pellicle formation does not occur.

(e). With small inocula, 1 mg. or less, the majority of strains produce progressive and often fatal disease in susceptible animals with the

formation of typical tubercles. The organisms invade and multiply in the tissues from which they can be recovered on both smear and culture even when extremely small inocula are used.

(f). Under defined experimental conditions, guinea pigs are allergically sensitized by these organisms, and they react strongly to tuberculin (PPD). Homologous tuberculins, in general, give more marked and persistent reactions than do heterologous ones.

b. The atypical chromogenic acid-fast bacilli.

(1). Type species.

Undetermined.

(a). On Corper-Cohn or similar glycerine-egg media the chromogens also show eugonic growth. Colonies are typically smooth[1], convex, mucoid or tenacious in consistency and intensely pigmented ranging in color from lemon yellow to deep orange.

(b). Development on standard tuberculosis media for most of the strains is also slow at 37 C.

(c). Growth on non-standard media with the majority of strains is slow, selective and variable.

---

[1] One chromogenic strain (M-2) assumed roughness after 13 months of subculture on Corper-Cohn medium. This may possibly be the result of environmental variation.

(d). In the liquid media used in this investigation pellicle formation usually does not occur[1].

(e). With large intraperitoneal doses (25 mgs.) pathologic lesions and even fatal disease may be produced in guinea pigs. Several strains are pathogenic in 1 mg. doses. The pathology is variable, tuberculoid in character but not identical with tuberculosis. With large (25 mgs.) and in some instances small (1 mg.) intraperitoneal inocula, organisms may also invade and multiply in the tissues from which they can be recovered on both smear and culture. As the period between infection and autopsy increases, however, it becomes correspondingly difficult to make such recoveries in animals which do not succumb to the infection.

(f). Under defined experimental conditions, the chromogens may sensitize guinea pigs to mammalian tuberculin (PPD), but the reactions for the most part are slight and transient in character. Homologous tuberculins, in general, give more marked and persistent reactions than do heterologous ones.

---

[1]Pellicle formation was noted only once with strain S-5. This was slight and developed only after prolonged incubation at 37 C.

c. The saprophytic mycobacteria.

(1). Type species.

M. smegmatis (*M. lacticola*).

M. phlei.

M. butyricum.

(a). On Corper-Cohn or similar glycerine-egg media the saprophytic strains grow rapidly and confluently. Colonies are typically rough, somewhat flattened, coarsely granular, often extremely friable and intensely pigmented. A range of colors may be noted such as white, yellowish-white, pink, lemon yellow, orange or brick red.

(b). Development on standard tuberculosis media is rapid over a wide temperature range (20 to 55 C. with an optimum for many strains around 20 C.).

(c). Growth on non-standard media is also rapid and non-selective.

(d). In the liquid media used in this study thick, rough, coarsely granular surface pellicles form within 24 to 48 hours.

(e). Under the conditions of these studies and with doses of 1 to 25 mgs. inoculated intraperitoneally, no evidence of disease was observed[1].

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[1] This observation does not exclude the possibility that other saprophytic strains can produce limited disease in animals.

No organisms were recovered from any of the tissues either on direct smear or culture.

(f). Under the conditions of these studies and with the doses and route indicated in (e) above, none of the saprophytic organisms were able to sensitize guinea pigs to mammalian tuberculin (PPD).

From the scheme presented above it can readily be seen that, in general, the various characteristics of each of these three groups of organisms are sufficiently distinctive to be of value in the differentiation of strains.

Colony morphology and pigmentation could be used to a limited extent by the study of growth on Corper-Cohn glycerine-egg or similar standard tuberculosis media. Thus, if a slow growing, rough, warty, coarsely granular, friable creamy or grayish-white colony were encountered, the presumptive evidence would be strongly in favor of the organism being a tubercle bacillus. If a slow growing, smooth, convex, mucoid or tenacious intensely yellow or orange colony were found, the evidence would point in the direction of one of the atypical chromogenic organisms. Similarly, a rapidly growing, rough somewhat flattened, coarsely granular, friable brick-red colony would suggest a saprophyte.

Temperature requirement and rate of growth on standard tuberculosis media would likewise be of some differential value, since both the chromogens and tubercle bacilli grow slowly at 37 C. Development of most of the former organisms also takes place at room temperature, but is much retarded. The latter organisms do not grow below 30 C. On the other hand, the saprophytic organisms multiply rapidly from 20 to 55 C.

with an optimum for many strains around 20 C. Additional experiments would be necessary to establish the exact temperature requirements of the chromogenic group.

On non-standard tuberculosis media Loeffler and thioglycollate may prove especially helpful, for it will be noted from the data in Tables 9 and 12, that the former medium supported the growth of only one chromogen (M-6, the least selective strain) and one tubercle bacillus (H37Rv). The M-6 strain required 7 days for development and produced a moderate number of colonies after 6 weeks of incubation at 37 C., whereas the H37Rv strain first showed evidence of growth after 19 days and produced only several small colonies by the end of the experiment. The thioglycollate supported the growth of 9 chromogenic organisms, but these grew poorly in 6 to 35 days. After 6 weeks, 8 strains showed + growth and 1 strain ++ growth. None of the tubercle bacilli were able to grow in this medium. The saprophytic strains, however, grew rapidly and luxuriantly in both media in 24 to 48 hours with heavy pellicle formation in thioglycollate. Differential growth in these two media, therefore, would be of value in segregating strains into the proper group. It should be recognised that cultural methods are also subject to certain limitations which must be considered to avoid error. The problems of metabolism and cultural characteristics of the mycobacteria have already been discussed in the general review of these organisms. As was pointed out previously there is great variability in the requirements for culturing acid-fast bacilli and many strains often exhibit marked heterogeneity in individual and colony morphology as well as metabolic activity. Therefore, any attempt at a cultural classification of a particular strain must, of necessity,

recognize the following variables.

The number of organisms inoculated is a limiting factor for growth on different media (see Part II). When the inoculum is large the growth requirements are less exacting. On the other hand, small inocula require more nutritious special media. Failure of small inocula to grow may not necessarily result from nutritional inadequacies, but may be due to inhibitory components either in the media or in contaminating substances.

When first isolated acid-fast bacilli may be atypical, but after a number of subcultures the organisms become more stable and manifest their true cultural characteristics.

The utilization of temperature of incubation as a means of grouping mycobacteria must consider that the optimum requirement for isolation of avian strains is 40 C., for human and bovine strains 37 C. and for cold-blooded strains 25 C. The human, bovine and avian strains do not grow below 30 C. The cold-blooded and saprophytic types grow readily at 20 C. Many saprophytes grow at 45 C. and a few even at 55 C. After a number of generations on culture media, the temperature requirements become less exacting.

The amount of glycerine present in the medium is also a limiting factor of growth. This component accelerates the multiplication of most strains of human and avian bacilli; it has little or no effect on the bovine strains, and it inhibits the growth of the vole bacillus. The degree of acceleration or inhibition varies somewhat depending upon the composition of the medium to which glycerine is added.

Pigment formation is chiefly characteristic of most of the saprophytic strains of the acid-fast bacilli, forming a wide range of

colors as already mentioned. Under the proper conditions the other types of organisms may also form pigments. Avian strains may manifest a pink pigment. Bovine strains which are usually colorless may also show a pink pigment. Mycobacteria from cold-blooded animals are generally colorless, but colonies of these strains may also be white, pink or yellow. Even human strains may be pigmented, and when grown on deeply colored ox serum, the colonies may be yellow or orange-yellow. The H37Rv strain of the tubercle bacillus which is normally creamy or grayish-white on Corper-Cohn medium not infrequently will develop orange colored variants morphologically indistinguishable from the parent colony as well as a number of saprophytes. Of interest also is the report of Reed and Rice[14] who found that a concentration of 0.02 per cent ferric citrate on glycerine agar medium stimulated the development of chromogenic colonies of human, bovine and avian tubercle bacilli and saprophytic acid-fast organisms. Pigmentation is best seen in cultures which have been incubated at 37 C. and then stored in the dark at room temperature. Most workers believe that pigment production depends upon a number of environmental factors, particularly oxygen supply and the chemical composition of the medium. The atypical chromogenic acid-fast bacilli studied in this investigation show a characteristic lemon-yellow to deep orange color on the standard glycerine-egg tuberculosis media, but on a number of non-standard media pigmentation is lost and the colonies appear colorless, creamy or grayish-white.

Animal pathogenicity has always been considered the best criterion for identifying the various types of mycobacteria. Corper[188] has called attention to the fact that as little as 0.000,001 mg. of tubercle

bacilli can consistently produce progressive and fatal disease in guinea pigs within three months. The experiments herein described were conducted with considerably larger doses, namely 1 and 25 mgs. of bacilli. With such amounts the chromogenic group manifested pathogenic qualities not shared by the saprophytes. Although the limits of dosage were not completely determined in this study, it would appear that the majority of chromogens (19 out of 26) are potentially pathogenic when given in 25 mg. amounts. A few of the strains were classed as non-pathogenic and still others (3) were lethal for animals in 1 mg. doses and possibly less. From these data, it seems unlikely that any of the chromogens would approach the virulence of tubercle bacilli. This characteristic then could be utilized for classification purposes, provided a large enough number of strains were carefully examined. The type of pathology produced by the chromogenic acid-fast bacilli would also be helpful in the characterization of strains because of the atypical nature of the lesions. Furthermore, as the period between infection and necropsy increased the chromogens were recovered from the tissues with increasing difficulty. Tubercle bacilli under the same conditions are readily found and cultured. Finally with doses of 1 to 25 mgs. of saprophytes inoculated intraperitoneally, no evidence of disease was observed. This observation, however, does not exclude the possibility that other saprophytic strains can produce limited disease in animals. None of these strains were recovered from any of the tissues either on direct smear or culture.

As in the case of cultural technics, differentiation of the mycobacteria from the standpoint of pathogenicity is also often extremely difficult because of the necessity for qualifying the term

"virulence" on the basis of strain characteristics, infective dose, route of inoculation, nutritional state of the animal, species susceptibility or resistance, the time interval between infection and necropsy and the other factors which must be considered in talking about the problem of pathogenicity. Further, there are no generally accepted criteria of what constitutes disease in animals.

One of the most troublesome problems in experimental tuberculosis is the lack of uniformity of infection and the variability in the extent, and often the character of disease in animals inoculated with tubercle bacilli and other acid-fast organisms. It has been observed by many workers that strains of human tubercle bacilli isolated from patients in a rapidly progressive phase of disease often exhibit low or no pathogenicity in guinea pigs and at times such strains show atypical biological activity, which makes typing difficult or impossible. The reverse effect may be observed, when organisms isolated from patients in a clinically inactive phase of tuberculosis produce serious or rapidly fatal disease in animals.

Certain organisms may lose their pathogenic potentialities after long, and sometimes even after very short, periods of cultivation. The pH of the medium is an important factor in pathogenicity for the optimal pH for maintaining the virulence of tubercle bacilli is 6.8. Cultures of human and bovine bacilli grown at pH 6.0 rapidly become attenuated. Other strains remain remarkably stable for years regardless of the environmental influences to which they are subjected. Serial animal passage may, but does not always, produce a recrudescence of their pathogenic capacities. In such cases the animal tissues afford the necessary environment for this accomplishment. With other strains,

virulence may be either maintained, reduced, increased or lost by culture on special media. With loss in pathogenicity, some bacilli may undergo remarkable alteration, manifesting changes in morphology, staining reactions, cultural and other characteristics. Conversely, some strains retain all their characteristics, or show only insignificant modifications. Some strains of the avian bacillus are as pathogenic for the rabbit as are some of the bovine strains, so the animal test under these conditions is of no value in typing these organisms. This is often true for other mycobacteria. There are numerous studies on the significance of environment in relation to pathogenicity emphasizing the role of temperature, moisture, sunlight, nutritional supply, communal association with other bacteria and the type of host, factors which indeed must be considered in understanding the mechanisms of the agents of disease.

Even the character of the lesions, thought by some to be only a manifestation of living virulent organisms, are not at all confined to such a group, for many workers have clearly demonstrated that similar reactions may be produced by dead organisms, avirulent living ones, combinations of either with certain chemical fractions and even by the chemical fractions alone. Take for example the work of Schwabacher(168) who demonstrated that saprophytic acid-fast bacilli, in sufficient dosage, inoculated subcutaneously or intracutaneously into the thigh of guinea pigs, may produce a local lesion with regional lymph gland enlargement from which acid-fast organisms can be recovered. Rabbits she showed may be killed by the intravenous inoculation of 10 mg. doses of these organisms, and further that both these effects may be accomplished by the use of heat-killed organisms, which she pointed out,

were mainly due to toxic components liberated from the bacilli rather than to their capacity to multiply in the tissues. Or take the results of other investigators who have shown that some saprophytic strains, injected intravenously into experimental animals, even as large as calves, may produce severe and even fatal disease. Of special interest is the fact that intraperitoneal infection of guinea pigs with saprophytes mixed with butter or mineral oil may produce extensive lesions which closely simulate those of tuberculosis. But to what extent the lesions are due to the organisms themselves has never been clearly demonstrated. Other workers insist that the saprophytic organisms may be clearly distinguished pathologically from other mycobacteria, for histologically the lesions which this group produces are more exudative than proliferative, and there is less tendency to caseation and more to suppuration; granulocytes are more numerous than epithelioid cells; and typical giant cells with peripheral nuclei are rare. Overlapping, however, does occur and may be confusing. It is further emphasized that saprophytic organisms do not multiply in the tissues, and for this reason true infection and progressive disease does not result. Symptoms of disease are believed to be due to toxemia from the liberation of endotoxins. Many investigators have pointed out that the toxic manifestations following intravenous inoculation of living or dead mycobacteria, whether saprophytic strains or virulent tubercle bacilli, may be very similar in character, and thus lead to considerable confusion in differentiation. Subcutaneous inoculations, however, generally do not present this difficulty.

Another factor in dealing with the problem of pathogenicity is the reliability of the animal test itself, and what criteria are essential

for the diagnosis of tuberculosis. This problem has been very well reviewed by Feldman and Magath[183] who pointed out that other infections, many of them of spontaneous origin, occasionally produce in guinea pigs, pathology which more or less resembles tuberculosis. It was also pointed out that the failure to find acid-fast bacilli in tuberculosis-like lesions in these animals is presumptive evidence that the disease is not tuberculosis, and that in histologic diagnosis, too much reliance should not be placed on the cellular aspects of the lesions, such as the finding of the Langhans type giant cell which apparently is not an essential or consistent part of the reaction. This cell may be present in normal tissues and is often found in infections other than tuberculosis. The presence or absence of acid-fast bacilli in the lesions, these workers felt, was the most dependable criterion. The danger of placing too much emphasis on the finding of acid-fast bacilli within the tissues, in the absence of additional criteria, has been emphasized by Thompson and Frobisher[184] and by Cooper and Petroff[185] who found acid-fast bacilli, morphologically similar to tubercle bacilli in the lymph nodes of over 30 per cent of normal guinea pigs. Another possible source of so-called spontaneous tuberculosis in guinea pigs was stressed by Sewall[186] who showed that it was possible for these animals to become infected by the ingestion of contaminated food obtained from tuberculous patients and even from diseased caretakers. Pseudotuberculosis is another condition which must be kept in mind to avoid error in pathologic diagnosis. The anatomic indistinguishability of the changes produced by this disease and those of tuberculosis has been pointed out by Reiman[187].

While the animal test is the most reliable method we have for

evaluating the pathogenic potentialities of tubercle bacilli and other mycobacteria, it must not be forgotten that pathogenicity for the animal and man may be quite different. It is, therefore, important to understand and appreciate the limitations of animal typing and the influences which operate to produce, among all the mycobacteria, variations from their typical characteristics.

In the scheme of classification presented it can be seen that tuberculin sensitivity is also sufficiently distinctive to be of some value in the differentiation of strains in these three groups of organisms. Under defined experimental conditions tubercle bacilli allergically sensitise guinea pigs to mammalian tuberculin (PPD). The atypical chromogenic bacilli may also sensitize these animals to mammalian tuberculin (PPD), but the reactions for the most part are slight and transient in character. Homologous tuberculins, in general, give more marked and persistent reactions than do heterologous ones. Under the conditions of these studies and with doses of 1 to 25 mgs. inoculated intraperitoneally, none of the saprophytic organisms were able to sensitize guinea pigs to mammalian tuberculin (PPD). Thus if a culturally typical tubercle bacillus, chromogen or saprophyte were encountered, and inoculated intraperitoneally into guinea pigs which were later tested with PPD and their homologous tuberculins it would be possible to gain additional presumptive evidence as to the type of organism with which one was dealing. But as with all the other tests discussed, tuberculin sensitivity has its limitations as well, and to also avoid the pitfall of ascribing too much significance to any one test, it is important that here too we understand and appreciate these limitations. The problems of tuberculin sensitivity variation and the

specificity of the reaction have also been discussed in the general review of this subject. As was emphasized previously a number of important variations in tuberculin sensitivity have been reported. Krause[122] has demonstrated that tuberculin sensitivity cannot be elicited until approximately 8 to 12 days after tuberculous infection, when caseation has occurred. With the healing of lesions, the intensity of the reaction diminishes. The cells nearest the focus of infection are more sensitive than the other cells, but Long[123] has shown that the germinal cells of the guinea pig testicle are exquisitely sensitive. In an acute miliary phase of disease where the animal is overwhelmed by infection, or in the terminal stages of tuberculosis the intensity of the reaction is greatly reduced or may be completely lost. Sensitivity does not manifest itself in the pre-allergic state. Repeated applications of tuberculin may reactivate a healing lesion and given at too frequent intervals may lead from a state of allergy to one of anergy. An animal which is too young or emaciated, even though successfully infected, will not react to tuberculin. Intercurrent infection, pregnancy and parturition have also been observed to temporarily diminish allergic capacity; but with return to a normal physiologic state, the allergy again becomes manifest.

The question of tuberculin specificity has always been one of great controversy. One group of workers believes that the reaction is specific, another group feels that it is not, and between these two extremes there are all degrees of opinion. There appears to be sufficient evidence to indicate that specificity is only relative. Many investigators have shown that in such types of tuberculosis as advanced

pulmonary, skin, genito-urinary and joint, the inoculation of non-specific proteins as egg albumin, milk, sera, vaccines, fungi, bacteria or their extracts, proteoses and glycerine can also produce all the tuberculin types of reactions. Other workers have called attention to the non-specificity factor by pointing out that positive reactions could be elicited in other inflammatory diseases. Petroff[124] showed that large enough doses of heat-killed tubercle bacilli could establish an allergic state. Baldwin[125] demonstrated that slight transitory degrees of hypersensitivity to tuberculin could be produced by repeated inoculations of tubercle bacilli proteins, but water extracts of the saprophytic timothy bacillus (M. phlei) failed to react in guinea pigs infected with the human strains. Long[126] referring to the work of Krause and Baldwin on cross sensitization and skin allergy pointed out that their experiments clearly indicated that tuberculous animals are hypersensitive to extracts of various non-pathogenic acid-fast bacilli, and conversely that animals infected with the avirulent strains respond to tuberculin in the same manner as tuberculous animals. In his own work he observed that extracts of acid-fast turtle bacilli could elicit an allergic response, though mild, in guinea pigs infected with human strains. In a later study with acid-fast bacilli he and Seyfarth[127] noted that extracts from the frog, grass and smegma bacilli were also capable of producing allergic phenomena in tuberculous guinea pigs. Elder and Lee[128] succeeded in producing local lesions in cattle with the avian tubercle bacillus, and they reported that these animals were able to react to mammalian tuberculin. Calmette[129] showed that inoculations of extracts of paratubercle bacilli in both tuberculous men and animals gave local or febrile reactions similar to those produced with

tuberculin. In an effort to determine the causes for tuberculin sensitivity in non-tuberculous cattle, Crawford[130] demonstrated that the timothy grass, the mist and hog-skin bacilli isolated from lesions of these animals could produce definite tuberculin sensitivity and since the reactions to mammalian tuberculin was usually atypical, he felt that this suggested a group rather than a specific mammalian-tubercle bacillus sensitization. Skin hypersensitiveness has even been described in cattle with no macroscopic lesions. Such allergy is thought to result from ingestion. Crawford[130, 131], Van Es[132], Frey and Hagan[133], Daines and Austin[134, 135] and Branch[136] have reported similar studies. The results in this investigation are in general accord with those described by some of these workers.

In spite of these extensive investigations there are those who still maintain that although it has been demonstrated that non-specific substances, fungi, certain non-acid-fast bacteria (colon, typhoid, brucella and diphtheria) or their products, and many acid-fast bacilli other than tubercle bacilli manifest allergic phenomena, the reactions are never as clear-cut or as intense as those obtained using equivalent amounts of tuberculin. The methods of preparing and standardising various tuberculins have been other factors of considerable debate. The major evidence that tuberculin allergy is based on tuberculous infection has been derived from the high correlation between tuberculin hypersensitivity and post-mortem findings. Thus, in the light of other supportive evidence, and with its limitations clearly in mind, the tuberculin test can also be used advantageously for the differentiation of the types of mycobacteria.

In summary the position of the atypical chromogenic acid-fast

bacilli in relation to the other mycobacteria is at present ambiguous. Some workers feel that these organisms belong in the saprophytic group, others believe that they are merely variant strains of true tubercle bacilli and need no special classification, while still others hold that some of these bacilli, at least, are probably new species which for the present cannot be placed into any of the groups. In view of their variability and because many strains have characteristics common to both the saprophytic and true tubercle bacilli, a midway position between these groups appears reasonable.

The studies on streptomycin sensitivity were of interest since it was found that the atypical chromogenic acid-fast bacilli respond to streptomycin in exactly the same way as do tubercle bacilli. All strains were found sensitive except three. One was isolated from a patient who had been treated for pulmonary tuberculosis with streptomycin for four months, and the resistance was undoubtedly due to the therapy. It was not known whether the other two had been subjected to the action of the antibiotic. Like tubercle bacilli, then, the chromogens are also initially sensitive to streptomycin, but following therapy in patients from whom these strains are isolated, they probably become resistant. Some strains are merely inhibited by the antibiotic, while others are killed by it.

Regarding the nature and relationship of these organisms to other mycobacteria, a number of workers feel that, some strains at least, may possibly bear a genetic relationship to true tubercle bacilli. That they have resulted from transmutation seems unlikely in view of the experimental evidence against this possibility, although according to other investigators indisputable evidence regarding rigid stability of

the mycobacteria as held by Calmette and others of his school is still wanting. There is good evidence from the reports of Miller[50], Griffith[182], Pinner[165], Larmola[65] and from the observations made in this investigation that some of the chromogenic strains are merely variants of human tubercle bacilli, which have resulted from various environmental influences. By serial animal passage they can often be made to revert to the parent type. In the variant phase some strains may be indistinguishable from many of the saprophytic mycobacteria, while others may be especially individualistic and exhibit unusually atypical characteristics. On a number of occasions during these studies it was noted that the standard H37Rv strain of the human tubercle bacillus on Corper-Cohn and Lowenstein media developed orange colored variants which were morphologically indistinguishable from the parent colonies, but exhibited atypical staining and pathogenicity. Guinea pigs inoculated intraperitoneally with 1 mg. of the chromogenic variant reacted strongly to both PPD and a homologous tuberculin. The first animal which survived this infection was sacrificed and autopsied at the end of the tenth week. Grossly all that was found were a few enlarged suppurating inguinal nodes. Cultures of the pus revealed the same organism that was inoculated into the first pig. Smears and cultures of the lungs, liver, spleen and kidneys were negative. The second pig, infected intraperitoneally with a heavy saline suspension of the pus from several of the nodes of the first pig expired at the end of the seventh week and showed typical progressive tuberculosis. Smears and cultures from the lesions and organs were positive and the colonies this time were almost identical to the parent H37Rv strain, and differed only in being somewhat darker grayish-white in color.

Another saline suspension of pus taken from several of the lesions of this animal was again inoculated intraperitoneally into a third pig, which has survived for eleven weeks to date. All three pigs have reacted strongly to PPD and the homologous tuberculin. Miller[50] described similar observations. Pinner[165] reported the isolation of 4 white smooth acid-fast strains, which he thought were probably relatively avirulent variants of human tubercle bacilli. By serial animal passage 3 of these strains were transformed into typical human colonies. The resulting rough-growing organisms behaved entirely like true human strains, and their characteristics remained stable for many generations. On the blood media described in Part II of this investigation four distinctly different types of colonies of the H37Rv strain have been observed, including a small, smooth grayish-white colony. Whether these are merely environmental variants, or true dissociants cannot be stated definitely, but in view of Alexander's work[53, 54], the latter colony probably is a dissociant. Using a similar blood medium she was able to dissociate both the human and avian tubercle bacilli into both rough and smooth variants, and in pathologic studies it was further demonstrated that the lesions produced by the latter strains differed radically from those produced by the rough ones. Larmola[65], in his studies of a large number of typical and atypical tubercle bacilli isolated from sewage, water tanks and forest ditches in the vicinity of a tuberculosis sanatorium and from sputum and gastric washings of tuberculous patients found great variability in staining, morphologic, cultural and pathogenic properties of these organisms. For example, he noted that organisms of low virulence on cultivation in the laboratory for long periods lost their pathogenicity. This was

associated with change in the characteristic mode of growth, staining and morphological appearance and colony pigmentation. Some strains of tubercle bacilli isolated from patients with slight activity exhibited low pathogenicity. Other strains with low pathogenicity for man produced overwhelming disease in guinea pigs, while others avirulent for these animals were still capable of sensitising them to tuberculin, and by animal passage they could again be made virulent. Still other strains which resembled true virulent human tubercle bacilli in all respects were not always pathogenic for guinea pigs.

The assumption of the variant concept is not at all untenable in view of the evidence which has been reviewed regarding the many resultant effects of environmental influences on all mycobacteria. For example, typical virulent human tubercle bacilli can become chromogenic by growing them on media containing minute amounts of ferric citrate or old ox serum, or by altering the pH. It is possible that a similar transformation could also occur in vivo. In this connection the observation made by Schiff and Tarshis[166] while investigating the inhibitory effects of streptomycin on human virulent tubercle bacilli is also suggestive. Nineteen per cent more chromogenic acid-fast bacilli were isolated from patients who were receiving or who had received streptomycin therapy than from those who had never had such treatment. It will be recalled from the work of Pinner[82], Wenkle et al.[110] and Schiff and Tarshis[166] that chromogens are invariably isolated from patients who were in a clinically improved phase of their disease. The observation on the possible influence of streptomycin may be merely a perfectly fortuitous experience, which has no significance, and in view of the limited number of cultures studied,

the observation, for the present at least, can only be considered suggestive. Perhaps streptomycin interferes in some way with the metabolism of virulent tubercle bacilli, so that atypical chromogenic variants are produced. In fact Dienes(189) has found that penicillin causes the production of "L" forms of Streptobacillus moniliformis and the bacteroides. Possibly the role of streptomycin is indirect, resulting merely in hastened clinical improvement, following which chromogenic variants appear. Further studies are necessary to determine whether streptomycin has such an influence on tubercle bacilli. Two lines of investigation are suggested. First, it would be interesting to extend the in vitro experiments such as described in this study, that is, to expose tubercle bacilli to varying concentrations of the antibiotic for different periods of time and then subculture the organisms to several different tuberculosis media. In this way it would be possible to determine whether exposure to streptomycin alone were responsible for changes in colony morphology, pigmentation, pathogenicity or other possible variations. Then to ascertain whether an in vivo mechanism was necessary for these changes, such as a combination of streptomycin with certain tissue components, it would be necessary to infect animals and treat them with the antibiotic. Following therapy isolations of the organisms would have to be made, either at different intervals from local or internal lesions, or after arbitrary periods of time following infection. If chromogens were isolated, it would then be necessary to repeat the experiments a sufficient number of times to determine whether the variation could be obtained at will, or whether it only occurred occasionally. This would also be necessary to rule out the error of possible contamination. With such variant strains the

studies could be further extended to include serial animal passage, such as described above regarding the H37Rv chromogen, to find out whether reversion to the parent type could be accomplished. At the same time tuberculin sensitivity and pathologic response could also be studied.

In speculations concerning the possible mechanisms which operate to produce atypical or variant strains of tubercle bacilli, many of the basic factors favoring variation are not clearly understood. From the numerous studies reported during the last 23 years[42-65] it has become increasingly evident that for the mycobacteria the dogma of monomorphism and stability can no longer be accepted. The previous review of this problem has indicated that there is little doubt that dissociation can be accomplished with a large variety of acid-fast bacilli. In addition all grades of pathogenicity are possible from single strains, depending upon the environmental and other factors which can be varied either experimentally or by chance. The true significance of these variants in relation to human and animal disease is even less clearly defined. In this connection one is impressed with the suggestive studies of L'Esperance[157, 158] who presented evidence that avian bacilli may be the cause of Hodgkins disease, and demonstrated that these organisms were non-pathogenic for the usual laboratory animals, except the chicken and the rabbit. However, if non-susceptible animals were first inoculated with dead human or bovine tubercle bacilli, they became susceptible to the avian strain. Thus it was possible to transform an animal relatively resistant to the avian bacillus into a more susceptible one by previous treatment with a dead heterologous strain of the tubercle bacillus. Such a result, whether due to the products of tubercle bacilli or to related chemical fractions from different sources, may possibly

explain the pathogenic characteristics of some of the atypical chromogenic or other acid-fast bacilli.

Finally it is again emphasized that atypical chromogenic acid-fast bacilli should not be interpreted as saprophytic strains, which unfortunately is so frequently done without some attempt at adequate verification. Such organisms are deserving of attention and may prove to be of significance in the pathogenesis of disease.

SUMMARY

1. A general description of the mycobacteria has been presented.

2. Twenty-six atypical chromogenic acid-fast bacilli isolated from patients either suspected of, having or dying from tuberculosis have been investigated. Their general bacteriological characteristics, the history of similar or related strains, their significance in disease and the difficulties of classifying these organisms have all been discussed.

3. The cultural studies indicate that these organisms are more closely related to the tubercle bacilli than to the saprophytic mycobacteria.

4. The streptomycin sensitivity studies have shown that the chromogens, like tubercle bacilli, are also initially sensitive to streptomycin, but following therapy they probably become resistant. Some strains are merely inhibited by the antibiotic, while others are killed by it.

5. The problem of virulence, the variability of the acid-fast bacilli in cultural behavior and pathologic activity, the limitations of animal tests for purposes of classification and differentiation of strains have also been discussed.

6. The findings of preliminary pathologic studies in guinea pigs, white Swiss mice and rabbits were in general agreement with those of other investigators. Following infection in guinea pigs with large inocula (25 mgs.), the strains could be classified into the three following groups:

Group I. Produce death with macroscopic lesions (7 strains).

Group II. Do not produce death, but produce macroscopic lesions (12 strains).

Group III. Do not produce death or macroscopic lesions (7 strains).

7. Guinea pigs infected intraperitoneally with 25 mgs., and in some instances 1 mg., doses of the chromogenic bacilli resulted in the death of a number of animals from whose lesions and tissues organisms were found on direct smear and recovered on culture. The same doses of control saprophytic strains produced no pathology, and no organisms could be recovered from the tissues, either on smear or culture. In these respects the chromogenic acid-fast bacilli differ from the latter organisms, which as a group, are characterized by their inability to invade and multiply in tissues or to produce progressive disease. Pathologically some of the chromogens behaved like the saprophytic bacilli. On the basis of both gross and microscopic studies, the lesions were classified as tuberculoid in character, but they are sufficiently different to make them distinguishable from true guinea pig tuberculosis.

8. The results of pathologic studies in white Swiss mice and rabbits infected with 2 chromogenic strains of Group I (M-6 and 886) were similar to those in the guinea pigs, but the reactions were less pronounced, especially in rabbits.

9. The studies on tuberculin sensitivity were also in general

accord with those of previous workers who found that guinea pigs infected with various acid-fast bacilli can be made hypersensitive to mammalian as well as other tuberculins, and conversely tuberculous animals react to tuberculins of other acid-fast bacilli in the same manner as the former pigs. The striking characteristic of tuberculin skin allergy was its variability, not only in animals infected with the chromogenic bacilli, but also in animals infected with the standard H37Rv strain of the human virulent tubercle bacillus. The results of the cross sensitization experiments presented suggestive evidence for the immunologic relationship between all the mycobacteria. It appears that they not only have antigenic properties in common, but some of the organisms may have specific components of their own. Further studies are necessary to elucidate these relationships. The limitations of the tuberculin test in animal differential diagnostic work have also been indicated.

10. The nature and relationship of the atypical chromogenic organisms to other mycobacteria have also been discussed and the importance of their identification when isolated from pathologic processes has been emphasized.

11. A scheme which may prove helpful in the differentiation of the types of mycobacteria and which might also serve as a basis for further studies along similar lines has been presented and discussed.

BIBLIOGRAPHY

1. Stitt, E. R., Clough, P. W. and Branham, S. E. Practical Bacteriology, Hematology and Parasitology, 10th ed., The Blakiston Co., Philadelphia, 1948.

2. Wilson, G. S. and Miles, A. A. Topley and Wilson's Principles of Bacteriology and Immunity, 3rd ed., The Williams & Wilkins Co., Baltimore, 1946.

3. Gay, F. P. and Collaborators. Agents of Disease and Host Resistance, 1st ed., Charles C. Thomas, Springfield, 1935.

4. Smith, D. T. and Collaborators. Zinsser's Textbook of Bacteriology, 9th ed., Appleton-Century-Crofts, Inc., New York, 1948.

5. Branch, A. Avian tubercle bacillus infection with special reference to mammals and to man. Arch. Path., vol. 12, pp. 253-274, 1931.

6. Smith, C. R. Survival of tubercle bacilli. Viability of dried tubercle bacilli in unfiltered room light in dark and in refrigerator. Am. Rev. Tubere., vol. 45, pp. 334-345, 1942.

7. Rhines, C. Longevity of tubercle bacilli in sewage and stream-water. Am. Rev. Tubere., vol. 31, pp. 493-497, 1935.

8. McCulloch, E. C. Disinfection and sterilization, 2nd ed., Lea and Febiger, Philadelphia, 1945.

9. Mudd, S. and Anderson, T. F. Pathogenic bacteria, rickettsias and viruses as shown by electron microscope. Their relationships to immunity and chemotherapy. Morphology. J.A.M.A., vol. 126, pp. 561-571, 1944.

10. Anderson, R. J. Chemistry of lipoids of tubercle bacilli. Physiol. Rev., vol. 12, pp. 166-189, 1932.

11. Kretschmer, O. S. The gram property of the acid-fast form of the tubercle bacillus. J. Lab. & Clin. Med., vol. 19, pp. 350-358, 1934.

12. Dubos. R. J. and Davis, B. D. Factors affecting the growth of tubercle bacilli in liquid media. J. Exper. Med., vol. 83, pp. 409-423, 1946.

13. Dubos, R. J. and Middlebrook, G. Media for tubercle bacilli. Am. Rev. Tubere., vol. 56, pp. 334-345, 1947.

14. Reed, G. B. and Rice, C. E. Influence of iron on pigmentation of acid-fast bacteria. J. Bact., vol. 17, pp. 407-411, 1929.

15. Long, E. R. and Seibert, F. B. The chemical composition of the active principle of tuberculin. I. A nonprotein medium

suitable for the production of tuberculin in large quantity. Am. Rev. Tubero., vol. 13, pp. 393-397, 1926.

16. Chargaff, E., Pangborn, M. C. and Anderson, R. J. Chemistry of lipoids of tubercle bacilli. Separation of lipoid fractions from timothy bacillus. J. Biol. Chem., vol. 90, pp. 45-55, 1931.

17. Pope, H. and Smith, D. T. Synthesis of B-complex vitamins by tubercle bacilli when grown on synthetic media. Am. Rev. Tubero., vol. 54, pp. 559-563, 1946.

18. Lesuk, A. and Anderson, R. J. The chemistry of the lipids of tubercle bacilli. LXII. Studies on mycolic acid. J. Biol. Chem., vol. 136, pp. 603-613, 1940.

19. Sabin, F. R. Cellular reactions to fractions from tubercle bacilli. Am. Rev. Tubero., vol. 44, pp. 415-423, 1941.

20. Sabin, F. R. and Joyner, A. L. Cellular reactions to defatted tubercle bacilli and their products. J. Exper. Med., vol. 68, pp. 853-868. 1938.

21. Tennent, D. M. and Watson, D. W. Molecular kinetic analyses and serological specificities of polysaccharides isolated from filtrates of the human tubercle bacillus and related species. J. Immunol., vol. 45, pp. 179-186, 1942.

22. Seibert, F. B. Chemistry of tuberculin. Chem. Rev., vol. 34, pp. 107-127, 1944.

23. Seibert, F. B., Pedersen, K. O. and Tiselius, A. Molecular weight, electrochemical and biological properties of tuberculin protein and polysaccharide molecules. J. Exper. Med., vol. 68, pp. 413-438, 1938.

24. Heilman, D. H. and Seibert, F. B. The effect of purified fractions of tuberculin on tuberculin-sensitive tissue. Quantitative studies on tissue cultures. Am. Rev. Tubero., vol. 53, pp. 71-82, 1946.

25. Tulloch, W. J., Munro, W. T., Ross, G. R. and Cumming, W. M. Bacteriological characteristics of 100 strains of tubercle bacilli isolated from sputum. Tubercle, vol. 6, pp. 18-27, 57-73, 105-123, 1924.

26. Cumming, W. M. Serology of bovine type of tubercle bacillus. Tubercle, vol. 7, pp. 105-111, 1925.

27. Wilson, G. S. Serological classification of tubercle bacilli by agglutination and absorption of agglutinins. J. Path. Bact., vol. 28, pp. 69-96, 1925.

28. Griffith, A. S. Serological classification of mammalian and avian tubercle bacilli. Tubercle, vol. 6, pp. 417-436, 1925.

29. Fürth, J. On the serological relationship of acid-fast bacteria. J. Immunol., vol. 12, pp. 273-292, 1926.

30. Kauffmann, F. Kulturelle und serologische Untersuchungen zum Zwecke der typentrennung der Tuberkelbacillen. Ztschr. f. Hyg. u. Infektionskr., vol. 114, pp. 121-123, 1932.

31. Seibert, F. B. Chemistry of proteins of acid-fast bacilli. Bact. Rev., vol. 5, pp. 69-95, 1941.

32. Freund, J. The agglutination of tubercle bacilli. Am. Rev. Tubere., vol. 12, pp. 124-141, 1925.

33. Mudd, E. B. H. and Mudd, S. Surface composition of tubercle bacillus and other acid-fast bacteria. J. Exper.Med., vol. 46, pp. 167-172, 1927.

34. Mudd, S. and Fürth, J. Antigenic properties and virulence among mammalian tubercle bacilli. J. Immunol., vol. 13, pp. 369-383, 1927.

35. Steenken, W. and Gardner, L. U. Vaccinating properties of avirulent dissociates of 5 different strains of tubercle bacilli. Yale J. Biol. & Med., vol. 15, pp. 393-402, 1943.

36. Trudeau, E. L., Baldwin, E. R. and Kinghorn, H. M. Studies on the tuberculin reaction. J. Med. Research, vol 12, pp. 169-189, 1904.

37. Zinsser, H. and Petroff, S. A. Tuberculin hypersensitiveness without infection in guinea pigs. J. Immunol., vol. 9, pp. 85-87, 1924.

38. Zinsser, H., Ward, H. K. and Jennings, F. B. Significance of bacterial allergy as sign of resistance. J. Immunol., vol. 10, pp. 719-723, 1925.

39. Petroff, S. A., Branch, A. and Steenken, W. A study of bacillus Calmette-Guerin (BCG). I. Biological characteristics, cultural "dissociation" and animal experimentation. Am. Rev. Tubere., vol. 19, pp. 9-46, 1929.

40. Opie, E. L. and Freund, J. Experimental study of protective inoculation with heat killed tubercle bacilli. J. Exper. Med., vol. 66, pp. 761-788, 1937.

41. Olson, B. J. Habel, K. and Piggott, W. R. Comparative study of live and killed vaccines in experimental tuberculosis. Preliminary note. Pub. Health Rep., vol. 62, pp. 293-296, 1947.

42. Oatway, W. H. and Steenken, W. The dissociation of tubercle bacilli. Am. Rev. Tubere., vol. 35, pp. 354-364, 1937.

43. Petroff, S. A. Microbic dissociation: The tubercle bacillus. Proc. Soc. Exper. Biol. & Med., vol. 24, pp. 632-634, 1927.

44. Petroff, S. A. Microbic dissociation. II. The tubercle bacillus. Proc. Soc. Exper. Biol. & Med., vol. 24, pp. 956-958, 1927.

45. Petroff, S. A., Branch, A. and Steenken, W. Microbic dissociation. III. BCG (Bacillus Calmette-Guerin). Proc. Soc. Exper. Biol. & Med., vol. 25, pp. 14-16, 1927.

46. Calmette, A. Preventive vaccination against tuberculosis with BCG and the Lübeck casualties. J.A.M.A., vol. 96, pp. 58-59, 1931.

47. Petroff, S. A. Dr. Petroff's reply to Dr. Calmette. J.A.M.A., vol. 96, pp. 59-60, 1931.

48. Petroff, S. A. and Steenken, W. Biological studies of the tubercle bacillus. I. Instability of the organism: microbic dissociation. J. Exper. Med., vol. 51, pp. 831-845, 1930.

49. Petroff, S. A. Report of research and clinical laboratory. Forty-Seventh Annual Medical Report of the Trudeau Sanatorium, 1931.

50. Miller, F. R. Non-acid-fast tubercle bacilli. Science, vol. 74, pp. 343-344, 1931.

51. Winn, W. A. and Petroff, S. A. Biological studies of the tubercle bacillus. II. A new conception of the pathology of avian tuberculosis with special reference to the disease produced by dissociated variants. J. Exper. Med., vol. 57, pp. 239-264, 1933.

52. Steenken, W., Oatway, W. H. and Petroff, S. A. Biological studies of the tubercle bacillus. III. Dissociation and pathogenicity of the R and S variants of the human tubercle bacillus (H37). J. Exper. Med., vol. 60, pp. 515-540, 1934.

53. Alexander, E. G. A method for obtaining stable S colonies of human tubercle bacilli. Proc. Soc. Exper. Biol. & Med., vol. 31, pp. 1101-1102, 1934.

54. Alexander, E. G. Developmental morphology of human and avian tubercle bacilli on Bordet-Gengou medium. Proc. Soc. Exper. Biol. & Med., vol. 31, pp. 1103-1104, 1934.

55. Tarshis, M. S. and Frisch, A. W. The use of simple human blood media for the bacteriologic diagnosis of tuberculosis. To be published.

56. Steenken, W. The influence of the pH on dissociation of B. Friedlander and M. tuberculosis. J. Infec. Dis., vol. 56, pp. 273-276, 1935.

57. Smithburn, K. C. The colony morphology of tubercle bacilli. I. The presence of smooth colonies in strains recently isolated from sources other than sputum. J. Exper. Med., vol. 61, pp. 395-410, 1935.

58. Smithburn, K. C. The colony morphology of tubercle bacilli. II. Influence of pH of culture medium on colony morphology and virulence. Proc. Soc. Exper. Biol. & Med., vol. 32, pp. 1336-1338, 1935.

59. Petroff, S. A. and Steenken, W. Biological studies of saprophytic acid-fast organisms. I. Dissociation of Mycobacterium phlei. J. Infec. Dis., vol. 56, pp. 277-287, 1935.

60. Ostwey, W. H. and Steenken, W. The pathogenesis and fate of tubercle bacilli produced by dissociated variants of tubercle bacilli. J. Infec. Dis., vol. 59, pp. 306-325, 1936.

61. Ostwey, W. H. and Steenken, W. The dissociation of tubercle bacilli. Am. Rev. Tubero., vol. 35, pp. 354-364, 1937.

62. Steenken, W. Effect of egg oil on tubercle bacilli. A study on the growth and macroscopical appearance of tubercle bacilli variants of H37, R_1 and undissociated strains. Am. Rev. Tubero., vol. 42, pp. 1-4, 1940.

63. Steenken, W. and Gardner, L. U. Vaccinating properties of avirulent dissociates of five different strains of tubercle bacilli. Yale J. Biol. & Med., vol. 15, pp. 393-402, 1943.

64. Middlebrook, G., Dubos, R. J. and Pierce, C. Virulence and morphological characteristics of mammalian tubercle bacilli. J. Exp. Med., vol. 86, pp. 175-184, 1947.

65. Larmela, E. On the pathogenicity and changeability of strains of Mycobacterium tuberculosis. Acta. tubero. Scandinav., vol. 21, pp. 292-322, 1947.

66. Maher, S. J. The origin of the tubercle bacillus. Am. Rev. Tubero., vol. 12, pp. 365-370, 1925.

67. Calmette, A. The question of the transmutation of tubercle bacilli and paratubercle bacilli. Am. Rev. Tubero., vol. 12, pp. 355-364, 1925.

68. Stockwell, W. M. Observations concerning the peculiar behavior of certain tubercle bacilli. Am. Rev. Tubero., vol. 12, pp. 371-378, 1925.

69. Sweany, H. C. The regeneration of acid-fastness by animal passage. Proc. Soc. Exper. Biol. & Med., vol. 25, pp. 452-454, 1928.

70. Sweany, H. C. The granules of the tubercle bacillus. Am. Rev. Tubere., vol. 17, pp. 53-76, 1928.

71. Kahn, M. C. A developmental cycle of the tubercle bacillus as revealed by single cell studies. Am. Rev. Tubere., vol. 20, pp. 150-200, 1929.

72. Kahn, M. C. and Monides, J. F. Non-acid-fast rods and granules in vertical sections of Mycobacterium tuberculosis colonies. Proc. Soc. Exper. Biol. & Med., vol. 30, pp. 577-582, 1933.

73. Dreyer, G. and Vollum, R. L. Bacillemia in experimental tuberculosis, together with experiments on mutation and pathogenicity of a human strain of B. tuberculosis. Lancet, vol. 220, pp. 1015-1021, 1931.

74. Wherry, W. B. Some chemical conditions influencing acid-proofness and non-acid-proofness in a saprophytic culture of B. tuberculosis. J. Infec. Dis., vol. 13, pp. 144-154, 1913.

75. Yegian, D. and Porter, K. R. Some artifacts encountered in stained preparations of tubercle bacilli. I. Non-acid-fast forms arising from mechanical treatment. J. Bact., vol. 48, pp. 83-91, 1944.

76. Much, H. Ueber die granuläre nach Ziehl nicht färbbare Form des Tuberkulose virus. Beitr. z. Klin. d. Tuberk., vol. 8, pp. 85-99, 1907.

77. Much, H. Die nach Ziehl nicht darstellbaren Formen des Tuberkelbacillus. Berl. klin. Wchnschr., vol. 45, pp. 691-694, 1908.

78. Kahn, M. Growth cycle of human tubercle bacillus as determined by single cell studies. Tubercle, vol. 11, pp. 202-217, 1930.

79. Pinner, M. and Voldrich, M. The disease caused by filtrates of tubercle bacillus cultures. Its alleged relation to filterable forms of tubercle bacilli. Am. Rev. Tubere., vol. 24, pp. 73-94, 1931.

80. Soltys, M. A. and Taylor, A. W. Filtration of Mycobacterium tuberculosis and Mycobacterium stercusis through gradocol membranes. J. Path. Bact., vol. 56, pp. 173-180, 1944.

81. Pierce, C., Dubos, R. J. and Middlebrook, G. Infection of mice with mammalian tubercle bacilli grown in tween-albumin liquid medium. J. Exper. Med., vol. 86, pp. 159-174, 1947.

82. Pinner, M. Pulmonary tuberculosis in the adult, 1st ed., Charles C. Thomas, Springfield, 1946.

83. Miller, J. The histogenesis of the tubercle. J. Path. Bact., vol. 10, pp. 1-49, 1905.

84. Krause, A. K. Studies on tuberculous infection. Am. Rev. tubere., vol. 14, pp. 271-305, 1926.

85. Freund, J. and Angevine, D. M. The spread of tubercle bacilli in the bodies of sensitized and immunized animals. J. Immunol., vol. 35, pp. 271-288, 1938.

86. Lurie, M. B. Studies on the mechanism of immunity in tuberculosis. The fate of tubercle bacilli ingested by mononuclear phagocytes derived from normal and immunized animals. J. Exper. Med., vol. 75, pp. 247-268, 1942.

87. Zacks, D. and Sartwell, P. E. Development of tuberculosis and changes in sensitivity to tuberculin in an institution for the feeble-minded. Am. J. Pub. Health, vol. 32, pp. 732-738, 1942.

88. Well, C. W., Flakiff, E. W. and Smith, H. H. Results obtained in man with use of a vaccine of heat-killed tubercle bacilli. Am. J. Hyg., vol. 40, pp. 116-126, 1944.

89. Rosenthal, S. R., Blahd, M. and Leslie, E. I. Ten years' experience with BCG (experimental and clinical). J. Pediat., vol. 26, pp. 470-480, 1945.

90. Levine, J. I. and Sackett, M. F. Results of BCG immunization in New York City. Am. Rev. Tubere., vol. 53, pp. 517-531, 1946.

91. Aronson, J. D. and Palmer, C. E. Experience with BCG vaccine in the control of tuberculosis among North American Indians. Pub. Health Rep., vol. 61, pp. 802-820, 1946.

92. Ferguson, R. G. BCG vaccination in hospitals and sanatoria of Saskatchewan. Am. Rev. Tubere., vol. 54, pp. 325-339, 1946.

93. Holm, J. BCG vaccination in Denmark. Pub. Health Rep., vol. 61, pp. 1298-1315, 1946.

94. Jensen, K. A. Practice of the Calmette vaccination. Acta. Tubere. Scandinav., vol. 20, pp. 1-45, 1946.

95. Birkhaug, K. BCG vaccination in Scandinavia. Twenty years of uninterrupted vaccination against tuberculosis. Am. Rev. Tubere., vol. 55, pp. 234-249, 1947.

96. Hilleboe, H. E. BCG vaccination. Plans of the United States Public Health Service. Am. Rev. Tubere., vol. 55, pp. 294-296, 1947.

97. Levine, M. I. Evaluation of BCG in prevention of tuberculosis in infants and children. Am. J. Pub. Health, vol. 37, pp. 1089-1096, 1947.

98. American Trudeau Society. Present policy of American Trudeau Society on BCG vaccination. Report of committee on chemotherapy. Am. Rev. Tubero., vol. 57, pp. 543-545. 1948.

99. Bøe, J. Variations in virulence of BCG. Acta. tubero. Scandinav., vol. 22, pp. 125-133, 1948.

100. Rosenthal, S. R. BCG vaccination and tuberculosis control in hospitals. Am. J. Nursing, vol. 98, pp. 783-786, 1948.

101. Rosenthal, S. R. Vaccination against tuberculosis by BCG (bacillus of Calmette and Guerin). Am. Pract., vol. 2, pp. 462-466, 1948.

102. Rosenthal, S. R., Leslie, E. I. and Loewinsohn, E. BCG vaccination in all age groups. Methods and results of strictly controlled study. J.A.M.A., vol. 136, pp. 73-79, 1948.

103. Seminar on BCG. Am. Rev. Tubero., vol. 57, pp. 95-113, 1948.

104. Weber, F. J. Further study of BCG vaccination. Pub. Health Rep., vol. 63, pp. 593-594, 1948.

105. Wallgran, A. BCG inoculation and BCG vaccination. Am. J. Dis. Child., vol. 76, pp. 485-491, 1948.

106. Jacox, R. F. and Meade, G. M. Variation in the duration of tuberculin skin sensitivity produced by 2 strains of BCG. Am. Rev. Tubero., vol. 60, pp. 541-546, 1949.

107. Opie, E. L. and Freund, J. An experimental study of protective inoculation with heat killed tubercle bacilli. J. Exper. Med., vol. 66, pp. 761-788, 1937.

108. Opie, E. L., Flahiff, E. W. and Smith, H.H. Protective inoculation against human tuberculosis with heat-killed tubercle bacilli. Am. J. Hyg., vol. 29, pp. 155-164, 1939.

109. Wells, A. Q. The murine type of tubercle bacillus (the vole acid-fast bacillus). Medical Research council Special Report Series, No. 259, London, His Majesty's Stationery Office, 1946.

110. Wenkle, W. C., Loomis, R. N. and Jarboe, J. M. Chromogenic acid-fast bacilli. Their use in vaccination against tuberculosis in the guinea pig. Am. Rev. Tubero., vol. 57, pp. 385-388, 1948.

111. Koch, R. Ueber bakteriologische Forschung. Cent. f. Bakt. u. Parasit., vol. 8, pp. 563-573, 1890.

112. Mantoux, C. Intradermo-reaction de la tuberculine. Compt. rend. Acad. d. sc., vol. 147, pp. 355-357, 1908.

113. Mantoux, C. Note sur la tuberculine pour intradermo-reaction. Compt. rend. Soc. de biol., vol. 56, p. 54, 1909.

114. Moro, E. Ueber eine diagnostisch verwertbare Reaktion der Haut auf Einreibung mit Tuberkulinsalbe. Munchen. med. Wchnschr., vol. 50, pp. 216-218, 1908.

115. Von Pirquet, C. Der diagnostische Wert der kutanen Tuberkulinreaktion bei der Tuberkulose des Kindesalters auf Grund von 100 Sectionen. Wien. Klin. Wchnschr., vol. 20, pp. 1123-1128, 1907.

116. Von Pirquet, C. Die kutane Tuberkulin probe. Med. Klin., vol. 3, pp. 1197-1199, 1907.

117. Calmette, A. On the etiology and vaccination of tuberculosis. J. Roy. Inst. Pub. Health, vol. 15, pp. 129-135, 1907.

118. Vollmer, H. and Goldberger, E. W. A new tuberculin patch test. Am. J. Dis. Child., vol. 54, pp. 1019-1024, 1937.

119. Vollmer, H. and Goldberger, E. W. Old tuberculin and purified protein derivative in the tuberculin patch test: A comparative study. Am. J. Dis. Child., vol. 58, pp. 527-528, 1939.

120. Seibert, F. B., Aronson, J. D., Reichel, J., Clark. L. T. and Long, E. R. Purified protein derivative. A standardized tuberculin for uniformity in diagnosis and epidemiology. Am. Rev. Tubere., vol. 30, pp. 713-768, 1934.

121. Long, E. R. and Seibert, F. B. Further studies on purified protein derivative of tuberculin (PPD). Its diagnostic value and keeping qualities in dilutions. Am. Rev. Tubere., vol. 35, pp. 281-295, 1937.

122. Krause, A. K. Studies in immunity to tuberculosis. Experimental studies on the cutaneous reaction to tuberculo-protein. J. Med. Res., vol. 35, pp. 1-23, 1916.

123. Long, E. R. Tuberculous reinfection and the tuberculin reaction in the testicle of the tuberculous guinea pig. Am. Rev. Tubere., vol. 9, pp. 215-253, 1924.

124. Petroff, S. A. Immunological studies in tuberculosis. II. Further observations on skin hypersensitiveness in experimental tuberculosis. Tr. Nat. Tubere. Assn., pp. 244-249, 1924.

125. Baldwin, E. R. Studies in immunity to tuberculosis. Hypersusceptibility or anaphylaxis. J. Med. Res., vol. 22, pp. 189-256, 1910.

126. Long, E. R. Tuberculous reinfection and the tuberculin reaction in the testicle of the tuberculous guinea pig. Am. Rev. Tubere., vol. 9, pp. 215-263, 1924.

127. Long, E. R. and Seyfarth, M. The testicle as an indicator of allergy in the hypersensitiveness of infection and anaphylaxis. Am. Rev. Tubere., vol. 9, pp. 254-263, 1924.

128. Elder, C. and Lee, A. M. Injection of cattle with B. tuberculosis (avian) and results of subsequent tuberculin tests. J.A.V.M.A., vol. 64, pp. 440-454, 1924.

129. Calmette, A. The question of the transmutation of tubercle bacilli and paratubercle bacilli. Am. Rev. Tubere., vol. 12, pp. 355-364, 1925.

130. Crawford, A. B. Tuberculin sensitization in guinea pigs caused by various acid-fast organisms. J.A.V.M.A., vol. 69, pp. 579-588, 1926.

131. Crawford, A. B. A sensitization method of differentiating avian from mammalian tubercle bacilli. Am. Rev. Tubere., vol. 15, pp. 111-126, 1927.

132. Van Es, L. The sources of tuberculosis. J.A.V.M.A., vol. 76, pp. 396-399, 1930.

133. Frey, C. A. and Hagan, W. A. The distribution of acid-fast bacteria in soils. J. Infec. Dis., vol. 49, pp. 497-506, 1931.

134. Daines, L. L. and Austin, H. A study of so-called skin-lesion and no-visible-lesion tuberculin-reacting cattle. J.A.V.M.A., vol. 80, pp. 414-433, 1932.

135. Daines, L. L. and Austin, H. A study of so-called skin lesions of tuberculin-reacting cattle. Am. Rev. Tubere., vol. 27, pp. 600-610, 1933.

136. Branch, A. A study of acid-fast organisms other than mammalian tubercle bacilli isolated from disease in man. The occurrence of avian tubercle bacillus infection of men and the diagnosis of avirulent avian tubercle bacilli. Tubercle, vol. 14, pp. 337-353, 1933.

137. Rich, A. R. and Lewis, M. R. Mechanism of allergy in tuberculosis. Proc. Soc. Exper. Biol. & Med., vol. 25, pp. 596-598, 1928.

138. Aronson, J. D. The specific cytotoxic action of tuberculin in tissue culture. J. Exper. Med., vol. 54, pp. 387-397, 1931.

139. Rich, A. R. and Lewis, M. R. The nature of allergy in tuberculosis as revealed by tissue culture studies. Bull. Johns Hopkins Hospital, vol. 50, pp. 115-127, 1932.

140. Moen, J. K. and Swift, H. F. Tissue culture studies on bacterial hypersensitivity. I. Tuberculin sensitive tissues. J. Exper. Med., vol. 64, pp. 339-353, 1936.

141. Heilman, D. H., Feldman, W. H. and Mann, F. C. Specific cytotoxic action of tuberculin. Quantitative studies on tissue cultures. Am. Rev. Tubere., vol. 50, pp. 344-356, 1944.

142. Chase, M. W. The cellular transfer of cutaneous hypersensitivity to tuberculin. Proc. Soc. Exper. Biol. & Med., vol. 59, pp. 134-135, 1945.

143. Cummings, M. M., Hoyt, M. and Gottshall, R. Y. Passive transfer of tuberculin sensitivity in the guinea pig. Pub. Health Rep., vol. 62, pp. 994-997, 1947.

144. Stavitsky, A. B. Passive cellular transfer of the tuberculin type of hypersensitivity. Proc. Soc. Exper. Biol. & Med., vol. 67, pp. 225-227, 1948.

145. Favour, C. B. Lytic effect of bacterial products on lymphocytes of tuberculous animals. Proc. Soc. Exper. Biol. & Med., vol. 65, pp. 269-272, 1947.

146. Fremont-Smith, P. and Favour, C. B. In vitro lysis of leucocytes from tuberculous humans by tuberculoprotein. Proc. Soc. Exper. Biol. & Med., vol 67, pp. 502-504, 1948.

147. Favour, C. B. Leucocyte blockade of in vitro tuberculin cytolysis. Proc. Soc. Exper. Biol. & Med., vol. 70, pp. 369-371, 1949.

148. Miller, J. M., Favour, C. B., Wilson, B. A. and Umberger, M. A. A plasma factor responsible for in vitro lysis of leucocytes by tuberculoprotein. Proc. Soc. Exper. Biol. & Med., vol. 70, pp. 738-740, 1949.

149. Miller, J. M., Favour, C. B., Wilson, B. A. and Umberger, M. A. Nature of the plasma factor responsible for in vitro lysis of leucocytes by tuberculoprotein. Proc. Soc. Exper. Biol. & Med., vol. 71, pp. 287-289, 1949.

150. Favour, C. B., Harrison, B. A. and Osgood, C. K. Increased in vitro tuberculin leucocyte cytolysis following a tuberculin skin test. Proc. Soc. Exper. Biol. & Med., vol. 73, pp. 122-124, 1950.

151. Eastwood, A. and Griffith, F. The characteristics of tubercle bacilli in human bone and joint tuberculosis. J. Hyg., vol. 15, pp. 257-309, 1916.

152. Griffith, A. S. Investigations of strains of tubercle bacilli derived from sputum. Lancet, vol. 1, pp. 721-724, 1916.

153. Cobbett, L. An acid-fast bacillus obtained from a pustular eruption. Brit. Med. J., vol. 2, pp. 158-159, 1918.

154. Rabinowitsch-Kemper, L. The types of tubercle bacilli in human tuberculosis. Am. Rev. Tubere., vol. 15, pp. 225-234, 1927.

155. Pibram, E. Remarks on atypical forms of the tubercle bacillus. Am. Rev. Tubere., vol. 15, pp. 235-236, 1927.

156. Mitchell, C. A. Bovine subcutaneous tuberculosis. J.A.V.M.A., vol. 73, pp. 493-506, 1928.

157. L'Esperance, E. S. The pathogenicity of the avian tubercle bacillus. J. Immunol., vol. 16, pp. 27-36, 1929.

158. L'Esperance, E. S. Experimental inoculation of chickens with Hodgkins nodes. J. Immunol., vol. 16, pp. 37-60, 1929.

159. Branch, A. Avian tubercle bacillus infection with special reference to mammals and to man: Its reported association with Hodgkins disease. Arch. Path., vol. 12, pp. 253-274, 1931.

160. Beaven, P. W. Infantile pulmonary tuberculosis due to an unusual type of tubercle bacillus. Am. J. Dis. Child., vol. 39, pp. 1270-1278, 1930.

161. Beaven, P. W. and Bayne-Jones, S. Mycobacterium (Sp. ?), Ryan strain, isolated from pleural exudate. J. Infec. Dis., vol. 49, pp. 399-419, 1931.

162. Tarshis, M. S. Unpublished data.

163. Pinner, M. Atypical acid-fast organisms. Proc. Soc. Exper. Biol. & Med., vol. 30, pp. 214-216, 1932.

164. Pinner, M. Atypical acid-fast microorganisms. III. Chromogenic acid-fast bacilli from human beings. Am. Rev. Tubere., vol. 32, pp. 424-439, 1935.

165. Pinner, M. Atypical acid-fast microorganisms. IV. Smooth growing tubercle bacilli. Am. Rev. Tubere., vol. 32, pp. 440-445, 1935.

166. Schiff, J. and Tarshis, M. S. Streptomycin in tuberculous meningitis. Report of case with recovery observed over three years with unusual bacteriologic findings. Northwest Med., vol. 49, pp. 451-455, 1950.

167. Thomson, H. M. Studies on saprophytic acid-fast bacteria. Am. Rev. Tubere., vol. 26, pp. 162-178, 1932.

168. Schwabacher, H. The occurrence of saprophytic acid-fast bacilli in human or animal blood cultures and their isolation from water, dust and comedones. Medical Research Council. Special Report Series, No. 182, London, His Majesty's Stationery Office, 1933.

169. Cummins, S. L. and Williams, E. M. An acid-fast other than Koch's bacillus cultivated from sputum. Tubercle, vol. 15, pp. 49-53, 1933.

170. Griffith, A. S. Observations on the "M" strain of acid-fast bacilli. Tubercle, vol. 15, pp. 53-59, 1933.

171. Steenken, W. and Landau, A. Dissociation of two unusual organisms isolated from human sources. J. Infec. Dis., vol. 58, pp. 247-258, 1936.

172. Lester, V. Saprophytic acid-fast bacilli as a source of error in diagnostic work. Acta. tubere. Scandinav., vol. 13, pp. 251-265, 1939.

173. Baldwin, E. R. Nonpathogenic acid-fast bacilli. I. A case of chronic pneumonitis associated with a nonpathogenic acid-fast bacillus. Am. Rev. Tubere., vol. 45, pp. 756-759, 1942.

174. Baldwin, E. R. II. The presence of acid-fast soil bacteria on raw fruits and vegetables. Am. Rev. Tubere., vol. 45, pp. 760-761, 1942.

175. Feldman, W. H., Davies, R., Moses, H. E. and Andberg, W. An unusual mycobacterium isolated from sputum of a man suffering from pulmonary disease of long duration. Am. Rev. Tubere., vol. 48, pp. 82-93, 1943.

176. Fisher, M. W. Sensitivity of tubercle bacilli to streptomycin. An in vitro study of some factors affecting results in various test media. Am. Rev. Tubere., vol. 57, pp. 58-62, 1948.

177. Kolmer, J. A. and Boerner, F. Approved Laboratory Technic, 4th ed., D. Appleton-Century Co., New York-London, 1945.

178. Corper, H. J. and Cohn, M. L. The nutrient quality of eggs for growing tubercle bacilli. Am. J. Hyg., vol. 18, pp. 1-25, 1933.

179. Diagnostic Procedures and Reagents, 2nd ed., American Public Health Association, New York, 1945.

180. Personal Communication. Communicable Disease Center, Tuberculosis Evaluation Laboratory, U. S. Public Health Service, Atlanta, Georgia.

181. Zinsser, H. and Bayne-Jones, S. A Textbook of Bacteriology, 8th ed., D. Appleton-Century Co., New York, 1939.

182. Griffith, A. S. Atypical tubercle bacilli in human and animal tuberculosis with special reference to those occurring in lupus. Tubercle, vol. 5, 569-586, 1924.

183. Feldman, W. H. and Magath, T. B. The reliability of guinea pig inoculation in the diagnosis of tuberculosis. Am. Rev. Tubere., vol. 24, pp. 312-325, 1931.

184. Thompson, W. P. and Frobisher, M. The filterability of the tubercle bacillus. Am. Rev. Tubere., vol. 18, pp. 823-828, 1928.

185. Cooper, F. B. and Petroff, S. A. Filterable forms of the tubercle bacillus. A critical review and personal observations. J. Infec. Dis., vol. 43, pp. 200-214, 1928.

186. Sewall, H. A possible source of so-called spontaneous tuberculosis in guinea pigs. Am. Rev. Tubere., vol. 18, pp. 829-831, 1928.

187. Reimann, H. A. Further studies on B. pseudotuberculosis. Am. J. Hyg., vol. 16, pp. 206-214, 1932.

188. Corper, H. J. Discussion of the common characteristics of the acid-fast bacteria. Tuberculosis and Leprosy - the Mycobacterial Diseases. A.A.A.S. Symposium Series, Vol. I., The Science Press Printing Co., Lancaster, 1938.

189. Dienes, L. The isolation of L type cultures from bacteroides with the aid of penicillin and their reversion into the usual bacilli. J. Bact., vol. 56, pp. 445-456, 1948.

CULTURE MEDIA AND METHODS OF PREPARATION

1. Agar Agar (Dehydrated, Baltimore Biological Laboratory)[1]
   Lot No. 19-793

2. Ascitic Brain Heart Infusion

Bacto Brain Heart Infusion.....	5 ml.
Human ascitic fluid.....	0.5 ml.

   Prepare brain heart infusion as directed below. Sterilize, cool to 40 C. and add ascitic fluid.

3. Bacto-Beef Blood Serum (Dehydrated, Difco).[2]
   Lot No. B138. Control No. 359305.

4. Bacto-Beef Extract (Difco).
   Lot No. B126. Control No. 392673.

5. Bacto-Blood Agar Base (Dehydrated, Difco).
   Lot No. B45. Control No. 392858.
   Ingredients per liter.

Beef heart, infusion from.....	500 g.
Bacto-tryptose.....	10 g.
Sodium chloride.....	5 g.
Bacto-agar.....	15 g.
Final pH 6.8	

   Suspend 40 grams in 1,000 ml. of distilled water and heat to boiling to dissolve the medium completely. Sterilize in the autoclave for 15 minutes at 15 pounds pressure (121 C.).

6. Bacto-Bordet-Gengou Agar Base (Dehydrated, Difco).
   Lot. No. B48. Control No. 394699.
   Ingredients per liter.

Potato, infusion from.....	125 g.
Proteose-peptone, Difco.....	10 g.
Sodium chloride.....	5.5 g.
Bacto-agar.....	20 g.
Final pH 6.7	

   Suspend 4 grams in 100 ml. of a 1 per cent solution of glycerine in distilled water and heat to boiling to dissolve the medium completely. Sterilize in the autoclave for 15 minutes at 15 pounds pressure (121 C.)

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[1]Baltimore Biological Laboratory, Baltimore, Maryland.

[2]Difco Laboratories Incorporated, Detroit, Michigan.

7. Bacto-Brain Heart Infusion (Dehydrated, Difco).
Lot No. B37. Control No. 395944.
Ingredients per liter.

Calf brains, infusion from	200 g.
Beef heart, infusion from	250 g.
Proteose-peptone, Difco	10 g.
Bacto-dextrose	2 g.
Sodium chloride	5 g.
Disodium phosphate	2.5 g.

Final pH 7.4

Suspend 37 grams in 1,000 ml. of distilled water. Sterilize in the autoclave for 15 minutes at 15 pounds pressure (121 C.).

8. Bacto-Peptone (Dehydrated, Difco).
Lot No. B118. Control No. 393243.

9. Bacto-Proteose No. 3 Agar (Dehydrated, Difco).
Lot No. B65. Control No. 397742.
Ingredients per liter.

Proteose-peptone No. 3, Difco	20 g.
Bacto-dextrose	0.5 g.
Sodium chloride	5 g.
Disodium phosphate	5 g.
Bacto-agar	15 g.

Final pH 7.3 without enrichment.

This basal medium was used for making blood agar slants. It was modified by adding an additional 5 grams of agar agar (Baltimore Biological Laboratory Brand). Suspend 4.5 grams in 100 ml. of distilled water and heat to boiling to dissolve the medium completely. Sterilize in the autoclave for 15 minutes at 15 pounds pressure (121 C.). Cool to 45-50 C. and add enough fresh oxalated human blood to give a final concentration of 15 per cent. The blood was oxalated with a solution of 1.2 per cent ammonium and 0.8 per cent potassium oxalate, 0.1 ml. being used for each 1.0 ml. of blood.

10. Bacto-Tryptose Agar (Dehydrated, Difco).
Lot No. B64. Control No. 388646.
Ingredients per liter.

Bacto-tryptose	20 g.
Bacto-dextrose	1 g.
Bacto-agar	15 g.
Sodium chloride	5 g.

Final pH 6.9 ±

Suspend 41 grams in 1,000 ml. of distilled water and heat to boiling to dissolve the medium completely. Sterilize in the autoclave for 15 minutes at 15 pounds pressure (121 C.).

11. Corper-Cohn Glycerol-Egg Yolk Medium[178]

Fresh egg yolk	100 g.
Distilled water	33 ml.
Glycerine C. P.	4 g.

Fresh eggs are placed in 70 per cent ethyl alcohol for 10 to 15 minutes and are then wiped dry. The yolk is separated from the white and placed in a sterile beaker on a scale and the desired weight obtained. The glycerine is next weighed and then the water is added. For each 100 ml. of medium 3 ml. of a 1 per cent aqueous solution of malachite green or congo red is added (final concentration = 0.03 per cent). The components are well mixed by stirring with a sterile glass rod (care being used to prevent beating which introduces undesirable air bubbles). The mixture is next filtered through sterile gauze and dispensed in desired amounts into sterile screw cap tubes. The tubed medium is placed in a slanted position and inspissated for 1 hour at 85 C. on three successive days. The medium used in this investigation was sterilized in the autoclave for 90 minutes at approximately 100 C. This is possible on certain types of autoclaves by special valve arrangement.
Mallinckrodt Glycerine, Analytical Grade, Lot No. 5088 was used during the entire study.

12. Dubos-Davis Liquid Medium (Modified)[176]

Asparagine	1 g.
$Na_2HPO_4 \cdot 12H_2O$	6.3 g.
KH_2PO_4	1 g.
Na_3citrate$\cdot 2H_2O$	1.5 g.
$MgSO_4 \cdot 7H_2O$	0.6 g.
Tween 80	0.5 g.
Distilled water	1,000 ml.

pH was not adjusted.

After sterilizing the above solution, bovine serum albumin, fraction V[1] was added to make a final concentration of 0.2 per cent. The albumin solution was made up in about 50 ml. of the medium and Seitz filtered. It was then added to the whole medium. The control and lot numbers of the chemicals when given are listed as follows: Difco-Bacto-Asparagine (Lot No. B1144, Control No. 390195), Tween 80 (Lot No. T-2)[2], Bovine serum albumin (Control No. C-1313), other chemicals (C.P. Grade)[3].

13. Extract Agar.

Agar agar	1.5 g.
Extract broth	100 ml.

[1]Armour Laboratories, Chicago, Illinois.

[2]Atlas Powder Company, Wilmington, Delaware.

[3]Eimer and Amend, New York, N. Y.

14. Extract Broth

Bacto-peptone	1 g.
Bacto-beef extract	0.3 g.
Sodium chloride	0.5 g.
Distilled water	100 ml.

Heat to dissolve ingredients. Adjust pH to 7.0. Filter and tube. Sterilise in autoclave for 15 minutes at 15 pounds pressure (121 C.).

15. Liver Tryptose Agar

Bacto-tryptose agar	41 g.
Liver infusion broth	1,000 ml.

Prepare liver infusion broth as follows:

Pure ground beef liver (free of fascia, fat, etc.)	1 part
Distilled water	4 parts
Adjust pH to 7.2	

Heat the mixture to boiling to dissolve the medium completely. Sterilize in the autoclave for 15 minutes at 15 pounds pressure (121 C.)

16. Loeffler's Medium

Bacto-beef blood serum	75 ml.
Meat infusion broth	25 ml.
Glucose	1 g.
Glycerine	0.5 g.

Tube medium and sterilise as described in the preparation of Corper-Cohn medium.

17. Lowenstein's Medium (Modified)(180)

Salt Solution:

Monopotassium phosphate	2.4 g.
Magnesium sulphate	0.24 g.
Magnesium citrate	0.6 g.
Asparagine	3.6 g.
Glycerine (twice distilled)	12 ml.
Redistilled water	600 ml.
Potato flour	30 g.
Eggs	1 3/8 kg.
Malachite green 2% solution	20 ml.

The salt mixture is dissolved in a flask and heated for 2 hours. The next day 30 grams of potato flour are added, and the flask is heated under continual shaking, in a water-bath, until the contents are clear. The flask is then boiled for 15 minutes, after which time it is left to stand in the bath for 1 hour at 56 C. Only fresh eggs are used, and from hens fed on greens. The eggs are washed in a 5 per cent soda and soap solution for 30 minutes, and then they are placed in a receptacle under cold running

water and allowed to remain there until the water becomes perfectly clear. After this the eggs are broken into a sterile flask, shaken well and filtered through sterile gauze. Next 1 liter (1 3/8 Kg.) of eggs is mixed with the potato-salt mixture and to this is added 20 ml. of the malachite green. The mixture is then allowed to stand for 1 hour before tubing. Finally the medium is sterilized at 85-88 C. for 40 minutes. The medium should be stored in the cold, protected from drying and exposure to sunshine, and should be used fresh.

18. Meat Infusion Broth(181)

Pure ground round beef	500 g.
Bacto-peptone	20 g.
Sodium chloride	5 g.
Distilled water	1,000 ml.

Infuse beef in water for 12 to 18 hours in ice box. Skim off all fat. Boil vigorously for one-half hour. Restore volume with distilled water several times during the boiling. Filter through gauze and then through paper. Restore volume with distilled water. To each 1,000 ml. of the infusion add 20 grams of peptone and 5 grams of sodium chloride. Heat to dissolve peptone. Adjust pH to 7.6-7.8. Boil for one-half hour. Allow to cool to about 30 C. Filter through paper. Distribute in tubes. Sterilize in the autoclave for 15 minutes at 15 pounds pressure (121 C.).

19. Petragnani's Medium(179)

Milk	900 ml.
Potato flour	36 g.
Peptone	6 g.
Pieces of potato (size of an egg)	6
Eggs	24
Egg yolks	6
Glycerine	72 ml.
Aqueous malachite green (certified) 2%	60 ml.

Cut the potatoes into thin slices. Add milk, potato flour and peptone. Cook in a double boiler for 2 hours, stir continuously until mixture becomes sticky and then stir occasionally. Break eggs and egg yolks into a 2 liter flask and shake well. Add glycerine and malachite green and shake. Cool potato mixture to 45-50 C. and add the egg-glycerine-dye mixture to the potato-milk mixture. Mix well. Filter through gauze, dispense into sterile screw cap tubes, and sterilize. This medium was sterilized as described in the preparation of Corper-Cohn medium.

20. Thioglycollate Medium (Dehydrated, Baltimore Biological Laboratory). Lot No. 1656.

Infusion from 37.5 grams of meat.

Peptone, thio	1 %
Dextrose	1 %
Sodium chloride	0.5 %
Dipotassium phosphate	0.2 %
Sodium thioglycollate	0.1 %
Agar	0.05 %
Methylene blue (1:500,000)	0.0002 %

Final pH 7.2 ±

Dissolve 3.85 grams in 100 ml. of distilled water. Heat until solution boils and allow to boil about 1 minute. Dispense in screw cap tubes to a height of about 7 centimeters, and sterilize in the autoclave for 15 minutes at 15 pounds pressure (121 C.). Keep at room temperature. Lower temperatures increases the solubility of atmospheric gases and decreases the keeping quality of the anaerobiosis.

21. Youmans Medium (Modified)(176)

Asparagine	5 g.
KH_2PO_4	5 g.
Mg_3citrate$\cdot 14H_2O$	1.5 g.
K_2SO_4	0.5 g.
Glycerine	20 ml.
Distilled water	1,000 ml.

Adjust to pH 7.2 with 40 per cent NaOH.

After sterilizing the above solution, bovine serum albumin, fraction V was added to make a final concentration of 0.2 per cent. The albumin was incorporated in the same manner as described in the preparation of Dubos-Davis medium. Anhydrous magnesium citrate was used instead of the hydrated salt and the weight adjusted to equal the latter.

PART II

BLOOD MEDIA FOR THE BACTERIOLOGIC DIAGNOSIS OF TUBERCULOSIS

INTRODUCTION

During the course of these studies it was observed that a large inoculum of virulent human tubercle bacilli often grew on blood agar base, tryptose agar and Bordet-Gengou agar. This fact was investigated further and it was found that the incorporation of blood into these basal media rendered them as suitable for the cultivation of tubercle bacilli from small inocula, as the more complex standard culture media. A search of the literature revealed that blood and serum were originally employed for the culture of tubercle bacilli by Koch[1]. The use of Bordet-Gengou base containing 25 per cent horse blood had been recommended as a primary diagnostic medium by Mishulow[2]. Her results were in part confirmed by Alexander[3, 4]. Pryce[5] found blood suitable for micro-culture technics. Most recently Dunphy and Fousek[6] have favored a liquid medium containing lysed, citrated, human blood and glycerine for the rapid cultivation of tubercle bacilli. The observations herein reported indicate that satisfactory culture of M. tuberculosis from small inocula may be readily accomplished by simply using commercial basal media incorporated with 15 to 25 per cent human bank blood.

METHODS

All basal media were obtained as dehydrated preparations and reconstituted according to directions given in the eighth edition of the Difco Manual for 1948. Bordet-Gengou agar base was used in 4 gram quantities instead of 4.5 grams as recommended. Samples of human blood were either defibrinated with sterile glass beads, oxalated with a

mixture of 1.2 per cent ammonium and 0.8 per cent potassium oxalate or citrated with 2 per cent sodium salt. Anticoagulants were sterilized by Seitz filtration and 0.1 ml. was added for each 1.0 ml. of whole blood. The fresh blood was always obtained from the same donor. Specimens of bank blood contained standard A.C.D. solution as follows: Sodium citrate U.S.P. 1.33 gm., citric acid U.S.P. 0.47 gm. and dextrose U.S.P. 3.30 gm. per each 100 ml. of transfusion solution. The control media included Corper-Cohn[7], modified Petragnani[8] and modified Lowenstein[9]. All culture media were checked for sterility prior to use.

A culture of the H37Rv strain of M. tuberculosis was obtained from the Trudeau Laboratory, Trudeau, New York. For the sake of uniformity this organism was grown in Proskauer and Beck liquid synthetic medium to maintain stock cultures and subplanted to Corper-Cohn medium prior to use. To obtain dispersed growth Dubos-Davis liquid medium (formula given by Fisher)[10] was inoculated and incubated for 14 days at 37 C. Following incubation the culture was adjusted to the No. 1 tube of the McFarland[11] nephelometer standard (300,000,000 organisms per ml.). Serial dilutions using a different pipette for each dilution were then made in 0.85 per cent sodium chloride so that each 0.1 ml. of inoculum contained 3,000, 300 and 30 bacilli respectively. These estimates were verified by colony counts which ranged from 2 to 20 in the last dilution. In several experiments the wet weight quantitative planting method described by Corper and Cohn[7] was employed. Primary isolation of tubercle bacilli from 24-hour sputum specimens was accomplished by preliminary concentration with trisodium phosphate[12] followed by serial dilution in 0.85 per cent sodium chloride. Each tube was then

inoculated with 0.1 ml. of each dilution.

Following inoculation all culture tubes were incubated in an inclined position for the first 6 days, after which time they were placed in the vertical position. The tubes were incubated at 37 C. and observed daily under a bright light for 6 weeks. The day growth first appeared was recorded numerically. After 6 weeks incubation the final degree of growth was indicated from + to ++++.

EXPERIMENTAL

The initial experiments were designed to determine if the basal media alone, or with added human blood could be enhanced by the addition of glycerine, egg yolk or both. These data are presented in Table 1. Fresh, defibrinated or citrated blood incorporated into Bordet-Gengou agar or blood agar base supported the growth from small numbers of tubercle bacilli as well as the control media. In fact, tryptose agar combined with blood in other experiments was equally good. The presence of glycerine did not significantly alter this result and even appeared to be slightly inhibitory. The addition of 25 per cent egg yolk, surprisingly, completely abolished the enhancing effect of blood in all instances. On the other hand, egg yolk alone supported the growth of the larger but not the smaller inocula. This observation was extended in other studies where it was found that a minimum of 25 per cent egg yolk and tryptose agar, with or without glycerine, was required to produce growth with an inoculum of approximately 300 tubercle bacilli. In no case, however, did the nutritive value of the yolk approximate that of blood.

On human blood media the colonies of the tubercle bacillus often

TABLE 1

Growth of H37Rv on Bordet-Gengou and Blood Agar Base: The Effect of Adding Blood, Glycerine and Egg Yolk

Medium	Number of Organisms Inoculated				
	3,000	300	30	30	30
B-G	0	0	0	0	0
B-G + 5% G	0	0	0	0	0
B-G + 25% EY	23+++	41+	0	0	0
B-G + 5% G + 25% EY	37++	0	0	0	0
B-G + 15% DB	10++++	10+++	13++	14++	13++
B-G + 15% DB + 5% G	10+++	11+++	20++	20++	15++
B-G + 15% DB + 25% EY	0	0	0	0	0
B-G + 15% DB + 5% G + 25% EY.	0	0	0	0	0
B-A + 15% OB	11++	12++	20+	14+	21+
B-A + 15% OB + 5% G	16++++	16++	25++	24++	23++
B-A + 15% OB + 25% EY	0	0	0	0	0
B-A + 15% OB + 5% G + 25% EY.	0	0	0	0	0
Corper-Cohn	12+++	13+++	15++	15++	16+
Petragnani	12+++	14++	18++	18+	16++

B-G = Bordet-Gengou agar base. B-A = blood agar base.
G = glycerine. EY = egg yolk. DB = defibrinated blood.
OB = oxalated blood.
Numbers represent the day growth was first observed.
Symbols + to ++++ represent final degree of growth after 6 weeks at 37 C.

become macroscopically visible as early as 6 days, particularly if the inoculum is heavy. At first they appear as tiny, gray, glistening, pin-point colonies which are easily recognized against the dark background of the blood when viewed under a bright light. They gradually increase in size and reach maximum growth in 5 to 6 weeks. Four distinctly different macroscopic types of mature colonies of human virulent tubercle bacilli have been observed on the blood media thus far. An irregular, warty colony gradually decreasing in diameter from base to apex (Fig. 1a). The second variety was a pyramidal, irregular, warty colony with a nipple-like apex (Fig. 1b). The third was a doughnut shaped, irregular, warty, somewhat flattened colony with a distinctly crateriform center (Fig. 1c). The fourth type was a small, smooth, dry, convex colony of various geometric shapes with little tendency to obliterate the boundary lines. These colonies appeared most frequently when growth was heavy in contrast to the rough forms which were found when growth was scanty (Fig. 1d). All four types varied greatly in diameter from approximately 0.5 mm. to 10 mm. The younger ones appeared gray, but most mature colonies assumed a tannish-gray color. Some were either darker or lighter than this and others acquired a slight greenish cast in addition.

In the following experiment quantities of freshly drawn blood from a single donor were defibrinated, oxalated and citrated. Each of these was incorporated in 15 per cent concentration into Bordet-Gengou agar base. The basal medium was hydrated in 1 per cent glycerine, sterilized and cooled to 50 C. before adding the blood. One half of the medium was dispensed into sterile screw cap tubes and slanted. The other half was left in the water bath and the temperature was slowly raised to 80 C.

Figure 1

Different types of mature colonies of human virulent tubercle bacilli observed on human blood media.

a. Irregular, warty colony gradually decreasing in diameter from base to apex.

b. Pyramidal, irregular, warty colony with a nipple-like apex.

c. Doughnut shaped, irregular, warty, somewhat flattened colony with a distinctly crateriform center.

d. Small, smooth, dry, convex colony of various geometric shapes with little tendency to obliterate the boundry lines.

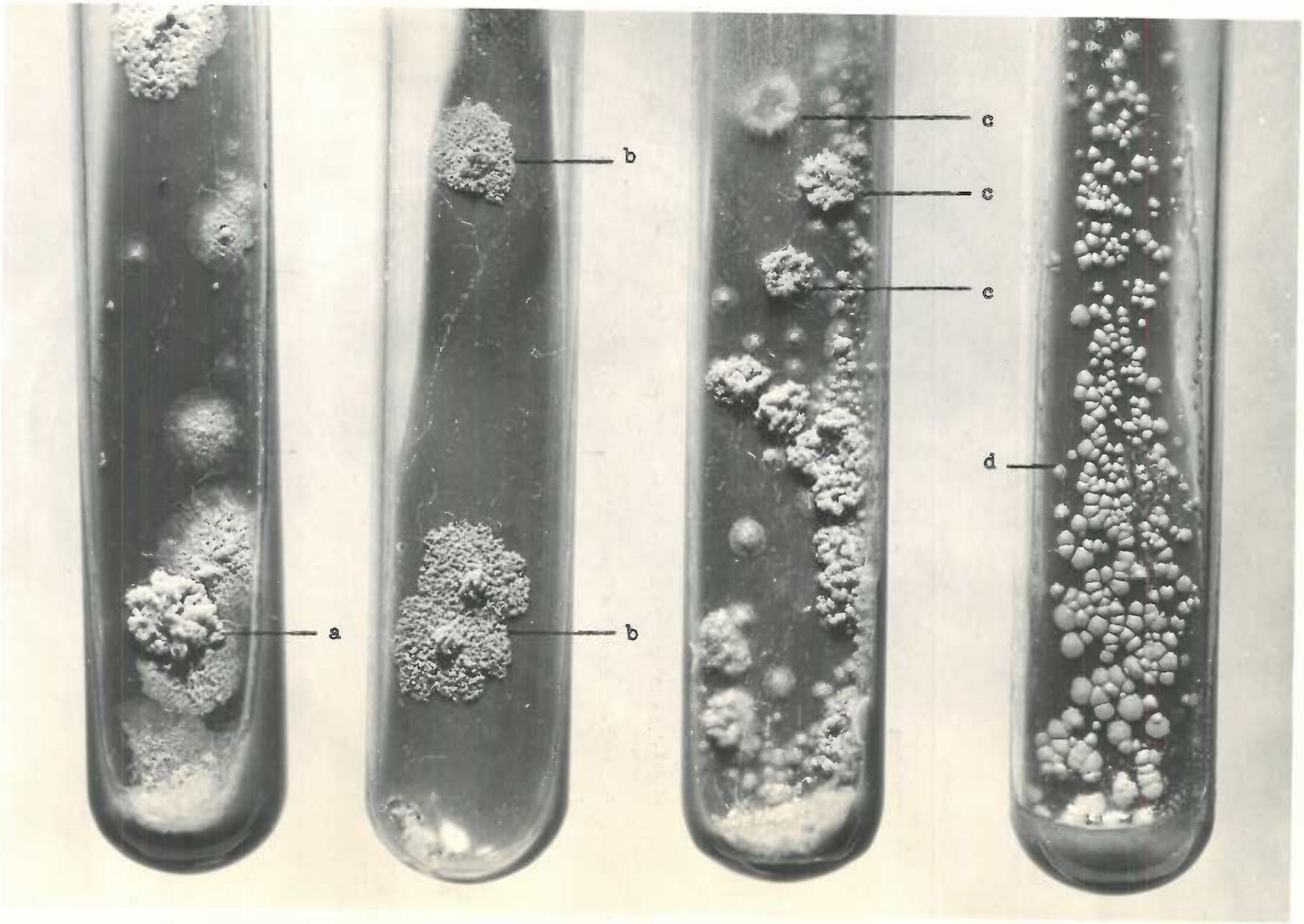

Fig. 1

When the blood assumed a chocolate color, the medium was dispensed into tubes and slanted. After checking for sterility, the media were inoculated with 300 or 30 tubercle bacilli. The results recorded in Table 2 demonstrate that the rate and final degree of growth was approximately the same regardless of the anticoagulant used. Heating of the blood, however, resulted in a slight but definite loss of nutritive value.

The observations regarding anticoagulants were repeated to determine if variations in the concentration of glycerine might influence the rate and degree of growth. The results in Table 3 indicate that 1, 3, and 5 per cent glycerine were equally effective in supporting the growth of H37Rv. Again the anticoagulants used in preparing the blood did not influence the result. Also included was a 46 day old sample of bank blood which, surprisingly, was as efficacious for growing organisms from small inocula as the freshly drawn specimens.

In view of the fact that 46 day old bank blood gave as good results as fresh blood, it appeared advisable to study a number of different bloods of varying ages. The results of such an experiment are found in Table 4 where it can be seen that bank blood kept as long as 126 days was still capable of initiating growth of as small an inoculum as 300 tubercle bacilli. The most striking results, however, were obtained with bloods ranging in age from 18 to 56 days. Of equal interest is the fact that of all samples of bank blood tested to date, none has failed to support the growth of small inocula of tubercle bacilli.

In the previous experiments blood in 15 per cent concentration had been arbitrarily selected as a standard. The following study was

TABLE 2

Growth of H37Rv on Bordet-Gengou Agar Base Containing Glycerine and Blood: The Effect of Anticoagulants and Heat

Medium B-G + 1% G + 15% Blood	Number of Organisms Inoculated 300	30	30
Unheated Blood			
Defibrinated	12+++	14++	13++
Oxalated	12+++	13++	13++
Citrated	6++++	11++	12++
Heated Blood			
Defibrinated	19+	21+	0
Oxalated	16+	0	17+
Citrated	16++	17++	17+
Corper-Cohn	15+++	16++	19+
Petragnani	13++	17++	18+

B-G = Bordet-Gengou agar base. G = glycerine.
Numbers represent the day growth was first observed.
Symbols + to ++++ represent final degree of growth after 6 weeks at 37 C.

TABLE 4

Growth of H37Rv on Bordet-Gengou Agar Base Containing Glycerine: The Effect of Adding Blood of Varying Ages

Medium B-G + 1% G + 15% BB	Number of Organisms Inoculated 300	30	30
Age of Blood in Days			
18	12+++	13+++	13++
38	12+++	15++	12++
46	12+++	12++	12++
56	13++++	13++	13++
96	15++	15+	22+
114	15++	15+	16+
124	15+++	16+	0
126	16++	0	0
Corper-Cohn	16+++	17+++	18++
Petragnani	14++	17++	17+
Lowenstein	13+++	14++	13++

B-G = Bordet-Gengou agar base. G = glycerine. BB = bank blood.
Numbers represent the day growth was first observed.
Symbols + to ++++ represent final degree of growth after 6 weeks at 37 C.

primarily designed to determine the effect of varying quantities of 2 day old bank blood on the growth of a recently isolated, virulent, strain of the human tubercle bacillus. Additional variations included 1 and 3 per cent glycerine and 0.03 per cent malachite green. The dye was incorporated into the blood medium to ascertain if the color contrast would aid in the early detection of colonies. In these experiments the quantitative wet weight planting technic[7] was used. The data recorded in Table 5 show that with the largest inoculum, the rate of growth was usually accelerated over what had been observed previously. In all instances the best results were obtained with concentrations of blood ranging from 15 to 50 per cent. The presence of malachite green proved to be inhibitory as evidenced by the delay in the appearance of colonies and the final degree of growth after 6 weeks. Glycerine in 3 per cent concentration demonstrated some growth restraining effects, particularly noticeable when smaller quantities of blood were used. The combination of malachite green and 3 per cent glycerine showed even greater inhibition than either alone. Of special interest is the fact that the inhibitory qualities of 3 per cent glycerine alone, or in combination with malachite green were partially overcome by increasing the concentration of the blood in the medium (Table 5).

The following experiment was undertaken to study the effects on growth of tubercle bacilli of varying concentrations of 2 day old bank blood and glycerine in combination with blood agar base. The results presented in Table 6 are in agreement with those given in Table 5. Here again, the presence of 15 to 50 per cent blood yielded the optimum growth of all inocula. Again glycerine in 3 and 5 per cent amounts

TABLE 5

Growth of a Recently Isolated Virulent Human Tubercle Bacillus on Bordet-Gengou Agar Base: The Effect of Adding Glycerine, Blood and Malachite Green

Bordet-Gengou Agar Base	Mg. Wet Weight 0.01	0.000,1	0.000,001
1% Glycerine			
5% BB	6++++	17++	0
10% BB	6++++	17+	19+
15% BB	6++++	12+++	17+
25% BB	6++++	12+++	16+
50% BB	6++++	10+++	12+
1% Glycerine + 0.03% MG			
5% BB	0	0	0
10% BB	13+	16+	19+
15% BB	10+	16+	19+
25% BB	6++	6+	16+
50% BB	6++	6+	16+
3% Glycerine			
5% BB	6++++	0	0
10% BB	6+++	0	0
15% BB	6++++	12+++	0
25% BB	6+++	12++	23+
50% BB	6++++	6+	23+
3% Glycerine + 0.03% MG			
5% BB	0	0	0
10% BB	0	0	0
15% BB	13+	0	0
25% BB	6++	12+	33+
50% BB	6++	16++	22+
Corper-Cohn	7+++	19+++	26+
Petragnani	10++	17++	0
Lowenstein	7+++	15+++	21++

BB = bank blood. MG = malachite green.
Numbers represent the day growth was first observed.
Symbols + to ++++ represent final degree of growth after 6 weeks at 37 C.

TABLE 6

Growth of a Recently Isolated Virulent Human Tubercle Bacillus on Blood Agar Base: The Effect of Adding Blood and Glycerine

Blood Agar Base	Mg. Wet Weight 0.01	0.000,1	0.000,001
5% BB	6+++	22+	0
10% BB	6+++	13++	36+
15% BB	6++++	13+++	20+
25% BB	6++++	13+++	15++
50% BB	6++++	12+++	13+
1% Glycerine			
5% BB	6++++	15+	33+
10% BB	6+	17+	33+
15% BB	6+++	13+	20+
25% BB	6++++	12++++	15++
50% BB	6++++	6+++	15+
3% Glycerine			
5% BB	6++	39+	0
10% BB	6++++	19+	0
15% BB	6++	23+	39+
25% BB	6+++	12+++	21+
50% BB	6++++	15+++	19+
5% Glycerine			
5% BB	12+++	20+	0
10% BB	12++++	19+	0
15% BB	7++++	13++	0
25% BB	7+++	13+++	21+
50% BB	7++++	13++++	16+
Gorper-Cohn	10++++	19+++	19++
Petragnani	11+++	17++	33+
Lowenstein	10+++	15+++	19++

BB = bank blood.
Numbers represent the day growth was first observed.
Symbols + to ++++ represent final degree of growth after 6 weeks at 37 C.

proved to be inhibitory with the lower but not with higher concentrations of blood.

In view of the fact that most of the above studies of human blood medium involved the use of the standard H37Rv strain of tubercle bacillus, it seemed desirable to determine if similar results could be obtained with other virulent strains isolated directly from sputum. For this purpose five 24-hour specimens from different patients were concentrated by the trisodium phosphate technic(12). The sediment from each was serially diluted using a different pipette for each dilution. Tubes of experimental and control media were inoculated with 0.1 ml. of each dilution and incubated in the manner previously described. The human blood medium was arbitrarily selected and consisted of Bordet-Gengou agar base containing 1 per cent glycerine and 15 per cent bank blood 14 days old. The results are presented in Table 7. It may be seen that in 17 out of 28 separate isolations the blood medium showed the earliest growth of any in the experiment. It was equal to or exceeded by the Lowenstein medium on 5 and 6 occasions respectively. In 3 instances organisms were isolated in a 10 fold greater dilution with the blood medium than with the others. Thus, the Corper-Cohn medium failed to grow tubercle bacilli 6 times out of 28 positive samples, the Petragnani 8 times and the Lowenstein medium failed on 3 occasions. The human blood medium, on the other hand, accounted for all 28 of the successful isolations. Evaluation in terms of degree of growth at the end of 6 weeks showed the blood and the Lowenstein media to be approximately equal, with the others considerably less effective.

TABLE 7

Growth of Virulent Human Tubercle Bacilli Isolated by Serial Dilution of Concentrated Sputum Specimens: Human Blood Medium Versus Corper-Cohn Petragnani and Lowenstein Media

Spec. No.	Medium	Specimen Dilutions 10^{-0}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}
1	B-G + 1%G + 15% BB..	10++++	12++++	13++	20+	21+	24+	24+	0
	Corper-Cohn.....	13++++	13+++	21+	21+	22+	0	0	0
	Petragnani.....	14++	15++	19++	20+	0	0	0	0
	Lowenstein.....	9++++	12++++	15++	15++	21++	23+	0	0
2	B-G + 1%G + 15% BB..	9++++	12++++	13+++	18++	21+	22+	27+	0
	Corper-Cohn.....	12++	18+++	21+	21+	24+	0	0	0
	Petragnani.....	10++	13+++	13++	17+	24+	0	0	0
	Lowenstein.....	12+++	13++++	13+++	16++	21+	24+	0	0
3	B-G + 1%G + 15% BB..	9++++	13+++	13+++	18+	0	0	0	0
	Corper-Cohn.....	15+++	20+++	24+	0	0	0	0	0
	Petragnani.....	14++	18++	21+	0	0	0	0	0
	Lowenstein.....	11++++	13+++	17+++	17++	0	0	0	0
4	B-G + 1%G + 15% BB..	11++++	12++++	12++	17+	0	0	0	0
	Corper-Cohn.....	13++++	15+++	22++	31+	0	0	0	0
	Petragnani.....	14++	15++	18+	18+	0	0	0	0
	Lowenstein.....	12+++	15+++	18++	18+	0	0	0	0
5	B-G + 1%G + 15% BB..	9++++	12++++	13++++	13++	24+	40+	0	0
	Corper-Cohn.....	15+++	22+++	22+	31+	37+	0	0	0
	Petragnani.....	13+++	15+++	15++	23+	0	0	0	0
	Lowenstein.....	12+++	13++++	18+++	18+++	23+	0	0	0

B-G = Bordet-Gengou agar base. G = glycerine. BB = bank blood.
Numbers represent the day growth was first observed.
Symbols + to ++++ represent final degree of growth after 6 weeks at 37 C.

DISCUSSION

The observations reported with human blood media may be coordinated with those of Mishulow[2] who also employed a Bordet-Gengou agar base and approximately 25 per cent horse blood. This medium had been previously studied by Dr. Williams who observed that it supported the growth of tubercle bacilli and some acid-fast actinomycetes. In Mishulow's experiments the number of organisms inoculated was based on crude estimates and her efforts were directed chiefly toward a differentiation of the bovine, avian and human colony types. However, she recognized the advantages of such a simply prepared medium and recommended its use for the routine isolation and differentiation of tubercle bacilli. Blood for the cultivation of tubercle bacilli, has also been employed by a number of other workers[1,3,4,5,6,13,14,15,16]. Relatively few of these were concerned with diagnostic problems but they reported good results with blood used either alone or combined with a variety of basal media. Horse[2], human[5,6,13], rabbit[15] and guinea pig[16] blood were utilized with success.

Pryce[5] observed that blood laked with saponin would grow the tubercle bacillus. This fact was investigated further by Dunphy and Fousek[6] who found that a liquid medium composed of saponin-lysed blood and glycerine yielded rapid growth of virulent organisms. Their experiments were repeated and confirmed in this laboratory. It was noted that inocula of 30 to 3,000 tubercle bacilli grew out in from 10 to 16 days. To avoid repeated microscopic examination of cultures, the liquid blood medium was incorporated into blood agar base and tryptose agar with equally good results. By this means it was possible

to observe growth macroscopically in from 12 to 16 days. The saponin laked preparations, however, proved to be no better than whole blood and were not investigated further.

The experimental data show that the concentration of blood incorporated into the basal media is a limiting factor for growth of the tubercle bacillus. A rather wide range of values proved to be suitable; in fact, as little as 5 per cent supported the growth of some but not all inocula. Elevation to 50 per cent, while giving excellent results, did not lend itself as well for the detection of colonies during the early phases of growth. Approximately 25 per cent of blood appeared to give optimal results. The method of preparing the blood, such as defibrinating, citrating or oxalating did not influence the sensitivity of the medium nor was evidence obtained that any of the anticoagulants enhanced growth. Similar observations were reported by Pryce(5) who favored the use of lysed blood. Heating of the blood resulted in an inhibitory effect which was slight but definite in character.

No evidence was obtained that old blood was either superior or inferior to freshly drawn blood. Of particular importance was the fact that bank blood as old as 126 days was still capable of initiating growth of small inocula. It appears that some loss in nutritive value of the blood occurs on standing but this is not evident for as long as 56 days. The growth supporting qualities of samples of blood from 10 separate donors remained remarkably homogeneous, although the possibility of encountering inhibitory specimens cannot, as yet, be eliminated. It was also interesting to have determined that tryptose agar, Bordet-Gengou and blood agar base combined with blood gave

comparable results. Other basal media may prove to be equally satisfactory but this problem requires further investigation.

The failure of an analytical grade of glycerine to enhance the growth of small inocula of tubercle bacilli was an unexpected result. In some cases actual inhibition was demonstrated, particularly with the higher concentrations. This effect was not always reproducible and and when present, was in part overcome by increasing the quantity of blood. Furthermore, equally good results were obtained in the absence of glycerine but 1 per cent was added to the blood media to encourage the growth of glycerophylic strains and to prevent loss of moisture. The use of malachite green did not offer any advantage for the early recognition of colonies. The inhibitory effect of the dye was, in part, overcome by increasing the concentration of blood. Bacteriostatic agents have not, as yet, been incorporated into the media with the idea of preventing contamination. Undoubtedly this could be accomplished but only after performing experiments designed to accurately measure the effect of such agents on the growth of small inocula. Perhaps the use of the trisodium phosphate technic[12] may destroy contaminants sufficiently to eliminate the necessity of using inhibitory substances. In any event, this problem requires further study.

In this investigation three types of planting technics were utilized. The turbidimetric method took advantage of the fact that dispersed growth of uniform density can now be obtained in Dubos-Davis medium. The smallest inoculum representing a 30 million fold dilution of the original suspension yielded consistent results which were verified by colony counts within the limits of experimental error. The wet weight quantitative planting technic described by Corper and Cohn[7]

was included for comparison, and the results of the two were in agreement. The third method was based on ten fold serial dilutions to the limits at which growth of tubercle bacilli could be obtained from concentrated sputum sediments when the experiment was compared with three control media under identical conditions. Here too, the data favored the human blood media where growth of tubercle bacilli was obtained in a ten fold greater dilution in three instances.

SUMMARY

1. In the experiments described it has been demonstrated that human blood media support the growth of very small inocula of tubercle bacilli. When comparisons with three standard tuberculosis media were made, the blood-containing media proved to be equal to and in many cases superior to the others from the standpoint of early detection and final degree of growth. These results were based upon experiments not only with a standard laboratory strain of tubercle bacillus (H37Rv) but also with other virulent human strains isolated directly from sputum under routine diagnostic conditions.

2. Whenever blood was present in adequate concentrations (15 to 50 per cent) the growth of small inocula of tubercle bacilli regularly occurred. This fact proved to be true regardless of the basal medium used, the presence or absence of glycerine, egg yolk or a combination of both. Actually most of these substances showed some degree of inhibition of growth when they were incorporated into the blood media, particularly when the concentration of blood was below 15 per cent.

3. It was also found that fresh defibrinated, citrated, oxalated and even 56 day old outdated bank blood were equally effective in

supporting the growth from small inocula of tubercle bacilli. Heating the blood, however, resulted in a slight but definite loss in nutritive value.

4. The blood media herein described should be thoroughly investigated for their diagnostic potentialities. For this purpose either Bordet-Gengou agar or blood agar base hydrated with 1 per cent glycerine and containing 25 per cent bank blood is recommended. The chief advantages of such media are their economy, simplicity of preparation and ability to grow tubercle bacilli from small inocula easily, recognizably and in a short period of time. Both the dehydrated basal media and the outdated bank blood are readily obtainable and small quantities of fresh media may be quickly prepared. All these factors should encourage a wider use of cultural technics for the isolation of tubercle bacilli.

5. Finally, the blood media may prove particularly suitable for measuring the sensitivity of tubercle bacilli to antibiotic and chemotherapeutic agents, an important procedure in the field of therapy.

BIBLIOGRAPHY

1. Koch, R. Die Aetiologie der Tuberculose. Berl. klin. Wchnschr., vol. 19, pp. 221-230, 1882.

2. Mischulow, L. Isolation and differentiation of tubercle bacilli on the Bordet-Gengou and Lowenstein mediums. J. Infec. Dis., vol. 51, pp. 416-427, 1932.

3. Alexander, E. G. A method for obtaining stable S colonies of human tubercle bacilli. Proc. Soc. Exper. Biol. & Med., vol. 31, pp. 1101-1102, 1934.

4. Alexander, E. G. Developmental morphology of human and avian tubercle bacilli on Bordet-Gengou medium. Proc. Soc. Exper. Biol. & Med., vol. 31, pp. 1103-1104, 1934.

5. Pryce, D. M. Sputum film cultures of tubercle bacilli: A method for the early observation of growth. J. Path. Bact., vol. 53, pp. 327-334, 1941.

6. Dunphy, D. L. and Fousek, M. D. A simple medium for growth of tubercle bacilli. Yale J. Biol. & Med., vol. 19, pp. 1013-1015, 1947.

7. Corper, H. J. and Cohn, M. L. The nutrient quality of eggs for growing tubercle bacilli. Am. J. Hyg., vol. 18, pp. 1-25, 1933.

8. Diagnostic Procedures and Reagents, 2nd ed., American Public Health Association, New York, 1945.

9. Personal Communication. Communicable Disease Center, Tuberculosis Evaluation Laboratory, U. S. Public Health Service, Atlanta, Georgia.

10. Fisher, M. W. Sensitivity of tubercle bacilli to streptomycin: An in vitro study of some factors affecting results in various test media. Am. Rev. Tubere., vol. 57, pp. 58-62, 1948.

11. Kolmer, J. A. and Boerner, F. Approved Laboratory Technic, 4th ed., D. Appleton-Century Co., New York-London, 1945.

12. Tarshis, M. S. and Lewis, W. G. Use of clorox and trisodium phosphate in demonstration of acid-fast bacilli in sputum. Am. J. Clin. Path., vol. 19, pp. 688-692, 1949.

13. Wright, A. E. New methods for the study of the pathology and treatment of tuberculous disease. Lancet, vol. 1, pp. 218-221, 1924.

14. Clough, M. G. The cultivation of tubercle bacilli from the circulating blood in miliary tuberculosis. Am. Rev. Tubere., vol. 1, pp. 598-621, 1917-18.

15. Wherry, W. B. and Ervin, D. M. The necessity of carbon dioxide for the growth of B. tuberculosis. J. Infec. Dis., vol. 22, pp. 194-197, 1918.

16. Hesse, E., Meissner, G. and Quast, G. Studien zur Chemotherapie der Tuberkulose. Arch. exper. Path. und Pharmakol., vol. 135, pp. 82-110, 1928.

ADDENDUM

The following protocols contain data which have been referred to in Part II of this investigation and these are included for completeness.

TABLE 1

Growth of H37Rv on Tryptose Agar: The Effect of Adding Glycerine and Egg Yolk

Medium	Number of Organisms Inoculated				
	3,000	300	30	30	30
TA	0	0	0	0	0
TA + 5% G	0	0	0	0	0
TA + 25% EY	31++	0	0	0	0
TA + 5% G + 25% EY	30++	0	0	0	0
Corper-Cohn	13+++	15++	15+	0	17+
Petragnani	14++	14++	15+	17+	18+

TA = tryptose agar. G = glycerine. EY = egg yolk.
Numbers represent the day growth was first observed.
Symbols + to +++ represent final degree of growth after 6 weeks at 37 C.

TABLE 2

Growth of H37Rv on Tryptose Agar: The Effect of Adding Glycerine and Varying Concentrations of Egg Yolk

Medium	Number of Organisms Inoculated					
	300	300	300	300	300	300
	Concentration of Egg Yolk					
	15%	20%	25%	30%	40%	50%
TA.....	0	0	31+	37++	27++	26++
TA + 5% G.....	0	0	29++	35++	26++	26++
Corper-Cohn.....	16++	15+++	14++	17++	11++	13+++
Petragnani.....	12++	11+++	15++	12++	18++	14+++

TA = tryptose agar. G = glycerine.
Numbers represent the day growth was first observed.
Symbols + to +++ represent final degree of growth after 6 weeks at 37 C.

TABLE 3

Growth of H37Rv on Dunphy-Fousek Liquid Blood-Glycerine Medium

Medium	Number of Organisms Inoculated				
	3,000	300	30	30	30
Dunphy-Fousek.....	10	12	17	14	16
Corper-Cohn.....	12+++	13+++	16++	14++	14++
Petragnani.....	10+++	11+++	14++	13++	17++

Numbers represent the day growth was first observed.
Symbols ++ to +++ represent final degree of growth after 6 weeks at 37 C.
Since growth cannot be observed macroscopically in the Dunphy-Fousek medium, the degree of growth is not recorded.
The medium was prepared exactly as described by the authors(6).

TABLE 4

Growth of H37Rv on Dunphy-Fousek Liquid Blood-Glycerine Medium Incorporated in Tryptose Agar

Medium	Number of Organisms Inoculated				
	3,000	300	30	30	30
TA + D-F.....	12+++	14+++	19+	17++	16+
Corper-Cohn.....	13+++	15+++	16+	19+	16++
Petragnani.....	12+++	12+++	18+	15++	17++

TA = tryptose agar. D-F = Dunphy-Fousek medium.
Numbers represent the day growth was first observed.
Symbols + to +++ represent final degree of growth after 6 weeks at 37 C.
The blood medium was combined with equal quantities of tryptose agar.

TABLE 5

Growth of H37Rv on Dunphy-Fousek Liquid Blood-Glycerine Medium Combined with Solid Blood Media

Medium	Number of Organisms Inoculated				
	3,000	300	30	30	30
B-G + 5% G + 15% BB + D-F..	10++++	15++++	15++++	17+++	15+++
B-A + 5% G + 15% BB + D-F..	10++++	16++++	15++++	16+++	16+++
TA + 5% G + 15% BB + D-F...	11++++	14++++	16+++	15+++	15+++
Corper-Cohn.....	12+++	15+++	17++	16++	17++
Petragnani.....	11+++	16++	19++	17++	18++

B-G = Bordet-Gengou agar base. B-A = blood agar base. G = glycerine.
BB = bank blood (47 days old). D-F = Dunphy-Fousek medium.
Numbers represent the day growth was first observed.
Symbols ++ to ++++ represent final degree of growth after 6 weeks at 37 C.
The solid blood media were prepared first, tubed and solidified.
Dunphy-Fousek liquid medium was then added to each tube in 5 ml. amounts.