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A METHOD FOR THE RAPID ISOLATION
OF
MYCOBACTERIUM TUBERCULOSIS

by

Roma Z. Hawirko

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Department of Bacteriology
McGill University,
Montreal, Canada

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I

Introduction

A. Introduction to the Problem

The demonstration of the tubercle bacillus by Robert Koch in 1882, as the etiological agent of tuberculosis led to developments in technique for the isolation of the bacilli from suspected material. Because of the scarcity of organisms in some specimens, concentration methods were devised to give a more delicate demonstration of the bacilli than was possible by direct smear or culture. The increasing importance of bacteriological methods for diagnosis in many cases of suspected tuberculosis has given a stimulus to the study of such procedures. In spite of the voluminous literature that has been published there is still a need for a more satisfactory method for the demonstration of the presence of the tubercle bacilli in pathological specimens. The development of nutritive media has made great progress since the time of Koch, but there still remains much to be desired from the point of view of time required for the appearance of colonies and the initiation of growth from minimal inocula.

A concentration method based on the flotation principle of Lange and Nitsche (1909) utilizing oil as the agent, and the medium described by Dubos (1945), using Tween and albumin were investigated for this problem, with the view of achieving a more satisfactory method for the demonstration of the tubercle bacillus.

B. Historical

Tuberculosis is a disease which antedates history. Important data accumulated during the twentieth century by Wood James, Eliot Smith, Armand Ruffles and others has revealed that tuberculosis was prevalent among the ancient Egyptians and the Pharoahs 6000 years ago. Psoas abscesses and tuberculous osteomyelitis have been found in Egyptian mummies (Ruffer) dating from the eleventh to the twenty first dynasty.

The first account of the disease is found in the Laws of Manu of India dating back to 1300 B.C. Puccinatti, in his history of medicine tells us, according to these laws pulmonary consumption was considered to be an unclean and incurable disease. Aristotle believed that the disease was contagious. Hippocrates regarded it as a suppurative process of the lungs. This concept was held by Areteus and Celsus who described its clinical phases. Galen considered it to be an ulcerative process and recommended a dry climate.

In the middle ages, Fracastorius (1478-1553) arrived at some remarkably accurate conclusions concerning the infectious nature of tuberculosis and its modes of transmission.

Not until the middle of the seventeenth century when the importance of anatomy first gained recognition and frequent autopsies were made did the knowledge of tuberculosis make any notable advance. Nodules were discovered in the lung and the genetic relationship between these and pulmonary phthisis was first maintained by Sylvius (1614-1672). He first used the

term tubercles (tubercula minora vel majora) to indicate the appearance of lesions found in the lungs of consumptives.

Morgagni (1682-1771) refused to do autopsies on consumptives because he considered the disease to be very contagious. So strongly did he and his teacher Valsova impress their teachings on the people of Italy, that isolation and segregation of tuberculous individuals were brought about. Thus for the first time in the history of medicine the disease was notifiable to the state.

Bayle (1774-1816) was the creator of the modern concept of tuberculosis as a morbid process. His work, not quite complete, was advanced through the masterly observation of his friend Laennec. Laennec (1781-1826) a distinguished and important internist of the early French school, was the first to describe the anatomical tubercle. He held the opinion that the tubercle is really a disease and that consumption is tubercle of the lungs; that, whenever tubercles occur, the disease is tuberculosis. Thereby he unified many conditions previously believed to be different. He described some of the macroscopic aspects of tuberculous lesions, stating that the lesions can occur both as isolated "follicular" (miliary tubercles) and as "infiltrative" forms (exudative tubercles). Laennec is justly regarded as a great pioneer in the history of tuberculosis and his description of the appearance and cause of the tuberculous process has become classic. His doctrines, revolutionary as they were for the time became the source of an enormous amount

of controversy but proved in the end to be correct.

Vichow (1850) directed attention to the pathognomic sign of the tubercle but regarded caseation as a non specific process. Experimental proof as to the etiology of tuberculosis was first successfully demonstrated by Klencke (1843). He infected rabbits with tuberculosis tissue and produced the disease by intravenous inoculation. His work while taking priority over that of Villemin, did not make an impression and remained without influence on the knowledge of the etiology of tuberculosis. J. A. Villemin (1827-1892) made his first important communication in 1865 on the cause and nature of tuberculosis and its inoculation from man to rabbit. His ingenious and broadly conceived experiments had a far reaching result and raised a storm in the medical world at the time of their publication. He inoculated rabbits with pus taken from tubercles of phthisical individuals and found that they regularly developed tubercles in the lungs. This brought him to the conclusion that phthisis was a specific disease, virulent in type and produced by an inoculable agent. His work was confirmed by Cohnheim and Salmonsens (1877). With these investigations, the belief in a specific virus for tuberculosis grew and a search for the specific agent began.

It was reserved for Robert Koch to bring the problem of the nature of tuberculosis to a final solution. On the 24th of March 1882, before the Physiological Society in Berlin, Robert Koch announced his discovery of the tubercle bacillus. In that epoch making communication he emphasized the long period

required for the tubercle colonies to become visible in contrast to the day or two required for colony formation of other organisms recognized at that time.

Koch succeeded in cultivating the tubercle bacillus when he applied coagulated serum media devised by John Tyndall, an English physicist. On the sixteenth day on the surface of the tubes he noticed many pin point white specks which under the magnifying glass appeared to be dry and wrinkled masses. The essential features of Koch's original publication were: proof of the constant presence of the bacillus, its isolation in pure cultures and the production of tubercles by the application of these pure cultures to the bodies of healthy animals. This classical demonstration was fortified and absolutely corroborated by numerous control experiments which were everywhere instituted. Koch's discovery was at once confirmed all over the world and it stamped him as one of the great investigators of his time. The question of the etiology of tuberculosis was finally settled; tuberculosis was classified among the infectious diseases and the tubercle bacillus was recognized as the sole cause of the disease.

The discovery of the tubercle bacillus was only made possible because Koch developed a method to stain it in the tissues and in other tuberculous material. He used an alkaline methylene blue which penetrated into the bacillus. An improved stain was devised immediately by Ehrlich (1882) and

was accepted by Koch as superior to his own. Ehrlich applied the fundamental acid fast property of the tubercle bacillus, staining it with basic dyes in the presence of aniline oil which retained the stain after treatment with strong nitric acid. The method currently used was adapted by Ziehl (1857-1926) who used carbolic acid instead of aniline oil and modified by Neelsen (1854-1894) with sulphuric acid and carbol fuchsin. The whole merit of staining the tubercle bacilli as used today is however due to Ehrlich.

The isolation and cultivation of the tubercle bacillus was repeated by others. It was a very slow procedure and by the time the growth appeared the medium on which the inoculation was made, dried out completely and became useless. To overcome this Nocard (1885) added a small amount of glycerine to the culture medium. It proved to be one of the most important accessory substances ever introduced in bacteriology. In the serum media to which he had added the glycerine he noticed the growth was profuse, while in the tubes without glycerine, the growth was scanty.

Nocard and Roux were probably the first to use glycerin beef broth to cultivate the organism. The introduction of this medium proved very useful later in the study of the metabolism of the tubercle bacillus. The successful cultivation on fluid media depends chiefly on the propagation of the organism on the surface of the fluid used. This was accomplished by floating the inoculum on the surface.

Pawlowsky (1888) introduced the glycerinated potato.

Dorset introduced the egg medium which is unquestionably the most reliable and easy to prepare. Long and Seibert (1926) described a synthetic medium which has been used to great advantage in the study of the chemistry of the tubercle bacillus.

There have been many improvements in the use of new media. Their principal ingredient is usually egg, (Petroff 1913 and Hohn 1926) or potato (Lowenstein 1931 and Corper 1928). Some authors find benefit in the addition of milk (Petragnani 1926), cream (Sweany 1928) or hematin (Hohn 1926). Petroff (1915) introduced the use of the property of bacteriostatic dyes to inhibit contaminants put in at the inoculation of the media. Petroff (1915) recommended gentian violet, Corper (1928) crystal violet and Petragnani (1926) malachite green. A most notable advance was made recently by Dubos (1945) with the description of an albumin tween liquid medium designed to produce submerged and diffuse growth of the tubercle bacillus.

The outstanding advantage of the cultural method as compared to animal inoculation is that less time is required for diagnosis. The principal advantage of the animal experiment is that it provides at the same time a virulence test of the bacilli.

Theobald Smith (1896) gave proof that the etiological agent of bovine tuberculosis was different from that of human tuberculosis. Rivalta (1899) and Maffucci (1890) discovered the related organism of avian tuberculosis.

C. A Review of Concentration Methods

The isolation of the tubercle bacilli from sputum or other contaminated material was for a long time one of the most difficult problems in bacteriology. Investigations were carried out to find a material which would have a maximum bactericidal or bacteriostatic effect on contaminants, a decided lytic effect on mucus, pus and protein and a minimum devitalizing effect on the tubercle bacilli.

Historically the first attempt was made by Biedert (1886, 1887, 1891) on the concentrating of specimens. He advocated boiling the sputum with a liberal amount of 0.2 per cent solution of sodium hydroxide. This procedure was obviously unsuitable. The laborious washing method of Kitasato (1892) which yielded very inconsistent results is now only of historical interest. Muhlhauser and Czaplewski (1891) reported encouraging results with a modification of Biedert's original method. They used sodium hydroxide for digestion followed by neutralization and centrifugation of the mixture and the examination of the sediment. Uhlenhuth and Xylander (1908) were the first to study the bactericidal property of antiformin and apply it to the sedimenting of the tubercle bacillus from the sputum and urine. They found that antiformin destroyed the contaminating organisms but left the tubercle bacilli intact. Other organic material such as cells were disintegrated. With their description of antiformin for this purpose the search for an ideal concentration

material began and has continued to the present day. The first important improvement was the antiformin modification by Griffith (1914) and the second was the description of the modified sodium hydroxide procedure by Petroff (1915).

The principal features sought in a concentration method are, maximum recovery of organisms, ease of operation and speed of performance. Most reagents were lauded because of their solvent effect upon tissues, sputum or pus. This very fact however made them a possible destructive agent injuriously affecting small numbers of tubercle bacilli. The isolation of the bacilli from contaminated material presents many difficulties. Steenken and Smith (1938) asserted that it was more profitable to cut down the growth of secondary contaminants by a proper digesting fluid than by the addition of a bacteriostatic substance such as gentian violet to the medium. Stanley Griffith (1914) made a study of the time factor relationship on concentration by digestion. He concluded that there is no standard fixed time since it depended on the constitution of the specimen. Other factors necessary for a successful method are that the final material should be available for examination.

(1) Methods Suitable for Direct Examination

These methods are not applicable to specimens to be cultured or to be used for guinea pig inoculation since most of the tubercle bacilli are killed by the treatment. However, they contribute to the advantage of giving immediate results.

There are two avenues of approach: (a) digestion (b) flotation

(a) DIGESTION is essentially the digestion of the proteins in the specimen. It is necessarily accompanied by the destruction of the histology of the specimen.

1. TERGITOL was described for this purpose by Petroff and Schain (1938). Tergitols are sodium secondary alcohols. Tergitol penetrant 08 is a sodium octyl sulphate and produces a marked reduction in surface tension, which factor is the key to a more complete concentration. It is a stable organic solvent which is a good digester of pathological fluid. Tergitols are powerful, wetting, penetrant and introfying compounds which affect the surface activity of water solutions and are potent surface tension depressors. This method gave a short digestion time, a small sediment and a high concentration. The addition of tergitol to sodium hydroxide did not enhance the action of the latter upon the sputum or tenacious material to be digested. More recently Schain, Magdalin and Russo (1948) advocated Tergitol Javelle water (40 grams chlorinated lime to 600 grams of sodium carbonate dissolved in 4000 cc of distilled water). Superiority using this method resulted from a greater degree of sputum solution with consequent concentration of organisms, a more even distribution of bacilli in the sediment and the fact that the reagent does not alter the capacity of the acid fast organisms to retain the fuchsin stain.
2. CLOROX was used as a digestant by Oliver and Reusser (1942). It demonstrated digesting properties which were even greater

than the tergitol sodium hydroxide mixture. The active agent was 5.25 per cent sodium hypochlorite and in this is similar to antiformin. It was very rapid, digestion being completed within two minutes. It required no neutralization or fixation to the slide. The mycobacteria were killed during digestion. It was simpler, more rapid and more sensitive than direct smear or sodium hydroxide methods. Among its disadvantages were incomplete digestion of sputa, and alteration of the capacity of the organism to retain the stain. As a method it was a satisfactory aid to diagnosis.

3. BROMINE was advised by Gaekel (1919) who used Rice's Bromine and Alkali reagent.

(b) FLOTATION is a method to facilitate the separation of the organisms from the digested specimen. The bacilli were extracted from the material by means of a suitable solvent. Volatile hydrocarbons were employed and almost always were preceded by digestion treatment. Kinyoun (1915) believed the mode of action was due to the hydrocarbon combining with the waxy envelope of the bacillus making it lighter and lubricating it. He also thought the hydrocarbon caused the bacilli to clump, which he considered an advantage. On the other hand Pottenger (1922) noting that the hydrocarbons used were fat solvents, attributed the action to be due to the resolution of the bacillary clumps by lipid solvents. Reed and Rice (1931) made a thorough investigation and found the same

separation of the organisms from a suspension with oils as with the volatile hydrocarbons. They attributed the action to be due to the presence on the surface of acid fast bacilli of mixtures of fatty substances which prevent their being wetted by water and allow them to be wetted by the non polar oils and hydrocarbons.

Hydrocarbons used for this purpose in order of their description:

1. ETHER was employed by Couratte-Arnaude (1903) utilizing the fundamental principles of this method. It was of little value.
2. LIGROIN a petrol ether mixture was employed by Lange and Nitche (1909). The digested material was shaken with ligroin and centrifuged. The bacilli were found in the soapy layer between the ligroin and the dissolved material. All the procedures using the hydrocarbon principle consist of a modification of the original Langes and Nitche. Haserodt (1909) applied ligroin following antiformin digestion. Kinyoun (1915) using this method showed an increased efficiency of 19% over direct smear examination.
3. CHLOROFORM was first used by Loeffler (1910) and later by Andrus and MacMahon (1924). The chloroform separated into the subnatant layer. Hanks and Feldman (1940) felt there were definite advantages to the heavier hydrocarbons in that the entire sediment may be used for smears. They also considered it superior to ligroin and gasoline since it could be recovered completely after removal of the supernatant. The

sediment formed a satisfactory material for staining and giving a good distribution of organisms.

4. GASOLENE was first used by Krause and Fleming (1916), the bacilli being recovered in the gasoline layer.

5. XYLOL was employed by Pottenger (1922,1932,1938), which showed the effective removal of tubercle bacilli from specimens by xylol, ligroin, chloroform and carbon disulphite. He felt the choice of the hydrocarbon was unimportant. His method employed extensive dilution, mechanical shaking and gravity separation. As discussed above he considered the mode of action to be due to the dissolving of the "interbacillary wax" thus setting free the individual organisms from the group in which they were found. Pottenger claimed that this dilution flotation method followed by picric acid as a counterstain gave an average of 60 times as many bacilli as could be found in direct smears counterstained with methylene blue. This efficiency was two times that of chemical flocculation and four times that of Petroff's method and as accurate as guinea pig inoculation. Dilution here was important due to the fact that the ease with which the groups were resolved into free cells was dependent upon the viscosity of the material.

(ii) Methods Suitable for Smear and Culture and Animal Inoculation

There are two basic procedures: (a) digestion (b) collection

(a) DIGESTION is the digestion of the proteins. Instability and toxicity towards small numbers of tubercle bacilli are the greatest drawback of the reagents used for this purpose.

1. ANTIFORMIN or Labbarroque's solution was used to dissolve the mucus and to destroy the secondary organisms; it is the patent name of an antiseptic introduced in 1900. The active principle is sodium hypochlorite, in addition, it contains caustic soda. Rapidly growing microorganisms were far more susceptible to its toxic action than were the wax protected tubercle bacilli. The activity seems to be due to its intense oxidation. It was first recommended for this purpose by Uhlenhuth (1908). Other workers modified their method. Brown and Smith (1910) demonstrated its value for culturing following antiformin treatment. Realizing the importance of this for the cultivation of tubercle bacilli direct from contaminated sources, Griffith (1914) undertook a study of this work. He showed that the presence of free antiformin in a tuberculous fluid was not detrimental to the growth of the tubercle bacillus. It was the method of choice until Petroff (1915) devised his sodium hydroxide method.

2. ALKALI

a. Sodium hydroxide was advocated by Petroff (1915) for the successful application of Biedert's principle, the homogenization of sputum by alkali and has found wide acceptance. In Petroff's method the sputum was mixed with an equal amount of 4% sodium hydroxide, a concentration which did not destroy the tubercle bacilli when incubated for 24 hours. His technique made good

progress possible. It was preferred to antiformin because it was more stable, more easily obtainable and less irritating. It exerted a double effect, dissolved the mucus and killed off other organisms and moulds. The main disadvantages were its action on staining qualities and viability, both being impaired on slightly prolonged contact at higher temperatures. Also pure solutions deteriorated by absorption of carbon dioxide. It is preferred to other reagents because it produced a smaller amount of sediment and hence made possible a more effective concentration of tubercle bacilli. It was universally accepted as a most satisfactory method for obtaining primary cultures from contaminated sources. Its efficiency is 55%. Corper (1927) reported that it prevented growth of all acid fast bacilli except the tubercle bacilli.

b. Ammonium hydroxide was first used by Corper and Uyei (1927). It was an efficient germicide and permitted the growth of only a small percentage of contaminators. However, ammonium hydroxide proved too toxic toward the tubercle bacilli in a concentration and time interval suitable for destroying the contaminators.

3. ACID

a. Sulphuric acid was first described for the treatment of tuberculous sputum by Bossan and Baudy (1922), but it is to Lowenstein and Sumoyashi (1924) to whom we owe the use of this method. Lowenstein's original procedure cannot be recommended because he used too high a concentration of acid. Corper (1927) recommended that 6% gave good results. Although its action

was not as rapid as sodium hydroxide, it had a wider range of being innocuous for the tubercle bacilli and its being capable of destroying undesirable contaminants. It gave a larger percentage of primary cultures and permitted the earlier appearance of colonies. Also the sediment did not need to be washed or neutralized which saved time and decreased the chance of contamination. The mucoid substances in the sputum are slightly soluble in strong acids but not in dilute acids. Therefore upon dilution of the sulphuric acid the liquid substances in the sputum coagulate and the coagulated materials on centrifugation materially assist in bringing down the tubercle bacilli to the bottom of the tube.

b. Oxalic acid was preferred to sulphuric acid by Corper and Uyei (1930) because of its stability in solution, purity and its crystalline property. Also, it was practically innocuous for small numbers of bacilli. It is a poor sputum solvent but a good differential antiseptic. It possesses a greater germicidal action for the contaminating organisms found in sputum. Whitehead (1939) noted that oxalic acid did not increase the number of positives but satisfactorily prepared the material for culture and injection into the guinea pig. Its main advantage lay in the fact that it was a crystalline solid of high purity and of stable composition.

c. Hydrochloric acid used by Corper and Uyei (1927) acted like sulphuric acid; the main difference being a narrower range of serviceable concentration in which the hydrochloric acid could be used. The optimum concentration was three per cent.

d. Citric acid was recommended by Herrold (1931) for urine and sputum but gave a high contamination especially molds.

4. SALTS

a. Ammonium carbonate, first used by Corper and Uyei (1927) destroyed many gram negative organisms and promoted the rapid decomposition with the formation of ammonia. Its neutralization rendered unnecessary treatment with acid. It liquified sputa readily and did not sterilize small inocula.

b. Ferrous sulphate used by Jungmann (1938) together with hydrogen peroxide and sulphuric acid (a trace). This mixture proved to be a very potent solvent for mucin. The reaction was dependent on the presence of all the three reagents and peculiar to all mucous secretions. The mode of action was thought to be by oxidation of certain chemical groups responsible for the mucinous character. The main advantages were speed, a small centrifugal deposit and effective elimination of secondary organisms. The liquefaction of the sputum was employed here in contradistinction to homogenization in most other methods. It is now known as the Acid Peroxide Iron method and has been used extensively by Nassau (1942).

c. Trisodium phosphate, a pure crystalline compound of standard chemical composition was introduced by Corper and

Stoner (1946) as an improved procedure for the dianostic culture of mammalian tubercle bacilli. It was a superior reagent for destroying contaminating organisms in pathological specimens for the cultural diagnosis of tuberculosis as compared with 3 per cent sodium hydroxide and 5 per cent oxalic acid. Ten per cent trisodium phosphate added to an equal volume of fine suspension of mammalian tubercle bacilli, not only proved innocuous but more protective than suspensions of bacilli in physiological saline for long periods of time. A 10 per cent solution trisodim phosphate (23 per cent of a $\text{Na}_3\text{PO}_4 \cdot 12\text{H}_2\text{O}$) could remain in contact with the bacilli up to a week at room temperature but it destroyed the undesirable contaminants in sputum within twenty four hours at 37° C.

5. ANIMAL FERMENTS

a. Pancreatin introduced by Spengler (1903) who suggested that enzymes were the logical alternatives as digestors for use in homogenizing sputum. However, they did not destroy the contaminating organisms.

b. Pepsin used by Jousset (1903). Pleural fluid was digested with pepsin and hydrochloric acid. The exudate was coagulated by the acid and digested by means of the pepsin. Gerundo (1940) applied this principle to sputum and urine using Jousset's liquid made of glycerine, hydrochloric acid, sodium fluoride and pepsin.

c. Trypsin described by Vogt, Zapposodi and Long (1940) for the treatment of specimens prior to culturing. In a

comparative study by Haynes (1942) eleven per cent more positives were found by the trypsin method than by the flocculation procedure of Hanks et al (1938). Its action however was not sufficiently vigorous in all types of specimens.

6. VEGETABLE FERMENTS

Papain (Caroid) was studied by Sullivan and Sears (1939). Although it did not digest pus actively it was more satisfactory than trypsin. It was more active in an alkaline medium and the digest may be allowed to stand for several hours without effect.

7. ORGANIC COMPOUNDS

a. Glycerine was recommended by Twort and Aberd (1922). Specimens if left sufficiently long in glycerine gave pure cultures even though containing very numerous contaminating microorganisms. Lurie (1924) reported that neither the highest concentration of glycerol, nor the most suitable time interval of exposure found satisfactory to still preserve the visability of the mycobacteria for culture, was found capable of destroying contaminants present in sputa. Corper (1927) likewise found it to be of little value.

b. Urea used by Dold (1908). The possibility of isolating tubercle bacilli from suspected materials by the use of urea to destroy contaminators was investigated. Urea possessed an excellent solvent action. It was unsuitable because it permitted frequent contamination of culture medium. The germicidal action of the urea upon contaminators was only moderate.

c. Formaldehyde used by Spengler (1903) never achieved a widespread use, since tubercle bacilli are susceptible to its action.

d. Sulfonamides were recommended by Schweitz (1944). This has been utilized for the treatment of sputum prior to the inoculation of media and guinea pigs.

(b) COLLECTION

i Flocculation is essentially the collection of the organisms by the flocculation of the protein by the entanglement of the organisms in a controlled precipitate. Flocculating agents were used to assist in the demonstration of bacilli from body fluids. Flocculated particles did not interfere with the cultivation of the tubercle bacilli when very small numbers of organisms were present. This method has been simplified by the incorporation of the flocculating agent in with the digester. This reduced the centrifugation time and permitted the preparation of very uniform rather thick films. The bacilli are more completely collected from the sputum or other specimens so that a unit amount of sediment contains from 3-7 or more bacilli than can be collected by direct centrifugation. The flocculated particles do not interfere with the cultivation of the bacilli when very small numbers of organisms are present. Robinson and Stovall (1941) in a study of concentration methods concluded that the use of a reagent to collect the organisms by flocculation of the proteins was of no advantage. They felt that the heavy

precipitate from a given quantity of digest greatly reduced the original specimen which could be used for examination.

Substances that have been employed for flocculation are:

- a. Sulphosalicylic acid recommended by Holm J. precipitated the albumin with a few drops of sulphosalicylic acid.
- b. Tannic acid used by Petroff (1915). This method depended on the efficiency of the precipitation. The precipitate carried down the organisms making them easier to find. Hanks and Felman (1940) showed that the precipitation produced by acidification and chilling the urine with or without tannic acid though bulky gave better concentrates than direct centrifugation. Also that the use of tannic acid was unnecessary and that acidification and chilling of the urine produced comparable results.
- c. Sodium carbonate first used by Ellerman and Erlandsen (1908). Greenfield and Anderson (1919) reported that even in a concentration as high as 40% findings showed that the development of contaminants was not prevented. They used it with phenol and showed that it possessed the advantages of rapidity, efficiency and did not destroy the cytology.
- d. Aluminium hydroxide cream used by Salhof (1924). The number of bacilli per field were slightly greater than in the untreated field.
- e. Ferric chloride investigated by Hanks et al (1938). The flocculated particles did not interfere with the growth of the organisms when very small numbers were present.

f. Alum described by Kinyoun and Hanks (1938). Flocculation by this substance was superior to that by ferric chloride. The preparation of the alum flocculated resulted in the complete removal of bacilli during very brief centrifugation.

Flocculation was inhibited in varying degrees in different urines. Alum precipitates gave a high incidence of growth, higher than the other sediments.

g. Antibody enable a harmless substance to be injected into the animal body.

h. Phosphates were introduced by Hanks and Feldman (1940). Flocculations resulting from neutralization or alkalization of urine were referred to as phosphate precipitates. This was a practical and efficient method.

ii Centrifugation was the procedure advocated by Crabtree (1916). Its efficiency depended on the different specific gravity of the acid fast organisms compared with the ordinary bacteria and the organic matter usually present, that is, pus, epithelial cells and mucus.

Four factors influence this method: a) specific gravity of specimen b) amount of pus present c) the length of centrifugation d) speed of centrifugation

Jeck and Munch (1927) concluded that best results were obtained for tuberculous urine at 2300 revolutions per minute for not less than 15 minutes. A longer time was unnecessary. Pus was an important factor as well as the centrifugal force in carrying down the tubercle bacilli.

Simple centrifugation was equal to an established concentration method and better than most of them. More recently, in a study of the collection of tubercle bacilli from aqueous suspensions of sputum Hanks et al (1938) and of urine Feldman and Hanks (1939), it has been shown that direct centrifugation is much less efficient than might be expected in view of the relative ease with which other bacteria are sedimented by this method. For example, chemical flocculations revealed that many bacilli remained in the supernatant fluid, after centrifugation of slightly positive sputa for one hour. The organisms could not be sedimented with any degree of completeness by prolonged centrifugation in machines of conventional design and speed, even when the bacilli were centrifuged in solutions which approximated the isoelectric point of the organisms pH 2.8.

iii Specific gravity of the specimen influences the collection of the organisms. Bezancon and Philibert (1903) attached great importance to the lowering of the specific gravity of the solution and advocated diluting with water. Robinson and Stovall (1941) concluded that dilution with water or alcohol to lower the specific gravity was of no value except in very viscous specimens.

iv Surface tension depressors were studied by Robinson and Stovall (1941) for the possibility that a lowering of the surface tension of the suspended medium might allow

the organisms to be more readily centrifuged. Various materials were added to the digested specimen for the purpose of lowering the surface tension. These included a stock soap solution aerosol 0.1 per cent, sodium glycholate 5 per cent, urea 5 per cent and 20 per cent dreft 0.1 per cent and 1.0 per cent. The results were so varied and inconsistent that no definite conclusions could be drawn. The optimum surface tension for this work was between sixty three and seventy three.

II EXPERIMENTAL

A PREPARATION OF INOCULUM

The difficulty of the preparation of suspensions of tubercle bacilli for quantitative experimental work is well known. This lack of precise methods has resulted in an inability to evaluate new developments in experimental work concerning the mycobacteria. The number of bacilli in an inoculum has been estimated in a number of ways, but mainly with a high degree of error. Of the several methods in use the most widely accepted is the estimation of organisms expressed gravimetrically. More recently, quantitative expression in terms of optical density is gaining favor.

Calmette (1920) advocated the estimation of the number of organisms per mg moist weight and this remained the classical method for some years.

Baldwin (1921) recommended a constant technique for smearing and staining. After counting the organisms in a microscopic field the amount of a suspension necessary to kill an animal in a given length of time was determined. This method did not tell how many organisms are being inoculated and has many sources of error.

Barber's method (1907) in which single bacilli are drawn up into capillary pipettes under the microscope, was a difficult technique and was inadequate for the inoculation of more than a few animals or cultures. One is not sure either of the viability of the organisms on account of the appreciably long exposure to strong light.

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The difficulty of the preparation of suspensions of tubercle bacilli for quantitative experimental work is well known. This lack of precise methods has resulted in an inability to evaluate new developments in experimental work concerning the mycobacteria. The number of bacilli in an inoculum has been estimated in a number of ways, but mainly with a high degree of error. Of the several methods in use the most widely accepted is the estimation of organisms expressed gravimetrically. More recently, quantitative expression in terms of optical density is gaining favor.

Calmette (1920) advocated the estimation of the number of organisms per mg moist weight and this remained the classical method for some years.

Baldwin (1921) recommended a constant technique for smearing and staining. After counting the organisms in a microscopic field the amount of a suspension necessary to kill an animal in a given length of time was determined. This method did not tell how many organisms are being inoculated and has many sources of error.

Barber's method (1907) in which single bacilli are drawn up into capillary pipettes under the microscope, was a difficult technique and was inadequate for the inoculation of more than a few animals or cultures. One is not sure either of the viability of the organisms on account of the appreciably long exposure to strong light.

Jennings (1926) used a Levy-Hausser counting chamber and obtained consistent results. The suspension was prepared by trituration followed by filtration through a Whatman #5 filter paper to remove large clumps. A pH of 8.7 was used since spontaneous agglutination did not occur at that point.

Corper and Cohn (1936) recommended the addition of a few drops of 0.5% sodium taurocholate. It materially assisted in speeding up the operation and gave more consistent results because of its surface tension reducing properties in initiating grinding.

Wilson and Schwabacher (1937) in a study of the relationship between moist organisms and number of total and viable organisms in a culture of tubercle bacilli, reported that only a portion is viable. A two week old culture of human mycobacteria contained a very large portion of dead cells and it is likely that many of these had undergone varying degrees of autolysis. This heterogeneity undoubtedly complicated the analysis of the factors affecting the rate of bacterial growth and the establishment of standard experimental infections. Their experiments showed that only 40-80% of a culture of avian bacilli was viable and the mammalian is much lower.

Hanks and James (1940) enumerated the bacilli by the microscopic method using dried smears on suitably measured glass slides.

Woodruff et al (1946) in a report from a committee

on the evaluation of laboratory procedures recommended that the inoculum be expressed in terms of dry weight. It was considered to be a most accurate measure of bacterial substance in suspension.

Dubos (1946) in his experiments on the effect of lipids and serum albumin on bacterial growth correlated the optical density with gravimetric determination (oven dry weight) for estimating bacterial growth. This correlation of the optical density and the weight of bacterial bodies was possible because diffuse growth was characteristic of cultures growing in the presence of water soluble esters of certain fatty acids. A very useful feature of the Tween albumin medium is that graded amounts of inoculum can be accurately prepared by simple dilutions; thus, avoiding the necessity of trituration prior to inoculation. It is apparent that this medium is well adapted to the quantitative study of the effect of nutritional factors and of bacteriostatic and bactericidal agents on the viability and growth rate of the tubercle bacilli.

Two methods for the preparation and estimation of inocula were used for experimental work in this problem.

1. Dry weight

- (a) Surface growth

- (b) Diffuse growth in Dubos liquid medium

2. Optical density correlated with microscopic bacterial counts.

Procedure 1.

- (a) Growth was scraped off a four week old culture of

Petragnani's medium and was triturated in an agate mortar with a minimum amount of physiological salt solution until a homogeneous paste was formed. This was then taken up in 10 cc of saline and centrifuged at low speed for two minutes to remove gross clumps of organisms. A portion of the suspension was removed to a weighed watch glass and evaporated to dryness in the incubator. With allowance for the salt content the weight of bacilli per cc in the remainder of the suspension was then calculated.

Results:

Weight of watch glass	8.5489 gms
Weight of saline in one cc	0.0085 gms
Weight of suspension	8.5511 gms

Suspension calculated to contain 2.2 mgs per cc. This was then diluted to give a suspension of 1 mg per cc.

(b) One cc of a 10 day old culture in Dubos medium was similarly estimated gravimetrically, there being no need for the preliminary trituration of the medium.

2. Optical density

Optical density standardized against the number of bacilli estimated by direct microscopic counts. A culture was prepared by planting in Dubos liquid medium containing Tween and incubation for 14 days. The density of the suspension and dilutions of the suspension were determined by an Evelyn photoelectric colorimeter. These results were correlated to numbers of bacilli per cc estimated by

a Thoma (Hawsley) counting chamber. Dubos (1947) reported that no significant proportion of cells in a seven day culture of Tween albumin media is sterile. This contrasts with the high proportion of sterile cells found in surface growth. These results were plotted on a graph using colorimeter readings against numbers of bacilli.

Determination of numbers of bacilli present could then be determined directly from colorimeter reading.

Results:

Table I

Correlation of Optical Density to Bacillary Counts

Inoculum	Dilution	Colorimeter Reading	Density	Count per cc
B.C.G.	-	85 ³	.0667	8 x 10 ⁸
	1:2	91 ³	.0374	6 x 10 ⁸
	10 ⁻¹	-	-	8 x 10 ⁷
	5x 10 ⁻²	96 ¹	.0166	5 x 10 ⁷
	1x 10 ⁻²	98 ³	.0055	8 x 10 ⁶
Virulent	-	94 ²	.0246	8 x 10 ⁸
Tubercle	1:2	96 ¹	.0166	5.6 x 10 ⁸
Bacilli	10 ⁻¹	99 ¹	.0033	1.2 x 10 ⁸
	5x 10 ⁻²	-	-	6 x 10 ⁷
	1x 10 ⁻²	-	-	1 x 10 ⁷
Virulent	-	92 ³	.0327	1.6 x 10 ⁹
Tubercle	1:2	96 ³	.0144	5.7 x 10 ⁸
Bacilli	10 ⁻¹	99 ⁰	.0044	1.3 x 10 ⁸
	5x 10 ⁻²	99 ²	.0022	6.6 x 10 ⁷
	1x 10 ⁻²	-	-	1.2 x 10 ⁷

The following method of inoculation was used. Pasteur pipettes prepared as described by Fildes (1931) were used for planting the bacilli. Four drops (0.1 cc) of the bacillary suspension or dilution were dropped from above the surface into the medium. This method was also utilized for the preparation of dilutions when it was necessary to use small amounts. The bacilli were shielded from direct rays when suspended and planted.

CONCLUSIONS

Two procedures for the estimation of an inoculum have been described. The estimation of the inoculum by optical density in Dubos' liquid medium has been found to be simply and sufficiently accurate for suspending and counting living tubercle bacilli. Synthetic fluids can be prepared in amounts which would permit the simultaneous testing of any number of experiments. By means of this approach it is possible not only to select a better method, but also, to indicate quantitative results.

B. COLLECTION OF ACID FAST BACILLI BY PARTITION WITH OIL

The efficiency of the culture methods depends on the method of concentration. Various procedures have been devised to render the examination more sensitive. Almost all of them include two processes: the digestion of the mucus and the cellular elements in the specimen, and, the collection of the organisms either by gravity, centrifugation or the use of hydrocarbons.

Methods of digesting the specimen have been subjected to intensive study as described in the review of the literature. The use of trisodium phosphate, advised by Corper and Stoner (1946) as a digesting agent has met with notable success. Much difficulty has been encountered in the collection of organisms for cultural work. This problem is dealt with here.

Many authors, (Pottenger, Kinyoun, Smith, Robinson and Stovall) agree that flotation by means of a hydrocarbon is a most efficient method for collecting tubercle bacilli from a suspension as compared with centrifugation and chemical flocculation. Most work done to date utilizing this method has been applied only to direct smear examination, owing to the inability to remove the hydrocarbon and to the lethal effect on the microorganisms. Pottenger (1932) stated that the efficiency of the method did not depend on the agent

used for collecting. Xylol, ligroin, chloroform and carbon disulphite served equally well.

Mudd and Mudd (1924, 1926) observed that non acid fast bacteria, in the water phase of a water oil emulsion, on reaching the interface, either remained at the interface or returned to the water but never passed into the oil. Acid fast bacteria however, which entered the interface from the water were snapped abruptly into the oil phase. This is explained on the theory that on the surface of the mycobacteria mixtures of fatty substances exist with many non polar groups which prevent their being wetted by water but allow them to be wetted by the non polar liquid oil. These phase boundary phenomena were observed microscopically.

Reed and Rice (1931) studied the behavior of acid fast bacilli in oil and water systems. Their method was to suspend the bacteria in water and overlay with oil. This was mechanically shaken for one hour. The experiment was concluded by centrifugation or by being allowed to stand. The concentration of the suspension of bacteria remaining in the aqueous phase was determined by comparing the opacity with that of standard suspensions of the same organisms or by microscopic counts using Breed's method.

They concluded that all the acid fast species partitioned almost completely into the oil, so that the aqueous layer was left clear. All the non acid fast species remained in the water phase. Considerable difference in the completeness of partition of the acid fast species into the oil was observed. This difference was correlated with the degree of acid fastness.

Olive oil was selected for partitioning of the acid fast bacilli from a suspension. Other oils, such as, boiled linseed, coconut, palm, whale, cod, mineral, melted tallow, castor fat, oleic acid and triolein were investigated. No significant difference from olive oil could be observed. Fat solvents were similar in their action to oils. Carbon tetrachloride, ether, xylol, carbon disulphide, kerosene, benzene, petroleum, chloroform, butyl amyl alcohol and iso amyl alcohol were tested. This agreed with the observations made by Pottenger and Kinyoun.

The acid fast bacilli must be brought into contact with the oil phase. The proportion of 1cc of water to 0.35cc oil gave approximately complete partition.

Adjustment of pH of water phase to pH 6.0 prevented the formation of soap and left the aqueous layer clear. Regardless of the small amount of soap formed in an alkaline solution, the acid fast organisms partitioned more or less completely into the oil.

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(i) Selection of type of oil for maximum efficiency

Preliminary experiments were carried out to select the type of oil most efficient for the collection or partition of tubercle bacilli suspended in saline.

The oil collecting capacity of a number of oils was investigated. Oils first selected for this purpose were castor, olive, cotton seed and peanut. Later corn and sesame oil were used.

Material and Methods

A suspension of B.C.G. was prepared as described under preparation of inocula. The inoculum was expressed quantitatively in terms of dry weight. The organisms were suspended in 10 cc saline and 0.5 cc oil. Various commercial oils were tested. This mixture was shaken for five minutes and centrifuged for ten minutes. 0.5 cc of supernatant was inoculated on Petraghani's medium to test for the presence of acid fast bacilli. Growth was recorded in terms of gross appearance. The experiments were repeated using pure oils, the inoculum being estimated with a Thoma (Hawksley) counting chamber. The results are recorded in Table II and Table III.

Table II

Effect of various oils on the partition of tubercle bacilli from a suspension

Inoculum in mg	Castor Oil	Olive Oil	Cotton Oil	Peanut Oil	Control
1×10^{-2}	^o ++	+	0	++	+++
2×10^{-2}	(+)	+	+	+	+++
1×10^{-4}	0	0	0	0	++
2×10^{-1}	+	+	++	++	+++
2×10^{-1} *	+++	+++	+++	+++	+++
1×10^{-2}	++	+++	++	+	+++
1×10^{-2}	++	++	++	+	+++
2×10^{-3}	+	(+)	+	(+)	+++

The number of +'s represents the gross appearance of growth obtained from 0.5 cc of subnatant after 28 days. (+) indicates a very slight growth. * Inoculum with Tween

Table III

Effect of various oils on the partition of tubercle bacilli from a suspension (continued)

Oil		Olive	Peanut	Sesame	Corn	Control
Inoculum No. of bacilli	Approx. mg					
8×10^4	10^{-5}	0	0	0	0	+++
8×10^7	10^{-2}	0	slight	(+)	+	+++
8×10^8	10^{-1}	(+)	+	++	+	+++

Summary of results:

No great difference in partition ability is apparent from the use of the various oils. There is however, some selectivity and olive oil followed by peanut oil is more efficient in its collecting capacity as compared with the other oils. Olive oil was used in most of the subsequent experiments.

- (ii) Determination of the size of inoculum on the collecting ability of the oil

Material and Methods

A preliminary experiment was conducted utilizing olive oil. The inoculum of B.C.G. was expressed gravimetrically. Saline was buffered to a pH 6.0 to prevent soap formation. The mixture containing organisms in 10 cc saline and 0.5 cc oil was shaken mechanically for thirty minutes and then centrifuged for twenty minutes. After removal of the oil, the 0.1 cc subnatant was planted on Petraghani's medium to determine the removal of bacilli by the oil. The experiment was repeated with the various oils to determine the influence of the size of the inoculum. The experiment was again repeated with several pure oils. The results are recorded in Tables IV, V and VI.

Table IV

Effect of the size of inoculum on the oil collection of the tubercle bacilli from a suspension

	Time of Growth	Inoculum in mg				
		10 ⁻²	10 ⁻³	10 ⁻⁴	10 ⁻⁵	10 ⁻⁶
Olive oil	14 days	+	+	0	0	0
	28 days	+++	+++	0	0	0
Control	14 days	+	+	+	(+)	(+)
	28 days	+++	+++	+++	+++	+++

Table V

Effect of the size of inoculum and various oils on the collection of tubercle bacilli from a suspension

Inoculum in mg	Castor Oil	Olive Oil	Cotton Seed Oil	Peanut Oil	Control
2 x 10 ⁻³	+	(+)	+	(+)	+++
1 x 10 ⁻⁴	0	0	0	0	+++
1 x 10 ⁻⁵	0	0	0	0	+++

Growth recorded after twenty eight days.

Table VI

Effect of the size of inoculum and various oils on the collection of the tubercle bacilli (continued)

No. Bacilli	Inoculum Approx mg	Olive Oil	Peanut Oil	Sesame Oil	Corn Oil	Control
8 x 10 ⁴	10 ⁻⁵	0	0	0	0	+++
8 x 10 ⁷	10 ⁻²	0	slight	(+)	+	+++
8 x 10 ⁸	10 ⁻¹	(+)	+	++	+	+++

Growth recorded after twenty eight days.

Growth in the above tables is obtained from the cultivation of 0.1 cc of subnatant after twenty eight days.

Summary of Results

The experiments in which the oil completely partitioned the acid fast bacilli from a suspension, that is in which the subnatant was sterile, were those in which the inoculum was small. There appears to be a critical point in the size of inoculum below which all the bacilli are efficiently collected from a suspension; when the size of inoculum is small the bacilli are completely taken up by the oil. It is around this critical point of size of inoculum that the advantage of type of oil manifests itself. These results can be expressed quantitatively. An inoculum of 10^{-4} mg or less is completely partitioned into the oil from an oil water system for the proportion of 10 cc saline to 0.5 cc oil. An inoculum of 10^{-3} mg or more is not taken up entirely by the oil in an oil water mixture.

The efficiency for a large inoculum depends to a greater extent on the type of oil used. There is definitely a selectivity of oils in this range with olive oil being outstanding in its action followed by peanut oil. The results in all three tables demonstrate effectively that the size of inoculum has a great influence

on the collecting capacity of the oil for the tubercle bacilli. This of course is not a drawback to this work since the prime purpose of a concentration method is to collect small numbers of acid fast organisms not demonstrable by other means.

(iii) Determination of the quantity of oil for maximum efficiency

Materials and Methods

The inoculum was prepared as described above and was expressed gravimetrically. An oil water suspension was made with ten cc saline containing the organisms and varying quantities of oil. The oil water suspension was shaken for five minutes. 0.1 cc of subnatant was planted on Petragnani's medium to test for the presence of mycobacteria. The results are recorded in Table VII.

Table VII

Effect of the quantity of oil for maximum efficiency on the collection by the oil of acid fast bacilli

Inoculum mg	Quantity suspension	Oil	0.1	0.5	1.0	1.5	2.0	Control
10 ⁻²	10 cc	Olive	+	(+)	+	-	+	+++
10 ⁻²	100 cc	Olive	++	++	++	-	+	+++
10 ⁻¹	10 cc	Olive	-	(+)	+	+++	+++	+++
10 ⁻⁴	10 cc	Olive	-	+	0	0	0	+++
10 ⁻⁴	10 cc	Peanut	-	+	(+)	0	0	+++

The number of +'s represents the gross appearance of growth obtained from 0.1 cc of subnatant after twenty eight days.

Summary of Results

The results obtained with two different quantities of suspension, showed the efficiency of the collection of bacilli by oil from a saline suspension, depended on the relative proportions of oil to saline. The quantity of oil remaining constant, the partition appeared to be more complete in a 10 cc saline suspension than in a larger suspension of 100 cc. This may be due to the chance contact of oil to organisms.

To determine if this factor was responsible for the added efficiency, increased dispersion by means of a detergent was investigated in a subsequent experiment.

When an inoculum was used at a critical level of 10^{-4} mg, that is, when the factors affecting the method can be more accurately evaluated, a quantity of 0.5 cc or over for olive oil, and 1.0 cc of peanut oil completely partitioned the acid fast bacilli from a water oil system. In conclusion, the quantity of oil used to obtain partition is not as important a factor as the size of inoculum.

(iv) Determination of the effect of dispersion by a detergent on the collecting capacity of the oil

Material and Method

Inoculum (B.C.G.) was suspended in 10 cc of buffered saline pH 6.0 Tween 80 was used as the source of a non toxic detergent in a concentration of 0.02 per cent

and was added to the buffered saline. Tween 80, a polyoxyethylene derivative of sorbitan monooleate is a viscous amber fluid, which is soluble in water and stable to autoclaving. After the addition of 0.5 cc of oil the mixture was shaken for ten minutes and centrifuged for twenty minutes. 0.05 cc of subnatant was planted on Petragnani's medium. Growth was recorded in terms of gross appearance. The results are reported in Table VIII.

Table VIII

Effect of dispersion by means of Tween 80 on the collection of oil

Inoculum mg	Time of Growth	Castor Oil	Olive Oil	Cotton Seed Oil	Peanut Oil	Control
Not centrifuged						
5 x 10 ⁻¹	14 days	+	+	(+)	(+)	(+)
	28 days	+++	+++	+++	+++	+++
Centrifuged						
5 x 10 ⁻³	14 days	(+)	slight	++	++	+
	28 days	+	(+)	+++	+++	+++
Centrifuged						
1 x 10 ⁻³	14 days	+	(+)	(+)	(+)	+
	28 days	+++	+++	+++	+++	+++

Growth represents the gross appearance obtained from 0.05 cc of subnatant.

Summary of Results

Tween 80 in a concentration of 0.02 per cent prevented the oil from accomplishing an effective collection.

These results are consistent with the theory of Mudd and Mudd (1926) that acid fast bacilli are wetted by the oil and if they are rendered dispersable in water, by the action of a detergent, they are no longer taken up by the oil. The effect of the Tween, may not however, interfere with partition as with maintaining the oil in suspension. The experiments show an increased efficiency with centrifuging which would indicate that Tween maintains a dispersion of the oil. The removal of the oil by filtration through a wet filter paper should be considered. A more thorough investigation of this aspect is required. Again it is noted that olive oil is more selective for this purpose than other oils. Although Tween 80 or other detergents were considered detrimental for this purpose, such agents might be used with advantage after collection, to remove the acid fast organisms from the oil system

- (v) To determine the effect of centrifugation on the partition of acid fast bacilli from an oil water suspension.

The procedure was similar to the foregoing experiments except that buffered saline pH 6.0 and 0.5cc of oil were used throughout. Centrifugation was carried out for twenty minutes. To determine the effect of centrifugation on the efficiency of the partition between water and oil, 0.1 cc of subnatant was planted on

Petragnani's medium. For the control experiments 0.1 cc of supernatant from a saline suspension of organisms was cultured. The results are summarized in Table IX.

Table IX (a)

Effect of centrifugation on the partition of acid fast bacilli by the oil

Inoculum	Castor Oil	Olive Oil	Cotton Seed Oil	Peanut Oil	Control
2×10^{-1} mg					
Not centrifuged	++	++	+++	+	+++
Centrifuged	+	++	++	(+)	+++
5×10^{-1} mg					
Not centrifuged	++	++	+++	++	+++
Centrifuged	++	++	++	+	+++

Table IX (b)

Inoculum 10^{-3} mg	Oil in cc				Control
	0.5	1.0	1.5	2.0	
Olive oil					
Not centrifuged	+	0	0	0	+++
Centrifuged	0	0	0	0	+++
Peanut oil					
Not centrifuged	+	(+)	0	0	+++
Centrifuged	0	0	0	0	+++

Growth represents gross appearance of culture after twenty eight days. (+) slight growth

Summary of Results

Centrifuging somewhat assists the efficiency of the method. This is more evident in the experiments in which

the inoculum approaches a size where maximum efficiency is possible. Another factor which influences the results is the type of oil used. It is noted that centrifuging at about 2000 revolutions per minute did not appreciably sediment the organisms in the control saline suspensions. This agrees with the results of Hanks et al (1938) that centrifuging is not of much value for the collection of tubercle bacilli, as referred to in the review of concentration methods. It would, however, seem advisable to carry out centrifugation to improve the separation of the oil and water.

Conclusions re the partition of acid fast bacilli by oil

The type of oil used for the partition of acid fast bacilli from a suspension was not of prime importance. Olive oil was the most effective, followed by peanut oil. The partition depended not only on the quantity used but also on the proportion of oil to water that is used.

The size of inoculum was the determining feature of the efficiency of this method. There appeared to be a critical level in the size of inoculum below which all the acid fast bacilli partitioned into the oil. This was not a disadvantage to this work since the purpose of a concentration method was the demonstration of minimal number of bacilli.

The use of the detergent Tween 80, in the oil water system seems to interfere with the bacilli being taken

up by the oil. Centrifugation improves the collection of the organisms by the oil, because it brings about a more complete separation of the oil and water.

When these factors are taken into consideration an efficient method of collection utilizing the partition of a saline suspension of acid fast bacilli into oil is possible.

C. DEGREE OF RESISTANCE OF TUBERCLE BACILLI TO VARIOUS FAT SOLVENTS

Most non sporing bacteria are considered to possess a high degree of susceptibility to the disinfectant action of fat solvents; the acid fast bacilli are believed to be relatively resistant to these agents (Topley and Wilson, 1947). Topley (1915) reported that some staphylococci survived five to twenty four hours exposure to ether. Yersin (1888) and Sternberg (1896) obtained experimental results that showed that tubercle bacilli were killed in ether in ten minutes.

The purpose of this investigation is to take up tubercle bacilli in oil from watery suspensions and to recover them by dissolving away the oil by means of a solvent. It is therefore necessary to select a fat solvent showing the least possible lethality to the tubercle bacillus.

Preliminary experiments were carried out using a strain of Bacillus Calmette Guerin as the inoculum and the results were confirmed using a strain of virulent tubercle bacilli.

Material and Methods

Fat solvents investigated for bactericidal action:

1. Ether - BDH absolute ethyl ether
2. Acetone - BDH analytical reagent

3. Benzin - Merck petroleum ether reagent (B.P. 30° - 60°)
4. Benzene - BDH analytical reagent

(1) Resistance of B.C.G. and virulent tubercle bacilli
to various fat solvents

A homogeneous suspension of B.C.G. was prepared. Sterile filter paper squares 1.5 mm were inoculated with 10^{-3} mg of culture and were evaporated to dryness at 37° c in air. These were immersed in the fat solvent for varying lengths of time and then evaporated to dryness at room temperature. The filter paper squares were then planted on Petragnani slopes. Growth appeared as a concentrated mass on the paper and was recorded in terms of gross appearance. Controls were set up with each experiment and readings were taken at the end of two and five weeks. The results are summarized in Table X. The experiments were repeated using virulent tubercle bacilli and acetone or benzin. These results are recorded in Table XI.

Table X

The resistance of B.C.G. to various fat solvents

Solvent	Test	Growth Time in weeks	Time of Exposure in Minutes							
			5	10	30	60	2x60	4x60	8x60	0
ETHER	A	2	(+)	(+)	0	0	0	0	0	++
		5	(+)	(+)	0	0	0	0	0	+++
	B	2	-	(+)	0	0	0	0	0	++
		5	-	+	(+)	0	0	0	0	+++
	C	2	0	0	0	-	-	-	-	++
		5	++	0	0	-	-	-	-	+++
	D	2	0	-	0	0	-	-	-	++
		5	+	-	0	0	-	-	-	+++
	A	5	+	(+)	+	+	+	0	0	++
		2	++	++	(+)	++	++	-	-	++
	B	5	+++	+++	+	+++	+++	-	-	+++
		2	(+)	(+)	0	(+)	(+)	-	-	(+)
ACETONE	C	5	+++	+++	0	+++	+++	-	-	+++
		2	(+)	(+)	0	(+)	(+)	-	-	(+)
	A	5	(+)	(+)	(+)	(+)	(+)	-	-	+++
		2	(+)	(+)	(+)	-	(+)	-	-	++
	B	5	+++	+++	+++	-	+++	-	-	+++
		2	+++	+++	+++	+	+++	+++	-	+++
	C	5	+++	+++	0	+++	+++	-	-	+++
		2	(+)	(+)	0	(+)	(+)	-	-	(+)
	A	5	(+)	(+)	(+)	(+)	(+)	-	-	+++
		2	(+)	(+)	(+)	-	(+)	-	-	++
	B	5	+++	+++	+++	-	+++	-	-	+++
		2	+++	+++	+++	+	+++	+++	-	+++
BENZINE	A	5	+	++	++	0	0	-	-	++
		2	+++	+++	+++	+	(+)	-	-	+++
	B	5	+++	+++	+++	+++	+++	-	-	+++
		2	+++	+++	+++	+	(+)	-	-	+++
	C	5	+++	+++	+++	+++	+++	-	-	+++
		2	+++	+++	+++	+	(+)	-	-	+++
	A	5	+	++	++	0	0	-	-	++
		2	+++	+++	+++	+	(+)	-	-	+++
	B	5	+++	+++	+++	+++	+++	-	-	+++
		2	+++	+++	+++	+	(+)	-	-	+++
	C	5	+++	+++	+++	+++	+++	-	-	+++
		2	+++	+++	+++	+	(+)	-	-	+++

+ indicates amount of growth after exposure to fat solvent
 (+) indicates a slight amount of growth

Table XI

The resistance of virulent tubercle bacilli to fat solvents

Solvent	Test	Growth Time Weeks	Time of Exposure in Minutes					
			5	10	30	60	2 x 60	0
ACETONE	A	2	+	+	+	+	+	++
		5	+++	+++	+++	+++	+++	+++
	B	2	+	+	+	+	(+)	+
		5	+++	+++	+++	+++	+++	+++
	A	2	+	+	+	+	(+)	+
		5	+++	+++	+++	+++	+	+++
BENZIN	A	2	+	+	+	+	(+)	+
		5	+++	+++	+++	+++	+	+++

+ indicates amount of growth after exposure to fat solvent,
(+) indicates a slight amount of growth.

Summary of Results

Four tests showed that ether exerted a definite bactericidal effect on the tubercle bacillus, even after an exposure of five minutes. Ether therefore, could not be used for this problem since the organisms were to be grown after collection. Three other solvents, acetone, benzene and benzin were then selected for testing purposes.

Table X showed acetone to be quite innocuous for at least two hours for the growth of tubercle bacilli. Benzin and benzene could also be used in that order of preference. Ether exerted a much greater inhibitory effect than any of the three others tested. The experiments were repeated with a strain of virulent tubercle bacillus using acetone and benzin and the results in Table XI indicate

that exposure to acetone or benzin for two hours exerts very little lethal action on virulent tubercle bacilli. It can be concluded that acetone is the most suitable fat solvent but that benzin may be used with little effect on the mycobacteria.

(ii) Solubility of olive oil in acetone as used in partition experiments

In order to determine the amount of acetone required for dissolving a given aliquote of olive oil and in order to determine the amount of acetone to dissolve the supernatant oil containing the acid fast bacilli, solubility experiments for a homogeneous solution were carried out.

- a. 0.5 cc of olive oil required 5 cc acetone.
- b. 0.5 cc of olive oil supernatant after shaking five minutes with 10 cc saline and centrifuging for twenty minutes, required 7 cc acetone.
- c. 0.5 cc olive oil supernatant, obtained from a saline suspension of 10^{-2} mg B.C.G. in 10 cc, required 15 cc acetone.
- d. 0.5 cc olive oil supernatant, obtained from a saline suspension of 10^{-2} mg B.C.G. in 10 cc, required 15 cc of water free acetone.

Summary of Results:

Fifteen cc of acetone are required to dissolve 0.5 cc of olive oil supernatant containing tubercle bacilli. The wetting of the oil interfered with solution by acetone. Analar acetone contains one per cent water and solubility experiments were repeated with this water removed. The results obtained showed that Analar acetone dissolved the oil as efficiently as dry acetone.

D. FILTRATION

The recovery of the acid fast bacilli by filtration from the oil, using a non toxic fat solvent was then investigated. The method depends on the use of a filter membrane on which the microorganisms could be collected during the process of washing away the oil. After filtration, the membrane would be transferred to a suitable medium. For this purpose a membrane was required, the porosity of which would retain the bacilli and allow the fat solvent to pass through as quickly as possible. The membrane, in addition, would need to be thin enough to allow the passage of nutritive substances from the medium to the bacilli retained on the membrane. In his review of filters for bacteriological work, Morton (1937) considered the use of sintered glass as the nearest approach to an ideal filter. It is however, not applicable to this problem. Therefore, three types of material were investigated, cellophane, gradacol membranes and Seitz filter pads. The apparatus used was adapted from a Jenkins filter (1927). A disc of the membrane to be tested was placed between two close fitting rubber sleeves, and the filter was set up in a Kitasato flask, to use suction by means of a water pump, which was attached to a mercury manometer so that the pressure during filtration could be

observed and regulated. The filtering material was investigated for, the ability to allow the fat solvent oil mixture to pass through in a suitable length of time, retention of the bacilli and the adaptability for the cultivation of the organisms.

INVESTIGATION OF FILTER MATERIAL

(a) Cellophane

C.I.L. No 600 PT cellophane discs were cut to fit the filter. Filtration did not occur with water or ether on application of a negative pressure of seventy two cm of mercury for two hours. The use of cellophane for this problem was discontinued.

(b) Gradacol Membranes

Gradacol membranes are thin collodion membranes which can be prepared with a given pore size as described by Elford (1931). These membranes were prepared for the investigation of the retention of acid fast bacilli following the removal of the oil by a fat solvent. The materials and proportions were selected to obtain an average pore size of 0.8 microns.

Material and Methods

The collodion used was Necol C.I.L. 356-A-9 (Nobel formula).

Quantities used:

Solution A	Weight
10 cc amyl alcohol	8.17 gm
Necol (20 gm Necol)	
(20 gm Acetone)	<u>40.00 gm</u>
	48.17 gm

Solution B

Alcohol	5.0 gm
Ether	<u>45.0 gm</u>
	50.0 gm

Equal quantities by weight made up Solution C. 75 cc Solution C were poured into a Petri dish 20 cm in diameter. This was allowed to dry for seventy five minutes at room temperature. The collodion set to a semi opaque film, which was then thoroughly washed in distilled water to further coagulate the membrane and to remove the excess solvents. Coagulation continued during the washing process and the film became quite white and opaque. Three sets of membranes were prepared: membrane alone, membrane embedded with Whatman # 2 filter paper and membrane embedded with filter paper support (Schleicher and Schull # 123). They were stored in distilled water.

Various Properties of the Membrane were Investigated

(1) Determination of the strength of the membranes
was done by filtering ether at determined pressure.

through discs cut to suitable size. The membrane that was not reinforced was too fragile and broke below 20 cm of mercury. The membrane with filter paper reinforcement withstood a pressure of 20-25 cm of mercury and broke at 30 cm. The membrane reinforced with filter paper support withstood a pressure of 60 cm of mercury. It was noted that a certain time elapsed before the filtration of the ether began.

(ii) Determination of the rate of flow of oil and ether through the membrane reinforced with filter paper. The experiments were carried out at a pressure of 20-25 cm of mercury. The rate of flow for 10 cc of ether with 0.5 cc olive oil was approximately 3.5 minutes per cc; for 20 cc of ether with 0.5 cc olive oil was approximately 2.2 minutes per cc. When 0.5 cc of olive oil was laid on the membrane and washed with 10 cc of ether, the rate of flow was approximately 6.0 minutes per cc. The results indicate that the dilution of the oil with larger quantities of solvent increased the rate of filtration. The results show that mixing of the oil and ether should be carried out prior to filtration period. Further studies were not made because of the solubility of the membrane in ether.

(iii) Determination of the retention of the microorganisms by the membrane. A staphylococcus suspension in saline was

filtered through a gradacol membrane. Good growth was obtained from the surface of the membrane and the filtrate proved sterile after two days of incubation.

(iv) Preparation of membranes with a larger pore size.

Filter paper reinforced membranes were prepared by the same technique described above, 1.5 cc of water was added to the solvent mixture to increase the average pore size to 1.5 - 2.0 microns. The filtration of a Staphylococcus saline suspension showed that both the membrane and the filtrate gave a good growth of Staphylococcus in 18 hours. Therefore, this membrane at this porosity was not applicable to this work.

(v) Determination of the solubility of membranes.

The membranes on removal from the apparatus, following the filtration of the ether appeared to be partly dissolved. The solubility of the membranes in various fat solvents was therefore investigated, and for this purpose the membrane, which was not reinforced with filter paper or filter paper support, was exposed to either acetone, benzene, benzin or ether for varying lengths of time. The results are recorded below.

Fat Solvent

Time of Exposure and Solubility

Acetone	2 minutes, completely dissolved
Benzene	2 hours, not affected
Benzin	2 hours, not affected
Ether	20 minutes, 75 per cent dissolved

Although acetone is the most desirable solvent for this work, it could not be used for the filtration through collodion membranes. It is probable that the ether filtration recorded in experiment (ii) was accomplished by the partial dissolving of the membrane. Benzoin and benzene did not appear to exert any solvent action on the collodion membranes and they also exert a very low lethal action on the mycobacterio.

(vi) Determination of the rate of filtration of fat solvents which did not dissolve the gradacol membrane. Benzoin and benzene would not filter through the collodion membrane even at a pressure of sixty cm of mercury for up to two hours. They appeared to be impermeable and the solvent seemed to exert a swelling action on the membrane. The addition of 0.5 cc of olive oil to 10 cc of benzene prior to filtration did not make the membrane permeable.

SUMMARY OF RESULTS

In order to determine the pore size of the collodion membrane for retention of microorganisms, it has been shown that a membrane with an average pore size of 0.8 microns retained Staphylococcus from a saline suspension. Staphylococcus was not completely retained by a membrane of an average pore size of 1.5 - 2.0 microns.

Collodion membranes are not suitable for the recovery of acid fast bacilli from oil by a non toxic fat solvent because the collodion is soluble in acetone and ether and is impermeable to benzine and benzene even at a pressure of sixty cm of mercury for two hours. Until these membranes can be altered either to withstand solution by acetone or ether, or to allow the filtration of benzene or benzine, they cannot be used for the recovery of the organisms from the oil.

(c) Seitz Pads

Seitz filter pads are made of asbestos and are obtained in discs in two grades of porosity. The E. K. (Entkeimung) grade is used in bacterial filtration.

Because of the difficulty of obtaining desirable filter membranes, Seitz filter pads were used to demonstrate the collection and growth of the tubercle bacilli until a more suitable material could be found; their disadvantage is that they cannot be applied to culturing on solid media since the pad is too thick. For experimental purposes the pads were cut down to fit a Jenkins filter funnel and following the filtration the pads were removed and planted in Dubos liquid medium. Filtration occurred rapidly with an oil and ether solution, on the application of a negative pressure of forty cm of mercury.

(1) The retention of viable acid fast bacilli by the Seitz pads, following the collection of the bacilli in oil from a saline suspension was studied. This was demonstrated by the cultivation of the pads in albumin tween liquid medium.

Material and Method

Five samples of 0.2 mg of B.C.G. were suspended in 10 cc of saline. 0.5 cc of olive oil was used for the partition of the bacilli from the saline suspension. The solutions filtered, together with the growth obtained, are summarized in Table XIII. The E.K. pads were removed from the filter and planted in Dubos liquid medium.

Table XIII

Solutions filtered and growth obtained from tubercle bacilli collected on Seitz pads

Sample	Solution filtered	Wash*	Growth in 14 days in Dubos medium
1.	Saline oil mixture	10 cc ether	0
2.	Saline oil mixture	10 cc acetone	(+)
3.	Oil supernatant	10 cc acetone	(+)
4.	Oil supernatant	10 cc acetone with 0.2% Tween	+++
5.	Saline suspension	none	+++

*Applied to the Seitz filter pad to dissolve the oil. The number of +'s represents the amount of growth obtained as estimated by the microscopic examination of the liquid medium. (+) indicates an occasional bacillus per field.

The experiment was repeated using an inoculum of 0.1 mg of B.C.G. in ten cc saline, and varying quantities of fat solvent were used in washing. The solutions filtered and the growth resulting is summarized in Table XIV.(a)

Table XIV (a)

Solutions filtered and growth obtained from tubercle bacilli collected on Seitz pads continued

Sample	Solution filtered	Wash	Growth in days in Dubos medium	
1.	Oil supernatant	10 cc acetone	rare (+) ++	7 days 14 days 21 days
2.	Oil supernatant	15 cc acetone	rare + ++	7 days 14 days 21 days
3.	Saline suspension	none	+ ++ ++	7 days 14 days 21 days

Summary of Results

It has been illustrated that the partition of acid fast bacilli from a suspension by means of an oil is feasible; it was possible to retain the organisms on a filter, by the removal of the oil with a fat solvent and then to grow the bacilli collected on the filter pad. The

absence of growth when ether was used as the fat solvent is probably due to the toxic action of the ether on the bacillus. The resultant growth from pads through which saline suspension was filtered indicates that there are no inhibitory substances present in the Seitz pads which exert a toxic effect on the growth of the tubercle bacillus. Tween 80, as discussed previously, probably serves to facilitate the separation of the microorganisms from the oil or dispersion of the organisms into the medium from the pad. There is no significant difference between the use of ten and fifteen cc of acetone for washing away the oil during filtration. The low growth without Tween might be due to retention of the organisms on the pad.

(ii) Quantitative estimation of growth of virulent tubercle bacilli.

Virulent tubercle bacilli were partitioned from a ten cc saline suspension into 0.5 cc olive oil. The oil containing the organisms was separated from the saline by filtration through a water wet filter paper Whatman #2. Thorough separation of the oil from the saline was determined by testing the filtrate with sodium hydroxide for the presence of soap; no soap was formed. The oil was then transferred to a Jenkin's filter containing a Seitz filter pad; this was washed with fifteen cc of acetone

to allow the bacilli to be separated from the oil and collected on the pad. This procedure was carried out for several dilutions of organisms. Control experiments were set up by filtering saline suspensions of organisms. The Seitz pads were removed from the filter and planted in Dubos liquid medium and the results are recorded in Table XIV (b).

Table XIV (b)

Growth of virulent tubercle bacilli from Seitz pads in Dubos liquid medium

Growth in days	10^{-3}	Inoculum in $\frac{\text{mg}}{10^{-4}}$	10^{-5}
7	+	0	0
14	+	0	0
21	++	0	0
Control			
7	+	0	0
14	++	0	0
21	++	0	0

++= 10-100 bacilli per field, += 1-10 bacilli per field, 10^{-5}mg = approximately 100,000 bacilli.

Summary of Results

The isolation of virulent tubercle bacilli was obtained by the partition of the bacilli in oil from a suspension, and their collection by filtration with the use of a non toxic fat solvent to remove the oil and shown by growth from the filter membrane in albumin tween medium. Seitz filter pads cannot be used to evaluate the collection of small numbers of tubercle bacilli by the oil partition method, because they are unsuitable for culturing on solid media.

E. DUBOS MEDIUM

Virulent tubercle bacilli are among the least exacting of the pathogenic microorganisms in their growth requirements. They are able to yield an abundant growth in simple synthetic media by synthesizing their structural and metabolic constituents from a few mineral salts and organic components. On the other hand it is often quite difficult to initiate the growth of small inocula of these organisms in vitro. Furthermore the strains of human, bovine and to a lesser extent avian bacilli, multiply extremely slowly even in media enriched with growth promoting substances present in serum, egg yolk, potato extract and milk. These characteristics render difficult the application of the tubercle bacilli to quantitative experimental procedures. The widely used egg media such as Lowenstein and Petraghiani lack the ability to promote both rapid growth and the multiplication of small inocula. In order to overcome some of these difficulties a study of Dubos medium was carried out.

Dubos (1945) described an albumin tween medium which produced diffuse and fairly rapid submerged growth of the tubercle bacilli. There have been several modifications described for use in different types of bacteriological work.

The property of diffuse growth of virulent and

avirulent tubercle bacilli was achieved by the addition of Tween, a synthetic detergent to a liquid base. Tween 80 had the remarkable property of wetting the surface of the tubercle bacilli and rendering them dispersible in water.

The concept of diffuse growth for the tubercle bacilli is not new. Besredka (1921) obtained rapid and diffuse growth by diluting sterile egg yolk unheated to ten volumes of distilled water. Boissevain (1931) grew tubercle bacilli diffusely in Long's synthetic medium containing cephalin or lecithin. Larson and Montauk (1923) obtained diffuse growth with soaps. This was however accompanied by a loss of pathogenicity.

The Tween albumin medium was developed to obtain more adequate methods for the bacteriological and immunological study of tuberculosis. Although among the least fastidious of the microorganisms in their metabolic requirements, in vitro growth of these organisms has necessitated relatively large inocula and long periods of incubation even in the presence of growth promoting substances such as serum, egg yolk and potato extract. The medium consists of a basal component to which Tween and albumin were added.

Basal Component

Briefly its composition contained mixtures of Na

and K phosphates which provided a satisfactory buffer system. A slightly acid reaction is desirable since initiation of growth appears to be optimum at pH 6.4 - 6.8. Enzymatic digests of casein were found to supply the most favorable source of nitrogen for the growth of the tubercle bacilli. The casein digest medium is fortified by the addition of asparagin, glucose or glycerol. These substances increase the total amount of growth but do not appear to facilitate its initiation. The basal medium also contained traces of the metallic salts, MgSO₄, CuSO₄, ZnSO₄ and CaCl₂.

The addition of certain water soluble lipids was found to exert two actions on the growth of the mycobacteria. First, the wetting of the bacilli thus rendering them dispersible in water which allows diffuse homogeneous growth to take place. It is thought that this may also stimulate the growth by facilitating exchanges between the organisms and the environment. Second, the enhancement of growth by a readily available non toxic source of long chain fatty acids. The wetting agents consist of long chain fatty acids which are used for certain industrial purposes because of their surface activity (detergency and wetting properties). Of the different preparations tested those marketed under the name of Tween 80 have been found to be most satisfactory and give the most reliable results. Tween 80, a

polyoxethylene derivative of sorbitan monooleate is a viscous amber fluid which is soluble in all concentrations in water and in the presence of other constituents of the basal medium. It is stable to autoclaving. However, aqueous solutions of Tween undergo very slow hydrolysis with the release of some free fatty acid. There is an inhibitory effect on the growth of small inocula due to this content of unesterified oleic acid. Three per cent of the oleic acid (0.6 per cent of the Tween by weight) in the commercial product Tween is unesterified. Davis (1947) described a method for the chemical extraction of the unesterified fatty acid by which 80-90 per cent is removed. The toxicity of the fatty acid is however generally abolished by the use of crystalline albumin.

Whole serum has been employed for many years in a variety of media designed for the cultivation of the tubercle bacilli. It is known that purified serum albumin enhances the growth of these organisms in synthetic media. This growth promoting activity is greater than that of extracts of egg yolk. The growth appears earlier, is more abundant and takes place from smaller inocula (Boissevain, 1940, Powelson and McCarter, 1944). Bovine albumin was selected for use in this media since it demonstrated the same effect as human and equine serum and was available as a commercial product. Bovine albumin can be obtained

as a desiccated product (designated as serum fraction V) prepared from plasma by alcoholic precipitation or as a more highly purified crystalline product. At first it was assumed that the albumin like most known growth factors contributed a nutritive which was absorbed by the bacilli. It has however been demonstrated that the serum albumin possesses a protective growth factor. By binding traces of fatty acid in the media, it permits initiation of growth by the smallest possible inocula of the tubercle bacilli. The bovine albumin fraction V exerts two actions. Firstly, it protects the tubercle bacilli against the bacteriostatic and bactericidal action of a variety of toxic agents. The detoxifying power of the albumin is destroyed by the enzymatic digestion or heating, as soon as the integrity of the molecule is destroyed. It is usually prepared by filtration of a five per cent solution through Sela's porcelain candles and added aseptically to the culture medium. Secondly there is present in serum and in bovine fraction V, a thermostable fraction soluble in ethanol which markedly increases the growth yield of the tubercle bacilli. This action is not exerted by highly purified crystalline albumin. Commercial albumin fraction V contains a small amount of lipase which releases free fatty acid in bacteriostatic concentrations from Tween 80. In one to two weeks the lipase can hydrolyze enough Tween

to exceed the binding capacity of the albumin. The lipase could be destroyed by heating for thirty minutes at 56° C. To avoid coagulation it is necessary either to neutralize the albumin pH 5.6 or to dissolve in two per cent saline.

Semisynthetic medium containing glucose, Tween and bovine serum albumin fraction V, sterile incubation for fourteen to twenty one days at 37° C destroyed the capacity of the medium to support the growth of even large inocula of the tubercle bacilli. Because of the interaction of the components of the medium, the albumin and Tween are generally added to the basal liquid just prior to use. Grown diffusely in submerged liquid culture in the presence of Tween 80, the mycobacteria retain their characteristic morphology and staining. This is true even when maintained over a year with repeated transfers in liquid media. Return of these cultures at any time to standard media (egg yolk) causes reversion to a granular type of growth. In addition mycobacteria grown in the presence of Tween 80 are extremely virulent for mice, guinea pigs, chick embryos and are able to elicit the production of agglutinins for the homologous cultures. No significant proportion of cells in a seven day culture in Tween albumin media is sterile. This contrasts with the high proportion of sterile cells found in surface growth. This feature alone would make its use advisable

for experimental procedures.

There are several drawbacks for its application to diagnostic primary isolation. Tween 80 is rapidly hydrolyzed in the presence of tissues or tissue fluids and there is no convenient technique for abolishing the lipolytic activity of these materials. Also, the growth requirements of the different strains of tubercle bacilli which occur in pathological material vary. Corper and Cohn (1948) maintained that the use of this medium was encumbered by a technique which might lead to erroneous deduction for practical clinical purposes. They did not consider that liquid culture media were as delicate or as expeditious for use in the cultivation of pathological material, as are the solid media.

Dubos (1947) stated that dispersed growth is not necessarily an advantage for diagnostic work. Virulent tubercle, inoculated into casein digest albumin media without Tween, grow in the form of long strands; because of the large size and characteristic morphology, the strands can be identified under the low power of the microscope.

The albumin Tween media were studied for the cultivation of tubercle bacilli with regards to growth rate and initiation of growth from minimal inocula; it was proposed that the tubercle bacilli collected on a suitable filter membrane by the method described in this thesis be cultivated on solid albumin tween media.

Dubos (1947) liquid media was used for the cultivation of tubercle bacilli. The formula and preparation of the medium is as follows:

BASAL MEDIUM

KH ₂ PO ₄	1.0 g
Na ₂ HPO ₄ .12H ₂ O	6.3 g
Asparagin	1.0 g

This was added to 100 cc of distilled water and was dissolved by heating.

To this was added

Distilled water	850 cc
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Enzymatic digest of casein (Trypticase - a pancreatic digest of casein)	1.0 g	(20cc of a 5 per cent solution)
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Ferric ammonium citrate	0.05 g
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MgSO ₄ .7H ₂ O	0.01 g	(one cc of a 1 per cent solution in distilled water)
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CaCl ₂	0.0005 g	(one cc of a 0.05 per cent solution in distilled water)
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ZnSO ₄	0.0001 g	(one cc of a 0.01 per cent solution in distilled water)
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CuSO ₄	0.0001 g	(one cc of a 0.01 per cent solution in distilled water)
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The pH was adjusted to 6.5 with N HCl and Brom cresol purple. The medium was autoclaved in 90 cc lots.

ALBUMIN

Bovine plasma fraction V was prepared in a five per cent solution in two per cent sodium chloride, to prevent coagulation, was filtered through a Seitz filter pad and heated to 56°C for thirty minutes to inactivate the serum lipase.

TWEEN 80

The detergent was prepared in a 10 per cent solution in distilled water and autoclaved.

GLUCOSE

A 10 per cent solution was prepared and autoclaved.

(a) Liquid media

The liquid medium was made up as follows:

90 cc of basal medium

10 cc of 5 per cent albumin

0.2 cc of 10 per cent Tween 80

5.0 cc of 10 per cent glucose

All four constituents were prepared separately and added together just prior to use, for reasons given in the discussion of the medium. It was dispensed in five cc lots in screw capped test tubes 18 mm in diameter.

The inoculum was made up as described in the preparation of inoculum and was expressed in terms of dry

weight or number of bacilli as determined by counting chamber. The medium was inoculated with calibrated Pasteur pipettes. Growth in liquid medium was examined microscopically and recorded in bacilli per high power field.

The media were investigated for the growth rate and the initiation of growth from minimal inocula of acid fast bacilli.

(1) The growth rate of tubercle bacilli in Dubos albumin tween liquid medium was compared to that obtained in Petragnani's egg medium. The results are recorded in Table XV.

Table XV

The growth rate of mycobacteria in Dubos albumin tween medium compared to Petragnani medium

Medium	Growth in days	B.C.G. mg						
		10 ⁻²	10 ⁻³	10 ⁻⁴	10 ⁻⁵	10 ⁻⁶	10 ⁻⁷	10 ⁻⁸
Dubos liquid	9	-	+++*	++	++	0	-	-
	14	-	+++	++	++	++	-	-
	21	-	+++	+++	+++	+++	-	-
Petragnani	9	-	+++°	+	+	0	-	-
	14	-	+++	+	+	0	-	-
	21	-	+++	+++	+++	+++	-	-
Dubos liquid	6	+	(+)	sl	0	0	0	0
	14	+++	+++	++	+	++	(+)	0
Petragnani	6	+++	0	0	0	0	0	0
	14	+++	++	++	++	++	0	0

*Growth +++ = 100 - 500 bacilli per field, ++ = 10 - 100 bacilli per field, +=1 - 10 bacilli per field, (+) = occasional bacillus per field. ° the number of +'s indicated the gross appearance of growth, (+) = slight, 0 = no growth, - = omitted.
of gro

Summary of Results

Although it is evident that a more rapid growth takes place on Dubos medium the initiation of growth from smaller inoculum leaves little to choose between the two media used. The results are not very encouraging as inocula with quite large numbers of organisms give no growth.

- (ii) The effect of the presence of Tween 80 on the growth rate of virulent tubercle bacilli was investigated.

The Tween allows a diffuse growth to take place, but dispersion is not required for our purpose in the cultivation of the tubercle bacillus. The medium was prepared by the standard formul and the inoculation of the medium was carried out as described previously. The results are recorded in Table XVI.

Table XVI

The effect of Tween 80 on the growth rate and size of inoculum of virulent tubercle bacilli

	Growth in days	inoculum in mg							
		10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}
Tween	7	-	0	0	-	0	0	0	0
	14	-	+	0	-	0	0	0	0
	21	-	++	0	-	0	0	0	0
No Tween	7	-	0	0	-	0	0	0	0
	14	-	+++	+	-	+	0	0	0
	21	-	+++	++	-	+	+	(+)	0
Tween	14	-	-	0	0	0	0	0	0
	28	-	-	+	(+)	0	0	0	0
No Tween	14	-	-	+	+	0	0	0	0
	28	-	-	++	++	+	(+)	0	0
Tween	10	-	+	0	0	0	0	0	0
	21	-	++	0	0	0	0	0	0
No Tween	10	-	+	+	+	0	0	0	0
	21	-	++	++	+	+	0	0	0
Tween	7	+	-	0	0	0	0	0	0
	14	++	-	++	+	0	0	0	0
No Tween	7	+	-	0	0	0	0	0	0
	14	++	-	++	+	+	(+)	0	0
Tween	10	0	-	0	0	0	0	0	0
	14	+	-	0	0	0	0	0	0
No Tween	10	+	-	0	0	0	0	0	0
	14	+	-	+	+	+	+	0	0

For key to readings refer to footnote of Table XV

Summary of Results

Growth in media containing Tween 80 was submerged and diffuse. In the media containing no Tween the growth occurred in granular masses somewhat resembling that of certain Streptococci; the liquid above remained clear and shaking did not bring about a homogeneous suspension. It is evident that the Dubos medium without the Tween supported the growth of the mycobacteria better, both from the aspect of rapidity of growth and the initiation of growth from small inocula. Dubos (1947) stated that submerged diffuse growth is not necessarily an advantage for diagnostic work. From the results, it would seem that the medium without the Tween would be preferred for this work. The antagonistic effect of the Tween is perhaps due to the hydrolysis of the Tween to a fatty acid which is toxic to the tubercle bacillus. While the toxic effect of the Tween makes it detrimental for inclusion in a diagnostic medium, it is still invaluable for many types of experimental work, especially for the production of an easily dispensable suspension of mycobacteria which grows fairly rapidly.

(b) Solid media

a. Agar

Because of the advantages of a solid medium for diagnostic primary isolation and the success of Dubos liquid medium for giving fairly rapid growth of minimal inocula. Dubos liquid medium with agar was investigated for use as a solid medium.

The following medium was prepared

Dubos Basal medium	90 cc (with 0.05 g ferric ammonium citrate per litre)
Agar (Difco)	1.5 g
Tween 80 10 oer cent	0.2 cc
Autoclaved and cooled to	60°C

Add:

Albumin	10 cc of 5 per cent
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This was poured into petri dishes and slants were prepared.

The growth rate of dilutions of tubercle bacilli on Dubos agar medium was compared with that of Petragnani's medium.

The results are recorded in Table XVII.

Table XVII

The effect of agar in Dubos medium on the
Growth of acid fast bacilli

Medium	Growth in days	B.C.G. in mg							
		10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}
Dubos Agar	7	+	+	0	0	-	-	-	-
	14	+	+	+	+	-	-	-	-
Petragnani	7	++	+	+	0	-	-	-	-
	14	+++	+++	+++	+++	-	-	-	-
Dubos Agar	14	-	++	-	+	+	0	0	0
	21	-	++	-	+	+	0	0	0
Petragnani	14	-	++	-	+	+	0	0	0
	21	-	++	-	+	+	+	0	0
Dubos Agar	7	-	++	++	0	0	0	0	0
	14	-	++	++	+	+	0	0	0
Dubos Liquid	7	-	++	+	(+)	0	0	0	0
	14	-	++	++	++	++	+	(+)	0
Petragnani	7	-	++	++	+	0	0	0	0
	14	-	++	++	++	++	0	0	0
Dubos Agar	7	-	+	(+)	0	0	0	0	0
	14	-	++	+	+	+	0	0	0
Petragnani	7	-	+	0	0	0	0	0	0
	14	-	++	++	+	+	+	0	0
Dubos Agar	7	-	+	-	(+)	(+)	0	0	0
	14	-	+	-	(+)	(+)	0	0	0
Petragnani	7	-	0	-	0	0	0	0	0
	14	-	0	-	0	0	0	0	0
Dubos Agar	7	++	0	0	0	0	0	0	0
	14	+++	++	0	0	0	0	0	0
Dubos Liquid	7	++	++	++	0	0	0	0	0
	14	+++	+++	++	+	(+)	0	0	0

For key to readings refer to footnote of Table XV

DISCUSSION

Dubos (1947) advocated the addition of 1.5 per cent agar to the constituents of the liquid medium for the production of a solid culture medium. It is well known that the addition of agar adds no beneficial material to the nutrient qualities of a medium for the cultivation of the tubercle bacilli and may even be detrimental. The inhibitory effect of the agar has been shown by Ley and Mueller (1946) to be due to long chain fatty acids which are removable by extraction with methanol. Dubos (1947) reported that the toxic effect could also be neutralized by the addition of 0.5 per cent serum albumin. The use of agar required a higher concentration of iron than is present in liquid media, since agar can bind the iron in a form which is no longer available to the microorganisms for growth. Dubos (1947) reported that growth of the bacilli present in human pathological material could be readily obtained on albumin tween agar media and evidence was accumulating that the use of these media would prove useful for routine bacteriological diagnosis.

Summary of Results

An inocula of 10^{-6} mg produced satisfactory growth in fourteen days in Dubos media containing agar. The agar

media did not disclose any advantages over that of the standard egg medium, Petragnani. In fact the egg medium is to be preferred since it supported growth up to an inoculum of 10^{-7} mg.

The experiments in which the inoculum was also set up with Dubos liquid medium establish that the use of the liquid medium is superior both for the rapidity of growth and the size of inoculum which initiates growth. In one set of experiments growth in Dubos tween albumin liquid media was demonstrated with an inoculum of 10^{-8} mg in fourteen days.

b. Whole egg and egg white

In an effort to modify the Dubos medium by solidification, without the impairment to the size of inoculum which would initiate growth, the use of whole egg and egg white was studied.

Schwabacher (1937) reported that egg white had no marked bactericidal action and that the failure of acid fast bacilli to grow in its presence was due to its high alkalinity; if this was neutralized the development of the bacilli occurred. However, the nutritive value of neutral egg white was much less than that of egg yolk.

The following medium was prepared.

1. The standard medium containing basal component with 5 percent albumin, 0.02 per cent Tween and 10 per cent

glucose was added to an equal quantity of whole egg slightly beaten. This was then tubed and inspissated at 80° C for fifteen minutes. The whole egg medium was prepared with and without Tween.

3. The standard Dubos medium containing the basic ingredients was added to an equal quantity of white of egg slightly beaten. This was prepared without adjusting the pH and with a pH of 6.5 It was inspissated at 80° C for fifteen minutes.

The growth rate of dilutions of tubercle bacilli on the whole egg and egg white media was compared with that on Petragnani's medium. Although there was no marked difference in the growth rate obtained between the whole egg Dubos medium with and without Tween the gross appearance of the colony varied. On the egg medium containing Tween the colony appeared as a soft discrete semitransparent colony resembling Streptococcus. On the egg medium without the Tween the colony was small dry, scaly with a corrugated surface. The results are tabulated in Tables XVIII, XIX, XX and XXI.

Table XVIII

The effect of whole egg and egg white in Dubos medium on the growth rate of the tubercle bacilli

Medium	Growth in days	B.C.G. in mg						
		10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}
Dubos	14	+++	++	0	0	0	0	0
Whole egg	21	+++	+++	+++	++	0	0	0
Tween	28	+++	+++	+++	+++	+++	+++	0
Dubos	14	(+)	0	0	0	0	0	0
Egg white	21	+	(+)	0	0	0	0	0
alk pH	28	+	(+)	0	0	0	0	0
Dubos	14	+++	++	(+)	(+)	0	0	0
Liquid	21	+++	+++	+	(+)	0	0	0
	28	+++	+++	+	+	0	0	0

For key to readings see footnote of Table XV

Table XIX

The effect of whole egg and egg white in Dubos medium on the growth rate of the tubercle bacilli

Medium	Growth in days	B.C.G. in mg							
		10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	5×10^{-8}
Dubos	7	+	+	+	+	0	0	0	0
Whole egg	14	+	+	+	+	+	+	0	0
No Tween	21	+++	+++	+++	++	+++	++	+	+
Dubos	7	+	0	0	0	0	0	0	0
Egg white	14	+	+	0	0	0	0	0	0
pH 6.5	21	+++	++	++	++	+	+	0	0
Petragnani	7	+	+	0	0	0	0	0	0
	14	+	+	+	+	+	+	0	0
	21	+++	+++	+++	+++	++	++	+	(+)

For key to readings see footnote of Table XV

Table XX

Comparison of the growth rate of acid fast bacilli in Dubos whole egg solid medium as compared to Petragnani

Medium	Growth in days	B.C.G. mg					
		10 ⁻²	10 ⁻³	10 ⁻⁴	10 ⁻⁵	10 ⁻⁶	10 ⁻⁷
Dubos	14	+++	++	++	++	0	0
Whole egg	28	+++	+++	+++	+++	+	+
Petragnani	14	+++	++	0	0	0	0
	28	+++	+++	++	+	+	0

For key to readings see footnote of Table XV

Table XXI

The effect of Dubos whole egg medium on the initiation of growth of minimal inocula

Medium	Growth in weeks	Numbers of virulent tubercle bacilli					
		50	40	30	20	10	5
Dubos	4	0	0	0	0	0	0
Whole egg	6	+	0	0	0	0	0
Petragnani	4	0	0	0	0	0	0
	6	++	0	(+)	0	0	0
Dubos	4	+	0	0	0	0	0
Whole egg	5	+	+	0	0	0	0
	6	+	+	+	+	0	0
Petragnani	4	0	0	0	0	0	0
	5	+	0	0	0	0	0
	6	+	0	0	0	0	0

For key to readings see footnote of Table XV

Summary of Results

The use of whole egg added to the Dubos liquid medium for solidification appears to be advantageous and would appear to be suitable for the routine diagnostic cultivation of the mycobacteria. An inoculum of 10^{-4} mg B.C.G. on the whole egg medium gave rise to growth in seven days, as compared with an inoculum of 10^{-2} mg on Petragnani's medium and 10^{-1} mg on the egg white medium for the same length of time. The whole egg incorporated in the Dubos medium can be used for the cultivation of minimal inocula, as demonstrated by the repeated growth obtained with 10^{-8} mg. The growth in the Dubos whole egg medium is usually accompanied by the appearance of the colony in a shorter time. This medium appears to have good possibilities but further investigations including pathological material are necessary for it to be accurately assessed.

The use of egg white for solidification gave a white slant on which the acid fast bacilli did not grow with great ease. Although the pH appeared to be an important factor, since much better growth of the tubercle bacilli was obtained when the pH of the media was adjusted to 6.5, it is probable that other factors have an important bearing on the efficacy of growth. The egg white medium is definitely not as promising as that of whole egg medium from the point of view of rate of growth,

10^{-7} mg requiring twenty one days, and the initiation of growth from minimal inocula.

F ANIMAL INOCULATION

Animal inoculation control experiments with reference to the partition of tubercle bacilli by oil from a saline suspension, were carried out to correlate the number of bacilli which cause an infection in an animal, with the size of inoculum which would yield growth in a culture medium. These experiments were carried out with minute inocula.

Procedure

Virulent tubercle bacilli, estimated quantitatively by counting chamber, were suspended in vaccine bottles containing ten cc saline to which 0.5 cc olive oil was added. This mixture was shaken for five minutes and centrifuged in an inverted position for ten minutes. The oil supernatant was withdrawn into a syringe by means of a long needle. Fifteen guinea pigs were injected subcutaneously into the groin with the oil supernatant. Five sets of inocula were prepared and three guinea pigs were injected with each set. After eight weeks the guinea pigs were autopsied and examined for tuberculous lesions. The amount of lesions in various organs is recorded in Table XXII. Cultures from the organs were planted on Petragnani's medium.

Number of Bacilli in Saline Suspension

Set 1.	1 - 10	organisms
Set 2.	10 - 100	"
Set 3.	100 - 1,000	"
Set 4.	1,000 - 10,000	"
Set 5.	10,000 - 100,000	"

Table XXII

Amount of lesions in various organs in Guinea Pigs from
injection of tubercle bacilli partitioned in oil

Number of bacilli	Guinea Pig #1	Guinea Pig #2	Guinea Pig #3
1 - 10	Spleen normal Liver normal Inguinal gland normal	Spleen normal Liver normal Inguinal gland normal	Spleen normal Liver normal Inguinal gland enlarged and caseous
10 - 100	Spleen several nodules Liver normal Inguinal gland swollen and caseous	Spleen enlarged many nodules Liver normal Inguinal gland swollen and caseous	Spleen normal Liver normal Inguinal gland normal
100 - 1000	Spleen enlarged many nodules Liver several yellow patches Inguinal gland swollen and caseous	Spleen enlarged many nodules Liver two or three patches Inguinal gland normal	Spleen enlarged several nodules Liver normal Inguinal gland caseous
1,000 - 10,000	Spleen enlarged many nodules Liver several yellow patches Inguinal gland swollen and caseous	Spleen enlarged many nodules Liver a few patches Inguinal gland swollen and caseous	Spleen enlarged many nodules Liver scattered areas of lesions Inguinal gland normal
10,000 - 100,000	Spleen many nodules Liver many patches Inguinal gland swollen and caseous	Spleen many nodules and enlarged Liver many lesions Inguinal gland caseous	Spleen enlarged many nodules Liver many patches Inguinal gland caseous

Summary of Results

The tubercle bacilli, partitioned in oil, from a saline suspension containing 10 - 100 organisms or more regularly gave rise to tuberculous infection in guinea pigs. One of three guinea pigs, injected with oil from a saline suspension containing 1 - 10 organisms caused a tuberculous infection, but if these animals had remained longer than eight weeks before autopsy, lesions might have developed.

The results confirmed the efficient collection of tubercle bacilli from a saline suspension by partition with oil. The production of tuberculous infection with such small numbers of organisms demonstrates that no inhibitory action was exerted by the oil on the virulence and visability of the tubercle bacillus.

III CONCLUSIONS

An efficient method for the isolation of tubercle bacilli, by their partition into olive oil from a saline suspension, has been adequately demonstrated by cultural and animal inoculation experiments. The consistent initiation of tuberculous infection in guinea pigs from injections of olive oil, which partitioned very small numbers of tubercle bacilli from a saline suspension, illustrates that the viability and the virulence of the acid fast bacillus is not impaired; and therefore, this method of isolation is applicable to cultural work.

The fat solvent, showing the least lethality on the tubercle bacilli, required for the recovery of the oil collected bacilli, was found to be acetone. Next best were benzin and benzene. Ether is toxic to tubercle bacilli.

The principle of the retention of tubercle bacilli partitioned in oil, by filtration through Seitz filter pads, with the use of a non toxic fat solvent, was established by the growth of the bacilli on the filter pad, when planted in Dubos albumin tween media. However, a more suitable filter membrane is required before this method can be used routinely. Further investigations are being carried out for a filter membrane which would be more convenient and allow the cultivation of smaller numbers of bacilli.

Dubos albumin tween medium solidified by inspissation with equal quantities of whole egg, allows the growth of small numbers of virulent tubercle bacilli; this medium could be used with advantage for the cultivation of the mycobacteria as isolated by the partition method.

A method for the isolation of tubercle bacilli has been demonstrated.

IV

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