THE CULTIVATION OF *M. TUBERCULOSIS* RECOVERED BY OIL PARTITION

by

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The widespread use of mass roentgenographic surveys in the past decade has led to an increase in the proportion of individuals having few or doubtful clinical signs and symptoms of tuberculosis. It is difficult and often impossible to demonstrate the presence of the tubercle bacillus in the excreta of such individuals by the use of cultural methods in common laboratory use. Since recovery of the tubercle bacillus admits of no further uncertainty in the diagnosis, its detection in doubtful cases has an importance which can scarcely be overestimated. Paucity of bacilli is an especial characteristic of the early stages of tuberculosis, and it is particularly in such cases that the conventional laboratory methods for their demonstration are unreliable. As a result, a negative culture is often regarded with doubt if clinical symptoms are suggestive of tuberculosis and no other pathogen has been isolated. A further disadvantage is the length of time required for a positive diagnosis to be made. What is needed, then, is a method by which one can unfailingly, and in as short a time as possible, make distinction between a suspect specimen which contains rare tubercle bacilli, and one which contains no bacilli.

A closer approach to this ideal is provided by the oil partition method, which was developed by Hawirko and Murray (1951, 1954) for the demonstration of the tubercle bacillus in

pathologic material. The essential feature of the method is the spontaneous migration of tubercle bacilli from the water to the oil phase of an oil-water emulsion, this spontaneous migration, or partition, being associated with the hydrophobic and lipophilic properties of the surface of the bacillus. It was demonstrated that oil partition provides a reliable method for the complete collection of small numbers of tubercle bacilli contained in a large volume of fluid, into a small compact volume of oil. Attempts to cultivate the bacilli collected in this manner were not entirely successful, and this was attributed, in part, to the experimental finding that exposure of tubercle bacilli to vegetable oils had a deleterious effect on their ability to proliferate in artificial media. However, it was possible to produce tuberculous lesions in guinea pigs by the injection of oil partitioned tubercle bacilli, which indicated that they were not irreparably damaged by exposure to oil.

This investigation was undertaken in order to find either (a) a means of reducing the damaging action of vegetable oils upon the tubercle bacillus, or (b) a means of restoring their viability after exposure to oil, so that they might be successfully cultivated. Thus, it was hoped, the remarkable capacity of oil partition to collect tubercle bacilli from aqueous suspension could be applied advantageously to the demonstration of small numbers of these organisms in clinical specimens.

REVIEW OF THE LITERATURE

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1. The Development of Methods for the Isolation of M. tuberculosis

Since Robert Koch isolated the tubercle bacillus in pure culture in 1882 and demonstrated that it was the etiologic agent of tuberculosis the technical methods advocated for isolation of the bacillus are remarkable for their number if not for their efficiency. Koch's successful isolation of the bacillus on inspissated blood serum served admirably when contaminating micro-organisms were absent from the material being examined, or were removed by passing the material through the guinea pig. However, as contaminating organisms are seldom, if ever, absent from the pathologic materials in which the tubercle bacillus is most often sought, and as preliminary animal passage prevented any attempt at cultural isolation for a month or more, other methods were sought which would allow the demonstration of the bacillus more rapidly and with more certainty.

The first successful isolation of the tubercle bacillus in pure culture after preliminary decontamination of pathologic material was achieved by Uhlenhuth & Xylander in 1908. These workers treated sputum with Antiformin, the patented name of an alkaline solution of "hypochlorites of soda" which was introduced in Sweden in 1900 as a cleansing agent for brewing vats. In 2-5% concentration, antiformin was lethal towards colon and paratyphoid bacilli, staphylococci, streptococci, meningococci, and pneumococci but tubercle bacilli

and a number of acid-fast saprophytes were refractory to this concentration. Thus, was the use of antiformin as a differential bactericidal agent suggested. It was further observed that antiformin had an excellent dissolving action on the mucoid and cellular elements in sputum and that centrifugation of the dissolved decontaminated sputum served to concentrate the bacilli in the sediment which collected as a result. Uhlenhuth's technique was to add antiformin to sputum in a final concentration of 15% and allow it to act for two to five hours. Following this, the material was centrifuged, the supernatant removed, the sediment washed with physiologic saline and injected into a guinea pig or onto coagulated serum. Uhlenhuth remarked that the viability of the bacillus was in no way depressed by this treatment as judged from the observation that positive cultures were obtained within two to three weeks, and he succeeded in isolating tubercle bacilli from a variety of infected materials.

Griffith (1914) improved this technique by reducing the concentration of antiformin to 5% and the time of exposure to thirty minutes in recognition of the fact that prolonged exposure to antiformin was detrimental to the viability of small numbers of bacilli. It is possible that Uhlenhuth did not recognize the need for this refinement, as his experimental materials most probably contained massive numbers of bacilli, viz.- "Auf diese Weise konnten in zahlreichen Versuchen Reinkulturen von Tuberkelbacillen erhalten werden. Auch aus tuberkulösen Organen - wie z.B. verfaulten

Hühnerlebern - gelang es, die Erreger der Geflügeltuberkulose in Reinkulturen zu züchten".

A second important improvement adopted by Griffith was the use of inspissated egg medium for the cultivation of the bacilli on primary isolation, egg medium having been introduced by Dorset (1902) as being most suitable for cultivation and study of the tubercle bacillus.

Griffith's modification of the antiformin method proved fairly successful in the hands of many investigators, and in particular in his own hands, for the massive study which he performed on the relative incidence of bovine and human types of tubercle bacilli in human disease. This was, indeed, the method of choice until Petroff (1915) devised the well known sodium hydroxide method.

The sodium hydroxide method was accorded almost universal acceptance and although modified slightly by individuals to meet their own requirements is used to this day in its original form. It consists essentially in exposing the material to be cultured for tubercle bacilli to an equal volume of 4% sodium hydroxide for thirty minutes at 37°C. This treatment serves a dual purpose: the specimen is homogenized and contaminating microorganisms are destroyed, leaving the tubercle bacillus intact. The digest is neutralized to free it from alkali and centrifuged to concentrate the bacilli in the sediment which results. The sediment is used for

seeding tubes of inspissated egg medium containing a bacteriostatic dye which is included for the purpose of inhibiting the development of contaminants which may not have been destroyed by the sodium hydroxide treatment. Petroff originally used gentian violet in the egg medium, but this has been superseded by malachite green, as the former dye imparts too deep a colour to the medium and is bacteriostatic toward small inocula of tubercle bacilli. This, and the introduction of more complex egg media have been the only significant changes suffered by Petroff's method since it was first announced in 1915.

It was soon apparent that sodium hydroxide treatment impaired the staining reactions and viability of tubercle bacilli, especially on prolonged contact or at higher temperatures. This fact spurred the efforts of numerous workers in the search for a material which would be truly selective with regard to its lethality for contaminating microorganisms and at the same time possess a lytic effect on sputum. In all these studies the principle borne in mind was that initiated by Uhlenhuth and improved by Griffith and Petroff respectively, i.e. chemical decontamination and homogenization of the specimen followed by centrifugation for enrichment of the inoculum.

Bossan and Baudy (1922) were the first to use sulphuric acid and Löwenstein (1924) improved their method. He advocated the addition of equal volumes of 15% sulphuric acid to sputum and

allowed it to act for fifteen minutes. Corper (1927) recommended 6% sulphuric acid. Its action was not as rapid as that of sodium hydroxide but it was relatively less lethal for the tubercle bacillus. Further, the mucoid substances in sputa are slightly soluble in strong, but not in dilute acids. Therefore, upon dilution of the sulphuric acid, a precipitate forms in the digest which aids in carrying down the tubercle bacilli upon centrifugation.

The use of sulphuric acid in 6% concentration has become fairly widespread but as it is a rather inefficient sputum solvent it is recommended chiefly for "thin" specimens which do not contain a large amount of pus or other tenacious material. It is also important that the exposure of the tubercle bacilli to the acid be kept as short as possible since prolonged contact is detrimental (Willis & Cummings 1952).

Many other materials have been investigated but have not achieved widespread use despite claims of superiority made by their advocates. This group includes oxalic acid, hydrochloric acid, ammonium carbonate and ammonium hydroxide (Corper 1927; Corper & Uyei 1927, 1930, 1934), a ferrous sulphate - hydrogen peroxide mixture with traces of sulphuric acid (Jungmann, 1938; Nassau 1942). Oxalic acid was preferred to sulphuric acid because it is stable in solution and can be obtained as a pure crystalline compound. Further, it is reported to be innocuous for small numbers of tubercle bacilli, is a good differential antiseptic, but

is a poor sputum solvent. Taylor (1950) reports that a simplification of the oxalic acid method proved superior to a number of other agents for the preliminary treatment of lymph nodes, lung and spleen tissue for the recovery of Myobacterium johnei. Hydrochloric acid in 3% concentration was recommended for thin clear sputa just as sulphuric acid. Ammonium hydroxide is an efficient germicide but too toxic toward the tubercle bacillus in a concentration and time interval necessary to destroy contaminants. Ammonium carbonate promoted rapid liquefaction of sputa and was reportedly not lethal to small numbers of bacilli. The combination of ferrous sulphate, hydrogen peroxide and a trace of sulphuric acid is a very potent solvent for mucus, the reaction being dependent on the presence of all three agents and peculiar to all mucous secretions. The mode of action was thought to be due to oxidation of certain chemical groups responsible for the mucinous character. Smears made from the centrifuged deposit after treatment with this agent contained from two to ten times more bacilli than the direct smear, and the staining properties of the bacilli were not impaired. One hour exposure to the agent was not lethal for the bacilli, while three hours exposure impaired the viability considerably.

A noteworthy improvement in the methods commonly used for preliminary treatment of sputa followed the introduction by Corper and Stoner (1946), of trisodium phosphate for this purpose. The most desirable features of this material are firstly, that it is relatively less lethal for small numbers of tubercle bacilli and

secondly, that it is a pure crystalline compound of standard chemical composition. For the cultural diagnosis of tuberculosis it was recommended that equal volumes of sputum and 10% trisodium phosphate (23% Na_3PO_4. 12 H_2O) be mixed and allowed to remain at 37°C for 18-24 hours. Corper and Stoner reported that small numbers of tubercle bacilli may remain in contact with 5% trisodium phosphate for as long as one week at room temperature without serious impairment to their viability. On the other hand, several days at room temperature or 24 hours at 37°C is sufficient to destroy most contaminants. Corper reported that 10^{-6} mgm of a virulent strain of the tubercle bacillus suspended in physiological saline was sterile after 4 days at 37°C while growth was obtained from a similar suspension after 2 weeks exposure to trisodium phosphate. In a comparison of the action of trisodium phosphate, sodium hydroxide and oxalic acid on Penicillium notatum, Actinomyces griseus and an unnamed acid fast saprophyte it was found that 10% trisodium phosphate killed P. notatum and the acid fast saprophyte after $1\frac{1}{2}$ hours at 37°C; A. griseus survived 24 hours exposure. The reverse was true when these organisms were exposed to 5% oxalic acid for one hour; A. griseus was killed but P. notatum survived 4 hours exposure. Three percent sodium hydroxide killed P. notatum and the acid-fast saprophyte but not A. griseus. From these findings, Corper suggests that it may be necessary at times to use alkali and acid treatment to destroy mould contaminants.

An obvious advantage in the use of trisodium phosphate is

that pathological specimens which must be transported over a distance to the laboratory may have the digestant agent added upon collection, thus saving time and preventing possible gross contamination of the specimen (Willis and Cummings 1952).

2. The Limitations of Conventional Methods

Corper (1946) has stated "The diagnostic culture test consists of several essential features, the two most important being the preliminary treatment of the specimen to destroy contaminating organisms and the choice of a good nutrient medium capable of supporting the growth of very small numbers of tubercle bacilli".

It is in both these respects that the common laboratory methods for the isolation of the tubercle bacillus are apt to fail when a specimen contains very small numbers of bacilli. Refinements in techniques of clinical diagnosis of recent years indicate the need for comparable refinements in the laboratory diagnosis, upon which the absolute proof of the presence or absence of tuberculosis depends.

(a) Digestion of pathologic material by chemical means:

It is generally agreed that the relatively harsh treatment to which the tubercle bacilli are subjected during preliminary processing of the specimen kills an undetermined number. The fewer the bacilli, the smaller are the chances that a sufficient

number of survivors remain to form colonies on an artificial medium.

The majority of reports in the literature which condemn one or another method as being inefficient when compared to some other method are based on studies with tuberculous sputa which contain an unknown number of bacilli, and are thus inadequate because of the lack of proper controls. From these studies, however, there appears to be general agreement that the commonly used sodium hydroxide exerts a considerable lethal effect on the tubercle bacillus. (Tarshis and Lewis 1949; Gifford et al. 1951). In a comparison of 4% sodium hydroxide and 6% sulphuric acid for preliminary treatment of sputa, Steenken and Smith (1938) found that the former was the more lethal for the tubercle bacillus and for a number of variants of low virulence. It was observed that the selection of medium for subsequent cultivation of the bacillus was important. After exposure to acid or alkali, the H37Rv strain grew better on an egg medium adjusted to pH 6.2 than at pH 7.2. It was also found that less virulent strains of the bacillus (H37Ra and R_I) were relatively more sensitive to the action of both acid and alkali.

A carefully controlled study conducted by Jensen and Bindslav (1946) demonstrated clearly the lethal effect of sodium hydroxide for tubercle bacilli in sputum. It was estimated that the proportion of tubercle bacilli germinating on Löwenstein - Jensen medium after treatment of the sputum with 4% NaOH for 20 minutes at 37°C was 2.1%. If the sputa were exposed to drying

or the action of light, the proportion germinating decreased to 0.25%. In a few clear sputa which contained so many tubercle bacilli that contaminants could be eliminated by dilution of the specimen, and which were, therefore, not treated with sodium hydroxide, the proportion of bacilli which germinated increased to 80%. The loss of bacilli would appear to be due chiefly to the homogenization process, and secondarily and to a lesser extent, to the centrifugation which follows. Jensen believes the culture test to be more sensitive than guinea pig inoculation, since the low, though not inconsiderable degree of resistance to infection which this animal possesses, will assert itself preferably with regard to bacilli which have been most injured by the pretreatment of the specimen.

Spendlove et al. (1949) compared the effect of a number of common digestants on the viability of tubercle bacilli in aqueous suspension. Viable counts performed after varying time intervals showed that the survival rate was 52% after 40 minutes exposure to 10% trisodium phosphate, and 46% after an equal exposure to 4% sodium hydroxide. However, after 72 hours exposure to these agents, the survival rate for trisodium phosphate was 45% while that of sodium hydroxide had fallen to 3%. No survivors remained after exposure to 5% sulphuric acid, 50% antiformin, 3% hydrochloric acid or 5% oxalic acid for 24 hours. Sputum had a slight protective effect on viability when tested in the same manner.

Yegian and Budd (1952) reported similar results in an examination of the toxic effect of sodium hydroxide for the H37Rv strain. The most significant finding was the high mortality rate during the first five minutes of exposure to sodium hydroxide - almost 50% being killed after this time interval. After the initial period of exposure, the death rate was significant but not proportionately as great.

It was also reported that a resting suspension of tubercle bacilli was neither more nor less susceptible to the action of sodium hydroxide than other cells, which would indicate that the physiologic state of the bacilli present in clinical material is not an important factor. Yegian concludes from this study that in instances where tubercle bacilli are too few to be found in stained preparations, no more than a very few will survive the action of the concentration process.

On the basis of the results reported by Spendlove et al. and by Yegian it appears that the mode of action of sodium hydroxide on the tubercle bacillus is similar to that of other injurious chemical and physical agents on bacteria in general; the first unit of time shows the largest proportion of deaths, while the last few survivors require a considerable exposure time before being killed (Topley and Wilson 1948).

Glover (1952) emulsified normal sputa with saline suspensions of M. tuberculosis, and added an equal volume of 4% sodium hydroxide

to these emulsions. After treatment at 37°C for varying periods, serial dilutions were prepared and inoculated into guinea pigs. His results showed that as the time of exposure increased, guinea pigs became refractory to infection by the higher dilutions. Only two of three guinea pigs showed slight lesions from a 10^{-6} dilution of the original material after 30 minutes exposure to sodium hydroxide, while the control emulsion (no sodium hydroxide) produced moderate lesions in guinea pigs in a 10^{-7} dilution. After 60 minutes exposure no dilution higher than 10^{-4} produced disease in guinea pigs.

Gray et al. (1954) carried out experiments to assess quantitatively the lethal effects of various concentrating agents for tubercle bacilli suspended in albumin water and in non-tuberculous sputum. The H37Rv strain was used and its use was justified by the demonstration that it was more sensitive to the lethal action of 4% sodium hydroxide than were four freshly isolated strains. Sulphuric acid, oxalic acid and Jungmann's ferrous sulphate-hydrogen peroxide agent were most lethal and killed in excess of 98% of the bacilli after 30 minutes exposure. 4% sodium hydroxide for 30 minutes and 11.5% trisodium phosphate for 24 hours killed about 85%. Trisodium phosphate in a concentration of 7.5% appeared to be less lethal than the higher concentration and was recommended for this reason. Further, in confirmatory experiments with tuberculous sputum, recovery of the bacillus was best when 7.5% trisodium phosphate was

used, as judged by the fact that a higher dilution of the sputum produced disease when inoculated into mice as compared to similar dilutions of sputum pretreated either with 11.5% trisodium phosphate or with 4% sodium hydroxide. In cultural experiments, growth was retarded by all agents in comparison with untreated bacilli. This effect was least marked with trisodium phosphate.

The results of these investigations are in good general agreement and focus attention on the fact that the methods adopted to homogenize specimens and destroy contaminants also kill a considerable proportion of tubercle bacilli. It need not be pointed out that almost any method for preliminary treatment of pathologic material will give positive cultures when there are sufficient tubercle bacilli to be easily revealed by direct microscopy. However, as the value of the cultural test lies in its usefulness in detecting small numbers of bacilli which are not revealed by direct microscopy, the question of selective toxicity becomes important.

(b) Concentration of tubercle bacilli by centrifugation:

Almost all methods devised for the cultural diagnosis of tuberculosis include essentially two processes: (1) homogenization or liquefaction and (2) concentration of the tubercle bacilli by centrifugation. The sediment which results from centrifugation of the sputum digest is retained for examination and the supernatant fluid is, as a rule, discarded. Various workers have noted that

tubercle bacilli are not completely sedimented by the ordinary centrifugal speeds of routine techniques of concentration. In a specimen which contains few bacilli this is a serious disadvantage.

Hanks et al. (1938) found that the supernatant fluid which remains after centrifugation, and which is usually discarded, may contain nearly as many viable tubercle bacilli as are found in the sediment.

Hata et al. (1950) in an investigation of the efficiency of centrifugation for concentrating tubercle bacilli, reported that of a series of 130 sputa with low bacillary content 38% were found positive by culture after centrifugation and 35% were positive without centrifugation.

Klein et al. (1952) were able to detect viable tubercle bacilli in the topmost layer of the supernatant fluid in 89% of a series of sputum digests of varying bacillary content, after centrifugation for 15 minutes at 3000 rpm. As a consequence of this observation the authors suggest that examination of the supernatant fluid by culture or animal inoculation should not be neglected. Gray et al. (1954) also showed that many viable bacterial units remain in the supernatant fluid. That the number remaining is appreciable was shown by performing viable counts on the supernatant fluid of a suspension of bacilli centrifuged for 30 minutes at 3000 rpm. in an angle centrifuge. Viable cells in the original suspension numbered 3.5×10^6/ml and in the supernatant after

centrifugation, 2.5×10^4/ml.

Several factors may influence the efficacy of centrifugation as a concentration method; these are the relative specific gravities of the tubercle bacillus and of the digest, and the speed and duration of centrifugation. Although there is a marked reduction in the number of tubercle bacilli found in the surface layer of the supernatant after centrifugation, the increase in the number of bacilli in the sediment is not proportionate. Thus, it appears that while the bacilli are thrown downward by centrifugal force, only those close to the bottom of the tube actually reach the sediment (Klein et al 1952).

Hanks & Feldman (1938) introduced the alum-flocculation technique as a means of improving the concentration by centrifugation. In this technique 0.2% potassium alum is added to the specimen simultaneously with the sodium hydroxide. Upon neutralization of the specimen, the alum is precipitated and entangles the bacilli. The flocculated sample is centrifuged for 5 minutes and the sediment can be used for preparing smears or inoculating culture media. It was also reported that the flocculated precipitate does not interfere with the cultivation of very small numbers of bacilli. The authors obtained results by this method which consistently excelled direct centrifugation at 1000 x g for one hour. Other workers did not favour the flocculation method as it was considered that the

heavy precipitate from a given quantity of digest greatly reduced the amount of original specimen which could be used for examination (Robinson and Stovall 1941; Nagy 1939).

More recently, another flocculation method has been reported (Weidmann 1952), in which the bacilli are entrapped in a precipitate of calcium phosphate which results when calcium chloride is added to sputa already digested by a mixture of sodium hydroxide and trisodium phosphate. After centrifugation the supernatant is discarded and the precipitate is dissolved in a solution of sodium citrate. This solution is used for inoculating culture media. It was reported that results were superior to direct centrifugation of sodium hydroxide digests of sputum.

Another factor which may be detrimental to tubercle bacilli during centrifugation is the heat developed during operation of the machine. It has been reported (Public Health Lab. Service, 1953) that the temperature of material being centrifuged in a machine which is operated intermittently for two hours may rise from room temperature to 40 - 50°C during a thirty minute period of centrifugation. The heat is largely produced by the air resistance within the centrifuge casing, and to a lesser extent by the motor. When neutralization of the digest is not performed until after centrifugation, and in a good many laboratories it is not (Willis and Cummings 1952), the higher temperature doubtless increases the lethal

action of the digestant on the bacillus. This is particularly disadvantageous to successful diagnosis when the specimen contains rare bacilli. Extreme temperature rises may be avoided by allowing the machines to cool between periods of operation or by the use of an angle-head centrifuge in which temperature rises are slight because the streamlined shape offers a lower degree of air resistance.

Kröger and Rosarius (1949) were not impressed by the use of a high speed centrifuge for the concentration of tubercle bacilli, though it had been reported by other workers to be superior to the usual laboratory centrifuges. A comparison between centrifuging specimens of sputum, pus, urine and puncture fluids at 30,000 rpm in a high speed centrifuge for thirty minutes and at 3,000 rpm in an ordinary centrifuge showed that high speed centrifugation did not produce a significantly greater number of microscopically or culturally positive specimens.

(c) The use of coagulated egg media:

The choice of a good nutrient medium capable of supporting the growth of very small numbers of tubercle bacilli is one of the several essential features in the diagnostic culture test (Corper 1946). Coagulated egg media, in various modifications, are widely used for the cultivation of M. tuberculosis on primary isolation. Earlier workers, particularly A. S. Griffith (1914), were most insistent on the value of Dorset egg medium, with or without glycerin, for the primary isolation of the human and bovine types of bacilli,

respectively.

Efforts to improve simple egg media by the incorporation of liver extract, casein, potato extract, starch, etc., have been made, but it is doubtful whether the advantages claimed for these more complex media are as significant as has been alleged. In some cases the addition of various substances to egg media has had an unfavourable effect. For example, it has been shown that the incorporation of glycerin-potato extract to an egg-yolk medium, officially recommended by the American Trudeau Society (1946), is not only unnecessary, but may result in a delay in the appearance of colonies (Glover 1952). Attention may be drawn to the observation of Taylor (1950) that a simple egg medium with a high yolk content has given a much higher percentage of positive cultures of M. johnei than the more complex media formerly used. Considerable dissatisfaction exists with respect to the use of complex egg media for the routine isolation of tubercle bacilli.. However, large scale comparisons of the relative merit of different media indicate that the Löwenstein - Jensen modification is the most satisfactory for this purpose (Reed and Morgante 1956).

The empirical use of egg media for cultivation of the tubercle bacillus has been justified in more recent years by the demonstration that the yolk of the egg contains a principle which enhances the growth of the bacilli. In fact, it has been reported

that a medium composed of 10% egg yolk in distilled water will support the growth of 10^{-8} mgm. of a freshly isolated strain of human type bacilli (Eggerth 1950). When egg yolk is present in Dubos' basal medium it need be present only to the extent of 1/2500 to allow growth of 10^{-8} mgm; the basal medium itself must receive a heavy inoculum before growth occurs. If egg yolk is extracted with hot alcohol, which removes the phospholipids, the residue will not support growth of the bacilli when added to the basal medium. Addition of the alcohol extract to the basal medium supports growth of a small inoculum. That the phospholipid fraction of egg yolk is largely responsible for the extraordinary capacity of the yolk to stimulate growth of the tubercle bacillus is supported by the investigations of other workers (Boissevain and Schultz 1938; Finlayson 1951). Dubos (1948) has shown that sphingomyelin, which constitutes about 0.3% by weight of the yolk, enhances growth of the tubercle bacillus. Recently, Hirsch (1954,a) has reported that egg yolk contains at least two heat-stable substances which promoted the growth of tubercle bacilli in vitro. One of these substances was identified as cholesterol, the other was of unknown nature, but intimately associated with the insoluble protein residue remaining after extraction of the yolk with organic solvents. Egg white has little or no nutritive value for tubercle bacilli.

In addition to the nutrient factors in egg yolk, there are

substances released on heating egg media which may retard growth of tubercle bacilli, or inhibit it completely, if the inoculum is small. This effect becomes more pronounced if old eggs are used in the preparation of the medium. Hudgins and Patnode (1952) report that media prepared from eggs more than three days old and used for the primary isolation of tubercle bacilli show a statistically significant reduction in their sensitivity, i.e., in their capacity to initiate the growth of small inocula. It was also noted that there was a decrease in the size of colonies as well as alterations in colonial morphology when older eggs were used. Corper and Clark (1946) recognized that the use of eggs introduces a variable directly affecting the sensitivity of the culture medium and postulated, without presenting detailed experimental evidence, that stored eggs, on heating, tend to liberate free fatty materials into the medium that retard the growth of the tubercle bacillus.

Cummings et al. (1948) showed that the amount of ether-soluble acidic material which could be extracted from coagulated egg media increased as the temperature and time of inspissation is increased. The rate and amount of growth of the H37Rv bacillus decreased as the amount of ether-soluble acidic material in the medium increased. It was recommended that the temperature and time of inspissation be kept at the absolute minimum necessary to obtain sterile media.

That free fatty acids may be highly bacteriostatic for the

tubercle bacillus has been demonstrated by Drea (1944) in his studies on the factors concerned in the successful cultivation of small inocula of tubercle bacilli in synthetic media. The prime condition for successful cultivation of small inocula is that the culture flask be absolutely clean. For example, flasks plugged with cotton and sterilized by dry heat give off growth inhibiting distillates of fatty materials which are absorbed by the walls of the flask and form films which are so thin as to be invisible and yet so antibacterial that an inoculum smaller than 10^{-2} mgm of tubercle bacilli will not grow.

3. Recent Trends in Laboratory Diagnosis

In the continuing search for improved methods of isolating the tubercle bacillus in pure culture from pathologic material there is a tendency to forsake the use of strong acids and alkalis for preliminary treatment of the specimen and of coagulated egg media for the isolation of the bacillus.

(a) Enzymes:

The use of enzymes for the preliminary digestion of sputum was advocated as long ago as 1903 by Spengler, who used pancreatin for this purpose. Trypsin, pepsin and papain have also been investigated by various workers over the years but have not found favour

because they did not digest purulent sputa as actively as did alkalis (Haynes 1942; Vogt et al. 1940; Sullivan and Sears 1939).

It was reasoned that enzymatic treatment of the specimen might be less injurious to tubercle bacilli than strong acids and alkalis, but it was also apparent that many non acid-fast microorganisms survived such treatment. Rawlins (1953) advocates the use of 1% pancreatin to homogenize sputum prior to the cultivation of the common pathogens of the respiratory tract and reports that H. influenzae, D. pneumoniae, Strep. viridans, E.coli and a number of non-haemolytic streptococci and Neisseria species are not killed by one to five hours exposure to this enzyme. Thus, a major obstacle to the development of an enzymatic method of sputum digestion has been the inability to eliminate contaminating microorganisms. Lack (1953) reports that treatment of thick tuberculous pus with streptokinase -desoxyribonuclease reduces its viscosity and gives better and more rapid growth of tubercle bacilli. However, no mention is made of the method used for elimination of contaminants. Middlebrook et al. (1954) use a mixture of pancreatic enzymes for digestion and a mixture of 0.5N sodium hydroxide and 0.05M trisodium phosphate for decontamination of sputa from which it is desired to isolate isoniazid-resistant strains of tubercle bacilli.

Saxholm (1954) has described an enzymatic method for digestion of sputum followed by differential decontamination with a

a quaternary ammonium compound. He adds pancreatin and Desogen (methyl-phenyl-dodecyl-trimethyl-ammonium-methosulphate) to the sputum simultaneously and concentrates the digest by centrifugation. He reports that the method compares favourable with the sodium hydroxide method and would prove better than the latter but for the slightly higher degree of contamination in cultures. Hirsch (1954) has also suggested that quaternary ammonium compounds might prove useful as decontaminating agents if used in conjunction with enzymes for the preliminary treatment of sputa. He reports that tubercle bacilli are not killed by exposure to moderate concentrations (0.1%) of the cationic detergent benzalkonium chloride (Zephiran), while a concentration as low as 0.005% exerts a rapid bactericidal action on non acid-fast microorganisms and on several saprophytic mycobacteria. Zephiran exerts a bacteriostatic action against the tubercle bacillus however, so that its use as a differential decontaminating agent would make necessary the washing or dilution of the material to be cultivated, in order that the concentration of the detergent be reduced below the bacteriostatic level. Hirsch also suggests that benzalkonium chloride might be useful in distinguishing saprophytes from avirulent or attenuated bacilli, which is a problem of current interest in connection with the bacteriologic study of pathologic specimens from tuberculous patients.

(b) Media:

The development by Dubos and his associates of the now well-known and widely used Tween-albumin fluid and oleic acid-albumin-agar media (Dubos and Middlebrook 1947) has stimulated, anew, interest in the factors essential for growth of the tubercle bacillus, especially upon primary isolation. The demonstration that visible dispersed growth could be obtained in the Tween-albumin fluid medium within ten to fifteen days of seeding with a small inoculum indicated its use as a diagnostic tool for more rapid results than had been customary up to this time.

Examination of the suitability of Dubos Tween-albumin fluid medium has been undertaken by a number of workers and has been shown to be as sensitive as the guinea pig for detection of small numbers of tubercle bacilli (Foley 1946; Roberts et al. 1950) Disadvantages were the frequent contamination by non acid-fast bacteria, and the necessity of confirming growth of the tubercle bacillus by the microscopic examination of smears prepared from the culture. Konowalchuk and Reed (1951) showed that the contamination problem could be overcome by the incorporation of 1/1,000,000 gentian violet in the medium; this concentration of the dye does not inhibit growth of the tubercle bacillus. They proposed that diagnostic cultures should be set up in Dubos' medium with gentian violet and on conventional egg medium, simultaneously. Tentative

conclusions could then be drawn from microscopic examination of fluid cultures after seven to ten days of incubation and confirmatory evidence obtained at a later date from the solid medium.

Hirsch (1954,b) has introduced a modification of Dubos' oleic acid-albumin agar medium in which the fraction V albumin is replaced with activated charcoal. The growth of mycobacteria on this medium is as good as that on egg or albumin media. Further, the charcoal medium has the same capacity as oleic acid-albumin agar to initiate the growth of the tubercle bacillus from very small inocula. The charcoal is thought to act by adsorbing toxic materials from the agar just as fraction V albumin.

Whalen and Mallmann (1955) have also introduced a charcoal-agar medium for consideration as a diagnostic culture tool. This is also a modification of the Dubos medium, and again activated charcoal replaces the bovine albumin. The charcoal preparation used in this medium was reported to be equivalent to human serum and oleic acid-albumin complex in stimulating the growth of recently isolated strains of M. tuberculosis. Ethyl violet in a concentration of 1/400,000, 100 units of penicillin per ml. and 100 micrograms of cycloheximide (Actidione) were included in the medium when used for the isolation of tubercle bacilli from sputa. This medium was superior to Petragnani egg medium for the primary isolation of the tubercle bacillus in that there was a lower incidence of contamination, a greater number of positive cultures and more

rapid appearance of growth.

In a series of recent communications, Tarshis et al. (1951, 1955) have reported on the use of a blood agar medium for the primary isolation of the tubercle bacillus. The medium consists of blood agar base hydrated with 1% glycerin and 25% outdated human bank blood, and can produce visible growth from small inocula of tubercle bacilli within ten to sixteen days. Penicillin and malachite green are added to the medium to reduce the growth of contaminating microorganisms. A comparison of this medium with Löwenstein - Jensen medium showed it to be superior to the latter with respect to the number of positive cultures obtained and the rapidity with which growth occurred. The advantages cited for the charcoal and blood media as compared to egg or albumin media include economy, reproducibility and ease of preparation.

The yolk sac of embryonated eggs has also been used for the primary isolation of tubercle bacilli. Pure culture studies have shown that the tubercle bacillus proliferates rapidly in the yolk sac of the chick embryo and also that it will support the growth of minute inocula. Brueck and Buddingh (1952) were able to demonstrate the presence of acid-fast bacilli in yolk sacs within six days of inoculation with specimens obtained from tuberculous individuals. The reports of others (McNealy and Riddel 1949; Dubos et al 1946; Hsiung and Haley 1954) indicate that the yolk sac of

the developing chick embryo provides exceptionally favourable nutritional and environmental conditions for the rapid proliferation of small numbers of tubercle bacilli. However, the use of the chick embryo has not yet been developed to the point where it constitutes a simple and rapid procedure for the routine isolation of the tubercle bacillus (Reed and Morgante 1956).

(c) Slide culture:

The slide culture technique, introduced by Pryce (1941), has provided a technique for relatively rapid diagnosis of tuberculosis. The method depends on detection of microcolonies of the tubercle bacillus by means of the low-power objective of the microscope before they become visible to the naked eye. Pryce made dried films of tuberculous sputa on microscopic slides and treated them with 6% sulphuric acid to destroy contaminants. The slides were submerged in equal parts of hemolysed blood and water in Petri dishes. From a number of sputa positive by direct smear, obvious signs of growth were quite distinct in three to six days.

Reed (1953) has reported the results of experiments with an improved slide culture method. Films of homogenized sputa are dried on slides, decontaminated with 6% sulphuric acid and incubated in Kirchner's fluid media with 10% human serum contained in special culture tubes. A comparison of the slide culture technique with routine culture on Herrold's egg-yolk agar for isolation of the

tubercle bacillus showed that slide culture was more sensitive than the latter. Much more striking was the fact that all slide culture positive specimens were positive within two weeks, while twelve weeks were required for all positive cultures to appear on Herrold's medium. A quantitative comparison of routine and slide culture methods showed that growth from as few as 50 bacilli of a recently isolated strain could be detected in two weeks by slide culture, while no growth could be obtained on Herrold's medium from fewer than 125 bacilli and that, only after four weeks.

In routine diagnostic trials of the slide culture method, Simpson and Reed (1955) report that of a series of specimens which were positive by examination of a smear prepared from the concentrated sediment, 95% were positive by slide culture as compared to 72% by routine culture. Again, the most striking feature was the contrast between the length of time required for positive cultures to be detected by each method.

(d) Molecular filter membranes:

The membrane (or millipore) filter, a paper-thin cellulose ester film which is devoid of fibrous structure and possessed of a high and uniform porosity, has been shown to be efficient for the collection of tubercle bacilli contained in relatively large volumes of fluid. The bacilli are collected on the surface of the membrane by filtration of the fluid and may then be demonstrated by staining

in situ or cultivated by placing the membrane on the surface of a suitable solid medium. This procedure is likely to be much more efficient than centrifugation for the concentration of small numbers of tubercle bacilli. Wayne (1954) states, "The possible advantages of the membrane should include recovery of every tubercle bacillus in a large volume of liquid such as urine or spinal fluid.....".

Membrane filters were first introduced by Zsigmondy (1918) and were utilized by Citron (1919) for the isolation of tubercle bacilli from urine. Tietz and associates (1950 a,b) demonstrated tubercle bacilli on membrane filters, following filtration of cerebro-spinal fluid from cases of tuberculous meningitis. After filtration of the cerebro-spinal fluid, the membranes were stained and examined microscopically. Kunzel (1950) cultivated tubercle on membrane filters from sputum, pus, and puncture fluids. Hawirko and Murray (1954) recovered tubercle bacilli from sputa by oil partition and collected the bacilli on membrane filters by dissolving the oil in a fat solvent to facilitate its passage through the membrane. The bacilli were demonstrated by microscopic examination of the stained membrane as well as by cultivation and guinea pig inoculation. Rogers et al. (1955) reported the successful cultivation of tubercle bacilli on membrane filters placed on the surface of either Löwenstein-Jensen medium or oleic acid-albumin agar medium. The bacilli were collected by the filtration of mouth wash gargle

specimens obtained from tuberculous patients whose sputum showed the presence of acid-fast rods on direct smear. Morgante and Murray (1955) introduced a membrane filtration-culture technique using Löwenstein - Jensen medium for the demonstration of tubercle bacilli in the cerebro-spinal fluid of persons suffering from tuberculous meningitis. This technique was shown to be much more effective in detecting tubercle bacilli when the meningeal reaction is minimal and the bacilli presumably few in number, than direct inoculation of Löwenstein medium with cerebro-spinal fluid.

Wayne (1954) has described a staining technique based on the neutral red reaction of Dubos and Middlebrook (1948) for the visualization of microcolonies of mycobacteria on membrane filters. This technique allowed more rapid detection of growth, as microcolonies of the tubercle bacillus were rendered visible with the low power objective of the microscope within six days of seeding with a relatively heavy inoculum.

Among the more recent developments was the demonstration by Hawirko and Murray (1954) that small numbers of tubercle bacilli may be collected effectively from watery menstrua by oil partition. This technique will be discussed in the following section.

4. Oils, Hydrocarbons and the Tubercle Bacillus

(a) Concentration by hydrocarbons:

That liquid hydrocarbons may be used to separate tubercle bacilli from a watery suspension was first observed by Lange and Nitsche (1909) and applied by them to the examination of sputum. They liquefied sputa with alkali, added water and shook the watery mixture vigorously with ligroin. After standing a time, separation of the ligroin and watery phases occurred and tubercle bacilli could be demonstrated in the foamy interface between the layers, by microscopic examination of a stained smear prepared from the foam. The authors claimed that by this method tubercle bacilli could be demonstrated without difficulty when it was impossible to detect them by other methods.

The method of Lange and Nitsche as well as other methods which followed embodied the principle that tubercle bacilli have a greater affinity for hydrocarbons than for water. Invariably, the digested or homogenized specimen was shaken with a hydrocarbon and after separation of the hydrocarbon and aqueous layers, smears were prepared from an undifferentiated "soapy" layer at the interface and examined microscopically for the presence of tubercle bacilli. These procedures were restricted to microscopic examination, as the hydrocarbon was usually lethal to the tubercle bacillus. This factor has been mainly responsible for lack of wider application, especially in more recent years, where emphasis is placed on the isolation of the

bacillus in pure culture and not on mere microscopic demonstration alone.

Kinyoun (1915) also used ligroin on sputa digested by Uhlenhuth's method. He claimed an increase of 19% positive specimens by the use of ligroin. Kraus and Fleming (1916) substituted gasoline for ligroin and introduced slow centrifugation to facilitate separation of the phases.

Andrus and MacMahon (1924) introduced chloroform and claimed that it was superior to other hydrocarbons and to centrifugation of sputum digests. The chloroform settled to the bottom of the tube upon centrifugation and in it were concentrated the tubercle bacilli originally present in the specimen. Successive applications of the chloroform residue to slides, with drying between each application, resulted in good concentration of the bacilli. It was reported that while centrifugation of the sputum digest concentrated the bacilli only 3 - 4 times as compared to direct smear, the chloroform method gave a concentration of 100 - 200 times that of direct smear. Further, the staining properties of the bacilli were not impaired, as was often the case with other reagents.

Pottenger (1931) demonstrated the effective collection of tubercle bacilli from specimens by the use of xylol, ligroin, chloroform, carbon bisulphide and gasoline, and concluded that the choice of hydrocarbons was unimportant. The essential features of Pottenger's method were the extensive dilution of the specimen with distilled

water and the use of picric acid as a counterstain. Dilution of the specimen until its viscosity approached that of water, enhanced the collection and aided the resolution of groups of tubercle bacilli into free single cells. The breaking up of groups of bacilli by the hydrocarbons due to its ... "solvent action on interbacillary wax-like cement substances" was considered important, as the chances of finding the tubercle bacilli were increased. He reported that the dilution - flotation method combined with picric acid as a counter-stain gave an average concentration factor of 60x over direct smears counterstained with methylene blue, and was able to detect as few as 1,000 organisms present in a 24 hour specimen of sputum. Nagy (1939) considered Pottenger's dilution - flotation method superior to chemical flocculation and the sodium hydroxide method.

Hanks and Feldman (1940) compared direct centrifugation, alum flocculation and chloroform for the concentration of tubercle bacilli in cerebro-spinal fluid. They reported that the use of chloroform and alum flocculation were superior to centrifugation. Concentration factors were 102x, 92x and 25x respectively, over direct smear. Robinson and Stovall (1941) also reported the superiority of chloroform as compared to the sodium hydroxide method.

Edwards et al. (1936) and Smith (1938) used modifications of Pottenger's dilution - flotation method in conjunction with a cultural technique for the demonstration of tubercle bacilli. Both showed that

dilution - flotation was not as sensitive as cultural methods for detection of bacilli but that many more positive results were obtained by a combination of the two techniques than by either alone, i.e., culture and flotation techniques were overlapping and supplementary. Smith concluded that flotation by hydrocarbons contributes the advantage of providing immediate results, culture that of being more objective and dependable. The combination of the two methods in a single technique was considered to be a definite improvement in sputum examination procedures.

(b) Concentration by oil partition:

Hawirko (1951) and Hawtirko and Murray (1954) reported the results of experiments which culminated in the development of a method for the demonstration of small numbers of tubercle bacilli in pathologic material by oil partition. Though water - insoluble materials had previously been used for the microscopic demonstration of tubercle bacilli (vide supra), this was the first attempt to isolate the tubercle bacillus in pure culture by the use of such materials. The oil partition technique is based on the fact that acid-fast bacteria, but not non acid-fast species, pass spontaneously into oils or hydrocarbons from water, in a finely dispersed emulsion. Upon centrifugation of the emulsion to separate the oil and water phases, myobacteria remain suspended in the former.

The number of tubercle bacilli in the suspension determines

the efficiency of collection by oil partition. Experiments showed that 10^5 or fewer bacilli were completely collected from 10 ml. of aqueous suspension by any one of a number of vegetable oils, while a number of bacilli remain in the aqueous phase (subnatant) if a greater number of bacilli were originally present. This was no disadvantage as the method was developed primarily for the purpose of revealing the presence of small numbers of bacilli in pathologic material. The selection of oil has no influence on the collecting capacity of the method when used to partition bacilli from aqueous suspension, though mineral oil proved more satisfactory than vegetable oils for the collection of tubercle bacilli from digested sputa.

For demonstration of the tubercle bacilli by staining or culture, the oil was dissolved in a mixture of acetone and petroleum ether to facilitate its passage through Cellafilter membranes (a modified membrane filter prepared for use with organic solvents), upon which the bacilli were retained. Cultivation of the tubercle bacilli on membranes was not uniformly successful especially when the inoculum was small. It was postulated that some factor in the process, either the oil or the fat solvent mixture, had a detrimental effect on the viability of the bacilli. However, it was possible to produce tuberculous lesions in guinea pigs by injection of oil into which tubercle bacilli had been partitioned, and this would indicate that the damage done to the bacilli by oil is not irreparable. It was

concluded that whereas considerable difficulty is encountered with the collection of small numbers of tubercle bacilli by centrifugation, oil partition provides a reliable method of collection.

(c) The behaviour of the tubercle bacillus in oil-water systems:

In the course of studies on the kinetics of phagocytosis, Mudd and Mudd (1924 a,b) demonstrated that acid-fast bacteria and non acid-fast bacteria behave differently when suspended in the aqueous phase of a two-phase water - oil film. Bacteria scraped from a culture were suspended in a drop of water on a slide and a cover-slip was laid over the suspension. A drop of a vegetable oil or other organic liquid was placed at the edge of the cover-slip, drawn under it by capillarity and on meeting the aqueous film formed with it a two-phase film between cover-slip and slide. The preparation was then examined with the dark-field microscope.

It was observed that non acid-fast bacteria on approaching the boundary between the oil and water phase were trapped in the interface and could not escape unless mechanical work was done on them. After a time, the interface was completely filled with trapped bacteria. If escape was effected it was almost always back into the water phase. No essential difference was noted in the behaviour of a number of Gram positive and Gram negative species, nor with a number of different vegetable and animal oils and organic solvents. Motile bacteria, e.g., S. typhimurium, were more readily able to

effect an escape from the interface.

On the other hand, acid-fast saprophytes passed through the interface and into the oil with a readiness which was in marked contrast to the behaviour of non acid-fast organisms, and as a consequence very few were found trapped in the interface. Tubercle bacilli did not pass into the oil with the same degree of spontaneity as the saphrophytic bacilli. They showed a measure of stability in the interface, but this only to a slight degree and not at all comparable to that displayed by non acid-fast organisms.

These phenomena were explained by a consideration of the interfacial surface tensions involved - (1) the interfacial tension between the two fluids (the more immiscible the fluids, the greater the interfacial tension); (2) the tension at the bacterium/oil interface; (3) the tension at the bacterium/water interface. With regard to non acid-fast bacteria, the tension between the bacterium and oil (2) was greater than the combined tensions between the bacterium and water and between the water and oil (1 + 3). Also, the tension between the bacterium and water (3) was greater than the combined tensions between the bacterium and oil and between the water and oil (1 + 2). Thus,

$$(2) > (1+3)$$
$$\text{and}$$
$$(3) > (1+2)$$

and the bacterium is in stable equilibrium in the interface due to the balance of interfacial tensions. Since motile bacteria may

leave the interface, it was deduced that these interfacial tensions were of the same order of magnitude as the force which could be generated by bacterial flagella. In this case, interfacial tension or its own motility may dominate the movement of a motile bacterium according to circumstances.

With acid-fast bacteria, which possess little or no stability in the interface and which pass spontaneously into the oil, the tension between the bacterium and the water (3), exceeds the combined tensions between the bacterium and oil and between oil and water (1 + 2), i.e., (3) $>$ (1+ 2). Thus, the acid-fast bacterium is enveloped by the oil.

The fact that the tension at the acid-fast bacterium/oil interface is less than that at the acid-fast bacterium/water interface is ascribed to the non-polar nature of its surface. The large amount of lipid material on the surface of an acid-fast bacterium causes it to be more lipophilic and hydrophobic than a non acid-fast bacterium, on the surface of which polar groups predominate, i.e., the former is the more readily wet by non-polar oil and the latter by water. The differences noted between acid-fast saprophytes and tubercle bacilli were attributed to quantitative differences in the relative amounts of lipid and protein or other polar component of the bacterial surface.

In subsequent communications (Mudd and Mudd 1927a,b), it was

shown that prolonged extraction of acid-fast bacilli with alcohol caused them to show resistance to wetting by oil comparable to that displayed by non acid-fast bacteria. It was also demonstrated that serum sensitization of acid-fast bacilli with homologous immune serum caused a definite change from a surface readily wet by oil to one more readily wet by water. The greater the concentration of immune serum, the more pronounced was this effect. Of interest, is the fact that Mudd used the interface reaction to detect the binding of antibody by tubercle bacilli. He found the reaction to be serologically specific, but not as sensitive as the complement fixation technique. The evidence indicated that the lipid material on the bacterial surface was coated by the serum antibody or by serum substances secondarily bound by antibody, which are apparently more hydrophilic than the unaltered bacterial surface.

Reed and Rice (1931) utilized a simpler technique to demonstrate the passage of acid-fast bacilli into oil from aqueous suspension. The organisms were suspended in water and shaken mechanically with a small quantity of oil or fat solvent. The resulting emulsion was centrifuged to separate the phases, and the number of bacteria remaining in the aqueous phase determined by comparing the opacity with that of standardized suspensions of the same organism. They found that acid-fast species were removed from the aqueous phase with varying degrees of completeness, depending on the degree of acid-fastness. For example, tubercle bacilli were more completely

removed from aqueous suspension than M. berolinensis. All non acid-fast species remained in the aqueous phase. It was also shown that serum sensitization of acid-fast bacilli with homologous immune serum reduced the degree to which the bacilli migrated into the oil. Emulsifying agents such as gelatin and sodium oleate had a similar effect, undoubtedly by reducing the interfacial tension between the water and oil.

In developing the oil partition method for collecting small numbers of tubercle bacilli from clinical specimens, Hawirko and Murray (1954) used a cultural method to determine the efficiency with which the bacilli were collected by oil from aqueous suspension. The aqueous suspension of bacilli was shaken with the test oil and subsequently centrifuged to separate the two phases. By inoculation of a portion of the aqueous subnatant on Petragnani medium it was found that no growth occurred when the original suspension contained 10^5 or fewer bacilli in a volume of 10 ml. When more concentrated suspensions were used, the cultural test invariably showed that viable organisms remained in the aqueous phase. There was no significant difference in the collecting capacity of a number of vegetable oils examined.

Neither variation of the pH of the suspending medium nor of the proportion of oil altered the collecting capacity of oil. However, it was found that the degree of partition was enhanced when the bacilli

were suspended in equal parts of a mixture of physiologic saline and 10% trisodium phosphate. In contrast to the findings of the Mudds and of Reed and Rice it was found that non acid-fast microorganisms are taken up by the oil under the conditions of the oil partition technique. This was demonstrated by culture of the oil and aqueous phases. Micrococcus epidermidis migrated completely into the oil and E. coli and Proteus mirabilis were distributed in both oil and water layers. It was assumed that the discrepencies of these results with those of Reed and Rice were due to the use of cultural techniques, which are more sensitive than the turbidimetric system used by the latter workers.

Another possible explanation for this discrepency may be found in the work of Mudd and Mudd (vide supra). Non acid-fast organisms are, as a rule, trapped in the interface in an oil - water system. However they can pass into the oil on the condition that mechanical work is done which will overcome the balance of forces which hold the bacterium in the interface. It is conceivable that upon shaking of an oil - water emulsion containing non acid-fast bacteria sufficient work will be done by agitation of the oil droplets to allow the escape of a proportion of these bacteria into the oil.

In a preliminary communication, Wayne and Juarez (1955) have proposed that the relative affinities of acid-fast bacilli for fat solvents may be used for taxonomic differentiation of species within

the genus Mycobacterium. A technique was outlined for differentiating between hydrophilic and lipophilic strains, based on the observation that certain strains remain in the aqueous phase when a broth suspension is extracted with petroleum ether. It was claimed that the partition affinity of a given strain of bacillus was reproducible and characteristic.

(d) The effect of oils on the viability and morphology of the tubercle bacillus:

It is well known that the growth and respiration of many microorganisms may be either stimulated or inhibited when exposed to the action of trace amounts of fatty acids incorporated in an aqueous medium. Which effect is manifest depends upon several features, e.g., the species of microorganism, the composition of the medium and the concentration of fatty acid. Many investigations have been carried out concerning the action of trace amounts of fatty acids, (amounts of the order of value of their solubilities in water i.e., 5 - 100 ppm) on the growth of bacteria (Nieman 1954). By contrast, little has been done with respect to the effect of direct exposure of bacteria to fatty acids or other oils. In fact, this field appears to have been neglected, perhaps because viability tests, devised for aqueous solutions, are less satisfactory with oils.

A good deal of attention has been devoted to the effect of natural oils on the viability of the tubercle bacillus. Much of this

has stemmed from the controversy concerning the therapeutic effect of vegetable and animal oils on human tuberculosis. In a few ancient civilizations diets rich in fat were presceibed for tuberculous individuals and cod liver oil attained a widespread use after its introduction for this purpose at the beginning of the 19th century. Several workers have claimed that experimental animals fed a diet rich in cod liver oil or other natural fats are more resistant to infection with the tubercle bacillus than are animals maintained on a fat-free diet; some refute this claim. Others, who admit the favourable influence of natural fats, attribute this action to the vitamins which they contain (Nègre 1932). Principally to settle this controversy, in vitro studies of fats and oils on the tubercle bacillus have been conducted by various workers.

In the majority of studies various oils were added to culture media or to grown cultures of the tubercle bacillus and the viability of the treated culture tested after varying exposure periods. Parallel experiments were, as a rule, performed with experimental animals infected with tubercle bacilli. Invariably, the oil showed bactericidal or bacteriostatic effects when tested in vitro, but conflicting results were reported for in vivo experiments (Calmette 1936). Crimm and Martos (1945) demonstrated that peanut oil and cod liver oil have an inhibitory action on the growth of tubercle bacilli and alter the colonial morphology but cause no change in virulence

or acid-fastness. Subcultures of tubercle bacilli grown on Long's synthetic medium containing either of these oils would grow only on egg medium and not on glycerin agar, which indicated that the viability of the bacilli was decreased by cultivation in the presence of oil and was restored only by the relatively more nutritious egg medium.

Solomides (1946, 1949, 1950) showed that the relatively feeble bactericidal power of cod liver oil could be increased by heating the oil to 180°C for 15 minutes. A distillate prepared from the oil by heating it to 250°C and condensing the vapours was still more active. Viable tubercle bacilli could be detected after suspension in unheated cod liver oil for 48 hours, while heated oil killed all bacilli in six hours and the distillate, in five minutes. The distillate prevented growth of tubercle bacilli in glycerin broth when present in a concentration of 1/10,000; the active principle was not identical with acrolein, and appeared to be associated with the unsaponifiable fraction. Injection of the distillate into infected guinea pigs caused the tuberculin reaction to become negative, but there was no favourable influence on the disease. Klip et al. (1952) showed that the unsaponifiable fraction of cod liver oil contained substances which inhibited the growth of tubercle bacilli in Sauton's medium in a concentration of 2 micrograms per ml; these substances were probably saturated and unsaturated α-glycerylethers. Addition of serum or dead mycobacteria to the

medium reduced the growth inhibition considerably.

In contrast, it has been demonstrated that fats may be utilized as nutrients by the tubercle bacillus. Sedych and Seliber (1927) reported that olive oil, triolein and butterfat could support growth of the tubercle bacillus in a basal synthetic medium. Quantitative estimation of the oil during growth of the culture showed that it was consumed by the growing bacilli after first being broken down to glycerin and fatty acid. It was also reported that the incorporation in nutrient agar of a few drops of olive oil, cod liver oil or an acetone extract of the tubercle bacillus rendered the agar able to support growth of tubercle bacilli (Nègre 1932). From these experiments it was concluded that neutral fats can be utilized as nutrients, in vitro, by the tubercle bacillus. Though less favourable than glycerin for the multiplication of the bacillus, they are capable of assuring its development.

Of greater significance are those studies which show that oils can alter the structural integrity of the tubercle bacillus. McJunkin (1923, 1926) reported that oleic acid and olive oil manifest a profound effect on the morphology of tubercle bacilli rapidly dehydrated by alcohol. It was observed that the bacilli became granular, developed polar bodies, became non acid-fast and disintegrated completely. This change took place only in living bacilli and was most rapid at 37°C, at which temperature the metabolic activity is optimal. This was interpreted as indicating that the loss of acid-

fastness was dependent upon some activity of the living organism. Boissevain (1926) reported that preliminary dehydration of living bacilli was not a necessary step, but did accelerate the reaction. He showed that the ability of fatty acids to destroy the acid-fast property of tubercle bacilli and cause disintegration was positively correlated with a long carbon chain, a free carboxyl group and unsaturated linkages in the molecule.

Solomides (1940, 1945, 1946) reported that peanut oil brought about considerable changes in the morphology, viability and pathogenicity of tubercle bacilli when added to fully developed cultures on glycerin - potato medium. The colony lost its rough contour and became smooth and flattened after 24 hours immersion in peanut oil. Serial microscopic examinations revealed that progressive degeneration of the bacilli occurred, with loss of acid fastness and eventual lysis. After 20 days the culture was composed entirely of blue granules, to which Solomides gave the name 'cyanophile' substance. Chaulmoogra oil acted in a similar fashion, while olive oil and liquid paraffin acted more quickly, bringing about the appearance of 'cyanophile' substance within 24 hours. Addition of oil to cultures killed by autoclaving was without effect, except upon extremely prolonged exposure.

Subcultures prepared from the so-called oil-lysed bacilli on glycerin-potato slopes led to the development of new colonies of

typical acid-fast tubercle bacilli. Inoculation of the 'cyanophile' substance into guinea pigs caused them to die of congestive and haemmorhagic lesions of the viscera. No acid-fast bacilli could be demonstrated in these lesions. It was concluded that when tubercle bacilli are exposed to oil under these conditions, their viability is gradually diminished, and a considerable modification, though not a suppression, of their pathogenicity is brought about.

Laporte and Bretey (1941) reported similar results when bacilli were exposed to the action of paraffin oil for three days at 37°C. The oil was turbid after separation of the bacilli by centrifugation, and the sediment consisted of normal acid-fast rods and a small proportion of non acid-fast granules. A second centrifugation of the oil at 80,000 rpm caused the deposition of material, which on microscopic examination was seen to consist entirely of blue granules similar to the 'cyanophile' substance of Solomides.

In studies on tuberculo - immunity, Choucroun (1943, 1947) extracted killed tubercle bacilli with paraffin oil and removed the bacilli by centrifugation at 80,000 rpm for 24 hours. After removal of the bacilli, the oil was found to differ in physical properties from the pure oil. Injection of the oil extract into rabbits caused the formation of antibodies and produced a definite sensitization to Old Tuberculin. Two fractions were isolated from the paraffin oil extract; a protein, and a carbohydrate-lipid complex. The former

appeared to be the 'sensitizing' antigen of the tubercle bacillus and the latter was a toxic principle.

To summarize, animal, vegetable and mineral oils have a bactericidal or bacteriostatic action on the tubercle bacillus. Animal and vegetable oils may be used as nutrients. The conditions of exposure determine to some extent which action becomes manifest. It is apparent that oils possess the ability to dissolve materials from the surface of the tubercle bacillus and to disrupt the integrity of its structure.

EXPERIMENTAL STUDIES

EXPERIMENTAL STUDIES

The Strains of M. tuberculosis and the Preparation of 'Single Cell' Suspensions

Strains of M. tuberculosis: The three strains of M. tuberculosis used throughout the investigation, and their sources, are listed below.

(1) M. tuberculosis var. bovis, strain BCG, was obtained from the Institut de Microbiologie et d'Hygiène, Université de Montréal.

(2) M. tuberculosis, strain HA, was obtained from the Clinical Laboratory, Department of Bacteriology, McGill University (#53-1530) and at the time of receipt had been recently isolated from the sputum of a tuberculous patient.

(3) M. tuberculosis, strain HS, was isolated by the author from the sputum of a tuberculous patient.

The HA and HS strains (author's designation) were not typed as to variety, but were regarded as human type strains because of their origin and also because they grew readily on primary isolation on glycerol-containing medium. Both strains were tested and found to be virulent for the guinea pig. Stock cultures of each strain were maintained on Löwenstein (Jensen) medium and subcultured at three-month intervals. Dubos Tween - albumin fluid medium was used for the routine maintenance of the strains, subcultures being made

once weekly. After the tenth serial subculture in Dubos medium, a fresh series was instituted by reverting to the stock culture for the inoculum.

Since the ultimate aim of this work was to isolate tubercle bacilli from pathologic material by oil partition, these freshly isolated strains were selected for use in the experimental work as it is likely that their properties are more characteristic of tubercle bacilli on primary isolation than those of a standard laboratory strain such as the H37Rv bacillus.

The preparation of 'single cell' suspensions of tubercle bacilli: In this work it was desired to use suspensions of tubercle bacilli consisting of single cells, in order to ensure that all bacilli in a given suspension were equally exposed to the action of oils and other materials which were to be investigated. Further, in many of the experiments suspensions containing very small numbers of bacilli were utilized, and equal dispersion of all cells in the suspension would allow greater accuracy in the preparation of higher dilutions.

The introduction of Tween-albumin fluid medium by Dubos and Middlebrook (1947) has overcome the difficulty of obtaining a well-dispersed suspension of growing tubercle bacilli. However, Tween - albumin cultures do not consist entirely of isolated bacterial cells, but also of small groups containing as many as ten to twenty bacilli. Fenner (1951) reported that slow centrifugation, or filtration through

paper, of cultures in Dubos' medium will result in a suspension consisting almost entirely of single cells.

These methods were tried with saline suspensions of tubercle bacilli which had been grown in Dubos' Tween - albumin medium and it was found that filtration of such a saline suspension through a double thickness of either Whatman #2 or Schleicher and Schuell #597 filter papers gave better results than either slow centrifugation or other types of filter paper. This procedure produced a suspension consisting almost entirely of single cells, with a few clumps of not more than 5 - 6 bacilli. The method finally adopted for the preparation and standardization of these single cell suspensions is as follows:

Cultures were grown in 5 ml. volumes of Dubos' Tween - albumin medium for 10 - 14 days, centrifuged, and the supernatant fluid withdrawn and replaced with an equal volume of 0.85% sodium chloride solution. The sedimented bacilli were resuspended by shaking and the suspension passed through a double thickness of Whatman #2 filter paper cut down to 2 cm. in diameter to fit the filtering apparatus to be described in the section on oil partition. The filtration was performed with a slight negative pressure and the resulting filtrate almost invariably had a faint, though definite, turbidity visible to the naked eye.

Suspensions prepared in this way were standardized by

performing a total count using a Thoma (Hawksley) bacterial counting chamber. In performing the counts, the bacilli were killed by exposing a small portion of the suspension to an equal volume of 10% formaldehyde for 15 minutes; this did not effect their dispersion. The count was made on the $\frac{1}{2}$ dilution of the suspension, using a 40x dry objective and a 10x eyepiece. The substage condenser of the microscope was racked down to provide contrast lighting, as the bacilli were unstained. This method is essentially similar to that described by Bonifas and Novel (1952) for the direct counting of bacteria.

When small clumps of bacilli were observed they were counted as one bacillary 'unit'. It was impossible to determine the exact number of bacilli in a clump, but, only on very rare occasions were clumps observed which would have been large enough to contain more than 5 bacilli. The practice of considering a small clump to be a viable or infective unit is well established in tuberculosis experimental work (Fenner 1951). If the filtered suspension was not turbid to the naked eye a fresh preparation was made, as such suspensions contained too few bacilli to permit an accurate count. Appropriate dilutions of the filtered suspensions were made in 0.85% saline to give the concentration of bacilli desired.

<u>The proportion of viable bacilli in filtered saline suspensions</u>: Dubos and Middlebrook (1947) reported that young cultures of tubercle bacilli in Tween - albumin fluid medium contain only an insignificant

proportion of non-viable cells. On order to determine the effect of the process for preparation of 'single cell' saline suspensions on viability, determinations of the proportion of viable cells in such suspensions were carried out by the pour plate method advocated by Yegian and Budd (1951). Parallel experiments with the drop-plate and pour plate methods showed that higher counts were obtained by use of the latter.

Immediately after preparation of the filtered saline suspension, a total count was performed and dilutions of the suspension were prepared in saline using separate pipettes for each dilution. One ml. aliquots of the appropriate dilutions were distributed in two Petri dishes and approximately 20 ml. of melted and cooled oleic acid-albumin agar, prepared according to the directions of Yegian and Budd, were poured into the dishes, which were then rotated to ensure mixing of the inoculum. The medium was allowed to solidify and the plates were incubated at 37°C. for 48 hours to permit the evaporation of excess moisture. They were then transferred to plastic bags which were sealed to prevent further drying of the medium and returned to the incubator. The maximum count was obtained in approximately four weeks.

The results of these separate determinations are summarized in Table 1 and show that only approximately 50% of the total number of bacilli is viable. This appears to be a rather low proportion and may be due to the fact that the bacilli are suspended in saline, which is not devoid of bactericidal action (Corper and Stoner 1946).

TABLE I

The Relation Between the Total and Viable Counts of 'Single Cell' Suspensions of M. tuberculosis

Strain	Total count	Viable count	Ratio of viable: total count expressed as percentage
BCG	4.0×10^7	2.0×10^7	50
HA	1.0×10^8	4.9×10^7	49
HA	2.4×10^7	1.3×10^7	54

Throughout this study, the experimental results refer to the total bacillary content of filtered saline suspensions and the assumption is made, on the basis of the viable counts, that only 50% of the bacilli in such suspensions are viable.

Investigations of Oils and Organic Solvents

In developing the oil partition method for the cultural demonstration of the tubercle bacillus, Hawirko & Murray (1954) demonstrated that the combination of oil and organic solvent necessary to effect collection of the bacilli from aqueous suspension, and subsequent deposition on a molecular filter membrane interfered with their capacity to grow on artificial media. An examination of various vegetable oils revealed that all exerted a harmful effect on viability. The findings of other workers with regard to the action of vegetable oils on the tubercle bacillus under various conditions of exposure have been cited in the review of the literature.

For the successful cultivation of tubercle bacilli after oil partition it is necessary to select a combination of oil and solvent which possesses a minimum inhibitory action. To this end, a reinvestigation of the influence of oils and organic solvents on the viability of the tubercle bacillus was carried out.

Procedure

Bacterial viability tests are devised for aqueous solutions or suspensions and are quite unsuitable when the material under test is immiscible with water. To test the effect of the exposure of tubercle bacilli to oils and organic solvents, the method adopted

consisted in immersing filter paper squares, previously impregnated with tubercle bacilli, in the material under examination. The method used throughout this portion of the work is detailed below.

Squares of Whatman #2 filter paper measuring 1 cm. were cut from larger sheets and distributed on a stainless steel wire mesh platform contained in a Petri dish of standard size. After sterilization by autoclaving and drying in a hot air oven, each square was inoculated with one drop of a 'single cell' saline suspension of bacilli, prepared as described in the preceding section. The inocula were delivered by means of glass pipettes prepared according to the directions of Fildes and Smart (1926), and calibrated to deliver drops of 0.025 ml. volume. The 1 cm. paper squares were able to absorb this volume of fluid completely, which ensured that each received the same number of bacilli. After inoculation the paper squares were dried at 37°C for one hour.

Vegetable oils# were sterilized by Seitz filtration and dispensed in a shallow layer in Petri dishes of 5 cm. diameter. Organic solvents, all of C. P. grade, were sterilized by filtration through Cellafilter membranes and similarly dispensed. Using aseptic technique, the dried, inoculated paper squares were transferred to the Petri dishes containing oils and solvents. Upon termination of the

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Obtained through Brickman and Co., Montreal

exposure period the paper squares were transferred to Löwenstein (Jensen) slopes (for preparation of this medium see Appendix A) and incubated at 37°C. All tests were performed in duplicate and the culture tubes examined at weekly intervals.

Results

The influence of oils and organic solvents on viability: In this experiment, each paper square was impregnated with 10^6 bacilli of the BCG strain (0.025 ml. of a suspension containing 4×10^7 bacilli per ml.). The bacilli were exposed to olive, corn, safflower and peanut oils for thirty minutes and then were transferred to sloped Petri dishes to allow excess oil to drain off, before implantation on Lowenstein slopes. A second series of inoculated paper squares were distributed in petroleum ether, acetone, benzene, diethyl ether and chloroform and exposed for 15 minutes. The paper squares were transferred directly to Löwenstein slopes as the residual solvent evaporated within a few seconds of removal from the fluid. Control papers were transferred directly to the medium after the one hour drying period.

Table II shows the effect of these materials on the viability of the BCG strain after two and four weeks incubation. No oil brought about sterilization of the paper squares under the conditions of this experiment. Peanut oil appeared most inhibitory while coconut and corn oils were least inhibitory. Fifteen minutes exposure to petroleum ether has no apparent effect on the viability of the tubercle

bacillus. Growth was as prolific as in the controls. Benzene displays a marked inhibitory action and acetone, chloroform and diethyl ether brought about complete sterilization of the paper squares.

Solomides (1940) has reported that peanut oil brings about changes in the morphology of the colonies and of the bacilli in growing cultures. In this experiment, colonies had developed in the presence of small amounts of oils, but no alteration in colonial morphology was evident. However, microscopic examination of Ziehl-Neelsen stained smears prepared from cultures grown after exposure to peanut and coconut oils revealed that a large proportion of the cells were beaded, though the acid-fast stain was retained and the bacilli were normal in other respects. This was observed only in young cultures; in older cultures the cells stained uniformly. This effect was not observed in cultures grown after exposure to other oils or to petroleum ether.

The combined influence of oils and petroleum ether: As petroleum ether appeared to have no inhibitory action for the tubercle bacillus its choice as a solvent is indicated. In this experiment, the filter paper squares were immersed in petroleum ether for 15 minutes after exposure to oil, in order to remove residual traces of the latter. Table III shows that the combined action of oil and petroleum ether allows macroscopic growth to appear earlier. Microscopic examination of Ziehl-Neelsen stained smears prepared from these cultures revealed no significant proportion of beaded cells, regardless of the age of

TABLE II

The Viability of M. tuberculosis (BCG) after Exposure to Vegetable Oils or Organic Solvents.

Oil or Solvent	Time of exposure	Growth# after incubation at 37°C for	
		2 weeks	4 weeks
None (Control)	0	++	+++
Olive oil	30 minutes	-	++
Safflower oil	30 minutes	-	++
Peanut oil	30 minutes	-	+
Corn oil	30 minutes	+	++
Coconut oil	30 minutes	+	+++
Petroleum ether	15 minutes	++	+++
Benzene	15 minutes	-	+
Acetone	15 minutes	-	-
Diethyl ether	15 minutes	-	-
Chloroform	15 minutes	-	-

+++
++ Extent of growth
+
- No growth

On Löwenstein (Jensen) medium

TABLE III

The Effect of the Combined Action of Vegetable Oils and Petroleum Ether on the Viability of M. tuberculosis (BCG)

(Growth on Löwenstein (Jensen) medium after exposure to oil for 30 minutes and petroleum ether for 15 minutes)

Oil	Growth after incubation at 37°C for	
	1 week	2 weeks
None (Control)	+	+++
Olive oil	-	+
Safflower oil	-	+
Peanut oil	-	+
Corn Oil	-	+
Cocomut oil	(+)	++

+++
++ Extent of growth
+
- No growth

the culture or of the oil to which the inoculum had been exposed. This was no doubt due to the fact that the oil was completely removed from the filter paper squares and so did not influence the growing bacilli. Coconut oil appeared to be least inhibitory, growth having appeared after one week of incubation.

Comparison of the influence of acetone and petroleum ether: The finding that acetone completely inhigits the growth of the tubercle bacillus after 15 minutes exposure is in agreement with Hawirko's finding that a mixture of petroleum ether and acetone is relatively more bactericidal than petroleum ether alone. It had previously been reported that acetone was relatively innocuous. In view of this discrepancy a comparison was made of the action of petroleum ether and of two different commercial preparations of acetone on the viability of the tubercle bacillus.

Table IV shows that both acetone preparations killed the bacilli after 10 minutes exposure, while petroleum ether had no effect after 2 hours exposure. Exposure to acetone for 5 minutes allowed growth of one colony in each of duplicate tests with preparation A, and three colonies in one of two duplicate tests with preparation B, after 4 weeks of incubation. This result confirms the finding that acetone is highly toxic for the tubercle bacillus.

TABLE IV

Comparison of the Effect of Two Acetone Preparations and Petroleum Ether on the Viability of M. tuberculosis (BCG)

(Growth on Löwenstein (Jensen) medium after 5 weeks at 37°C)

Time of Exposure	Acetone		Pet.ether	Control
	Preparation A	Preparation B		
0				+++
5 minutes	+	+	+++	
10 minutes	-	-	+++	
15 minutes	-	-	+++	
30 minutes	-	-	+++	
60 minutes	-	-	+++	
120 minutes	-	-	+++	

+++
++ Extent of growth
+

\- No growth

The relative inhibitory action of oils: In the preceding experiments, there did not appear to be a great deal of difference between oils with regard to their effect on the viability of the tubercle bacillus. To obtain a better measure of the inhibitory action of oils, two approaches were considered - (1) the use of inocula of decreasing size exposed to oil for a fixed time and (2) the use of a fixed, large inoculum exposed to oil for varying time periods. Preliminary trials showed that the latter method was the better for this purpose. With the first method, results were not reproducible when the inoculum was small.

In this experiment liquid paraffin, oleic acid and glycerol were examined in addition to the vegetable oils. The bacilli were exposed to oil for periods increasing by two-fold increments from one hour to ninety-six hours. The BCG, HA and HS strains were used as it was also desired to test the relative sensitivity of different strains to the action of oils.

In order to standardize the conditions of exposure, the inocula consisted of filtered saline suspensions prepared from nine day Tween - albumin cultures of each of these strains. Fourteen inoculated paper squares were placed in each oil and two were removed after 1, 3, 6, 12, 24, 48, and 96 hours of exposure. Oil was removed from the filter paper square by immersion in petroleum ether for two to three minutes. The glycerol was removed by immersion in sterile

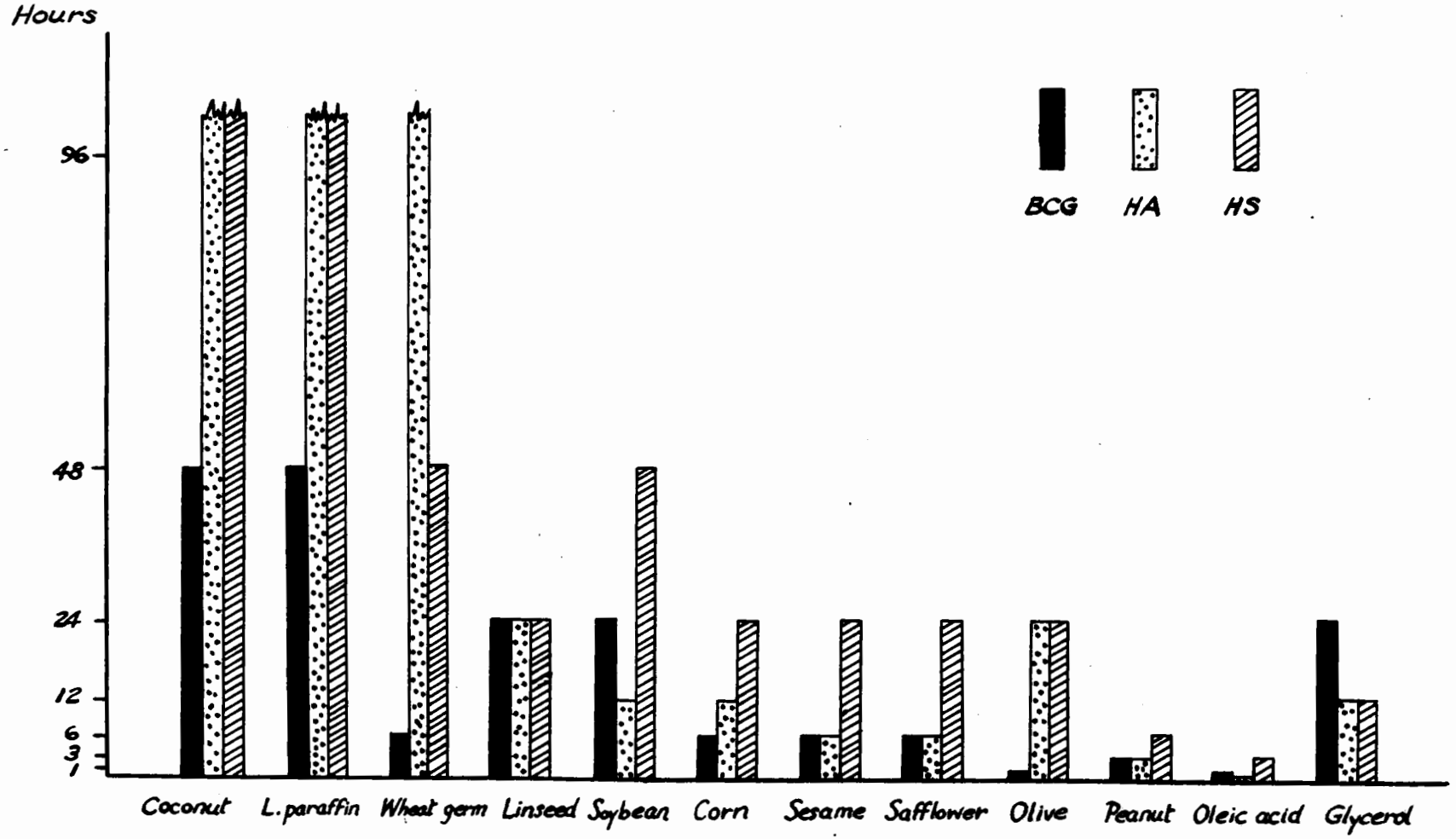
distilled water heated to 45°C, as it is insoluble in petroleum ether. Final readings were taken after 8 weeks of incubation.

The results of this experiment are illustrated in Figure I. In the figure the results have been arranged in order of increasing inhibitory action and represent the time of exposure tolerated by each of the three strains of tubercle bacilli under the conditions of the experiment. Coconut oil and liquid paraffin were the least inhibitory of the oils tested; each allowed growth of the BCG strain after 48 hours exposure while the two human strains, HA and HS, survived 96 hours exposure. Peanut oil and oleic acid were most inhibitory and the other oils fell between these extremes. The marked inhibitory action of peanut oil was masked in preceding experiments, to some extent, by the short exposure period employed.

It is also demonstrated in Figure I that differences exist between the three strains of tubercle bacilli with regard to their sensitivity to the action of oils. The BCG strain is most susceptible and the HS strain least, while the HA strain occupies an intermediate position. These differences are mostly of degree rather than of kind.

FIG. I

THE VIABILITY OF M. TUBERCULOSIS AFTER EXPOSURE TO OILS



The results of these experiments show that tubercle bacilli are damaged by exposure to vegetable oils and organic solvents. This confirms the findings of Hawirko and Murray (1954). Petroleum ether is the only solvent, among those examined, which is not lethal. The finding that petroleum ether is innocuous, even after two hours exposure, is in agreement with the findings of Bloch (1950), who showed that a component of the tubercle bacillus, apparently necessary for virulence, may be extracted from living cells by petroleum ether without impairment to their viability.

Tubercle bacilli more readily tolerate exposure to coconut oil than to the other vegetable oils tested. Coconut oil is unique among this group of oils in that 90% of its total fatty acid content is made up of long chain saturated acids. The other oils contain only 10% - 20% of saturated acids, the balance being made up of long chain unsaturated molecules (Deuel 1951). It is interesting to note that olive oil, which contains a large proportion of triolein, is not nearly as inhibitory as free oleic acid. These observations would indicate that free carboxyl groups and unsaturated molecules are responsible, in part, for the lethal action displayed by an oil for the tubercle bacillus. In this regard, Boissevain (1926) has reported that the ability of a fatty acid to cause disintegration of tubercle bacilli is positively correlated with a long hydrocarbon chain, a free carboxyl group and unsaturated linkages in the molecule.

As cocomut oil and liquid paraffin are the least lethal of the oils tested, and petroleum ether the least lethal of the solvents, these materials were considered to be of most value in oil partition experiments.

Preliminary Investigations of Oil Partition

In the preceding section it was shown that tubercle bacilli are more tolerant of coconut oil and liquid paraffin than of the other oils tested. Accordingly, they were selected for the first trials of the oil partition technique. The low toxicity of petroleum ether and its non-polar properties indicated that it would possibly be a good choice as a vehicle for collection of tubercle bacilli from aqueous suspension. A number of earlier workers have used organic solvents such as ligroin, chloroform, gasoline etc., in flotation methods for the concentration of tubercle bacilli in pathologic material (Pottenger 1931, Andrus and MacMahon 1929, Lange and Nitsche 1909). These materials were investigated for their ability to collect tubercle bacilli from aqueous suspension and to determine whether bacilli so collected can be grown in artificial media. The BCG strain was employed as the test organism in the preliminary trials.

Procedures

The technique of oil partition: The essential feature of this process is the emulsification of an aqueous suspension of tubercle bacilli with a small volume of oil (during which process the bacilli migrate from water to oil), and subsequent separation of the phases of the emulsion by centrifugation. The oil may be used for the injection of animals, or processed further for cultivation of the

bacilli *in vitro*. After much experimentation, the technique finally adopted for the cultural demonstration of the bacilli is herewith described in detail:

Five millilitre aliquots of a 'single cell' saline suspension of known bacillary content were distributed in screw-capped test tubes measuring 15 x 150 mm. One ml. of oil was added to each tube and the tubes were shaken for 10 minutes on a mechanical shaking machine#. The resultant emulsion was transferred to a conical centrifuge tube of 15 ml. capacity and centrifuged at 1500 - 2000 rpm for 20 minutes, which brought about separation of the phases of the emulsion. If it was desired to examine the lower aqueous layer for the presence of viable elements, the material was transferred to a separatory funnel constructed from a test tube measuring 15 x 150 mm. The lower aqueous layer was then drawn off and transferred directly to the filtration apparatus described below. As this technique was cumbersome, it was used only when an examination of the aqueous layer was desired. Otherwise, the aqueous subnatant layer was withdrawn by means of a long, narrow-stemmed capillary pipette having a bulb of 10 ml. capacity. By holding the centrifuge tube in a sloped position it was possible to admit the tip of the pipette into the aqueous layer so that there was only minimal contact with the upper

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Laboratory model shaker, J.W.Towers and Co., Ltd., Liverpool England

oil layer. Careful manipulation allowed complete removal of the aqueous layer, which was then discarded.

The oil remaining in the centrifuge tube was dissolved in 10 ml. of sterile petroleum ether and passed through a membrane filter 2 cm. in diameter mounted in an apparatus#, the construction of which was similar in principle to that of the Seitz 'Technico' filter; filtration was aided by the application of a slight negative pressure and was complete in approximately one minute. The membrane was subjected to a final washing with 2 - 3 ml. of petroleum ether in order to remove any residual oil and was then transferred to the culture medium, with its collecting surface uppermost. Aseptic precautions were observed throughout.

This represents a slight departure from the technique of Hawirko (1951) who used oil in the proportion of 0.5 - 1 ml. to 10 ml. of the bacterial suspension. However, it was demonstrated by Hawirko that the relative proportion of oil and aqueous suspension is unimportant insofar as the degree of partition is concerned. In the practical application of oil partition it is important to keep the volume of oil as small as possible, but as this portion of the

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Constructed by Milos Srb., Optical Engineer, Montreal, of stainless steel and brass. The stainless steel barrel is 7.5 cm. in length and has an internal diameter of 1.5 cm. The effective collecting surface of the membrane is thus 1.5 cm. The capacity of the barrel is approximately 12 c.c.

investigation is concerned only with the cultivation of tubercle bacilli which have been exposed to oil during the process of oil partition, the relative proportions are not important. For ease in manipulation, it was found most convenient to use 5 ml. quantities of aqueous suspension and 1 ml. of oil.

Membrane filters and Cellafilter membranes: These membranes are prepared by a special method for processing nitrocellulose which was developed by Zsigmondy (1918) and since patented and put on the market#. They are white, opaque, of glossy consistency and devoid of fibrous structure.

Membrane filter and Cellafilters are intended for use with aqueous and organic solutions respectively. They may be obtained in a variety of sizes and of varying porosity. For this work, the membranes selected were 2 cm. in diameter and of #5 porosity; membranes of this porosity are intended for the sterilization of suspensions containing bacteria. Cellafilter membranes were received in wet pack and it was the manufacturer's recommendation that they be kept wet at all times. If allowed to dry these membranes shrivelled and disintegrated, which rendered them useless. When used for the filtration of an organic solvent which is immiscible with water, it is

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Membranfilter Gesellschaft, Sartorius-Werke, A.G. & Co., Gottingen, Germany.

necessary to displace the water from the capillary system of the membrane by preliminary treatment with a solvent which is miscible both with water and the organic solvent; alcohol is usually used for this purpose. As it was desired to use these for filtration of a mixture of oil and petroleum ether, they were sterilized and stored in 95% alcohol. Before use they were transferred to the filter apparatus which had been sterilized previously by autoclaving. Membrane filters were received in dry pack, with no restriction as to the method of storage. They were mounted in the filter apparatus and sterilized by autoclaving.

Results

The suitability of membrane filters: Preliminary experiments revealed that tubercle bacilli were able to grow on the surfaces of membrane filters and Cellafilter membranes which were stored in water when placed on Löwenstein (Jensen) medium. If the Cellafilters were stored in 95% ethanol, however, the membranes would not allow growth of smaller numbers of bacilli, which indicated the toxicity of ethanol for the tubercle bacillus, and the necessity for its removal from Cellafilter membranes before the filtration of suspensions containing bacilli. When the membranes stored in alcohol were first washed with water or with petroleum ether to remove the alcohol from the pores of the membrane, growth was as prolific as on membrane

filters. Thus, in using Cellafilter membranes for the filtration of an oil-solvent mixture, it is necessary to remove ethanol before use or otherwise, to store the membranes in a non-toxic solvent, such as petroleum ether.

It had been observed earlier that membrane filters were soluble in alcohol, acetone and ether, but not in benzene or petroleum ether; benzene caused the membrane to become translucent however, while petroleum ether brought about no apparent change. It was considered that the petroleum ether might alter the porosity of membrane filters, and to determine this the following experiment was performed.

Ten millilitres of petroleum ether and 10 ml. of a petroleum ether-coconut oil mixture were passed through each of two groups of membrane filters and dried by the prolonged application of a slight negative pressure. When dry, a heavy saline suspension of BCG was passed through all the membranes and through control membranes; all membranes were then transferred to slopes of Lowenstein (Jensen) medium. The filtrates from each of the two groups of treated membranes were passed through another set of untreated membranes in order to detect the presence of viable elements and these were also placed on Löwenstein slopes.

The results showed that the preliminary passage of petroleum ether or of a mixture of petroleum ether and coconut oil in no way interfered with the capacity of membrane filters to retain tubercle

bacilli, nor did it interfere with the capacity of the bacilli to grow on the surface of the membrane. Full advantage was taken of this fortuitous circumstance and membrane filters were used in all experiments for the cultivation of tubercle bacilli collected by oil partition. Though Cellafilter membranes are intended for the filtration of organic solvents, their use is precluded by the extra labor involved and by the suitability of membrane filters.

Comparison of coconut oil, liquid paraffin and petroleum ether:
Saline suspensions of the BCG strain, prepared as previously described and of known bacillary content, were distributed in three groups of test tubes. Within each group of tubes, successive decimal dilutions were prepared which extended from 5×10^7 to 50 bacilli per 5 ml. of suspension. One group was subjected to oil partition by coconut oil, the second by liquid paraffin and the third by petroleum ether. No centrifugation was necessary after shaking of the petroleum ether group; the solvent separated spontaneously from the aqueous layer. The coconut oil and liquid paraffin were dissolved in 10 ml. of petroleum ether after withdrawal of the aqueous subnatant layer and passed through membrane filters. The aqueous subnatant layers were withdrawn by means of the separatory funnels, as described earlier, and passed through membrane filters, as it was desired to ascertain whether or not they contained viable bacilli. The absence of viable bacilli in the aqueous subnatant would indicate

that they had been completely removed from the original suspension by the process of oil partition. On the other hand, the presence of viable bacilli would indicate they had not been completely removed. All membranes were placed on slopes of Löwenstein - Jensen medium, incubated at 37°C. and examined once weekly.

The results after five weeks of incubation are shown in Table V. Coconut oil appears to be the most efficient as a vehicle for oil partition; no viable bacilli were detected in the aqueous subnatants of suspensions which originally contained fewer than 5×10^4 organisms. By contrast, viable bacilli were detected in the aqueous subnatants derived from even the most dilute suspensions, when liquid paraffin or petroleum ether were used. No growth appeared on membranes through which the liquid paraffin or petroleum ether had been passed, except when the original suspensions contained more than 5×10^5 bacilli; this finding further demonstrates the inefficiency of these materials as agents for oil partition.

The Table also shows that although coconut oil is efficient as a vehicle for oil partition, its inhibitory action appears to be considerable when the number of bacilli is small; after collection by coconut oil, the growth of less than 5×10^3 bacilli is completely inhibited.

The lethal action of coconut oil for small numbers of tubercle bacilli: In a number of experiments in which coconut oil was used to partition

TABLE V

The influence of the Bacillary Content of Aqueous Suspensions on the Distribution# of M. tuberculosis (BCG) Between Oil and Aqueous Layers Subsequent to Oil Partition

Bacillary content of suspension	Coconut oil		Liquid paraffin		Petroleum ether	
	Oil layer	Aqueous layer	Oil layer	Aqueous layer	Oil layer	Aqueous layer
5×10^7	+++	+++	x	x	+++	+++
5×10^6	+++	+++	+++	+++	x	x
5×10^5	+++	+	+	++	-	+++
5×10^4	+	-	-	+++	x	x
5×10^3	+	-	-	++	-	+
5×10^2	-	-	-	+	x	x
5×10^1	-	-	x	x	-	-

As shown by growth of bacilli collected on membranes after five weeks cultivation on Löwenstein (Jensen) medium

+++
++ Extent of growth
+

\- No growth

x Omitted

tubercle bacilli of the BCG strain, growth was never obtained from the oil when the original suspension contained fewer than 5×10^3 bacilli. In this series, suspensions of the bacilli were also passed directly through membrane filters; these served as controls. The results of two such experiments, which were conducted at different times, are shown in Table VI. In both experiments, growth occurred within four weeks on membranes through which suspensions containing as few as 50 bacilli had been filtered. This finding proves that the membrane itself is not contributing to the inhibition suffered by the organisms. Further, in the control series growth always appeared earlier and was more prolific.

Though the results reported in the previous section indicated that coconut oil is relatively less lethal than other oils for the tubercle bacillus, it is not devoid of lethal action. This is given expression when the exposure time is prolonged (see Fig. I), or when the number of bacilli exposed is small (Tables V and VI).

The finding that coconut oil completely collected fewer than 5×10^5 bacilli from aqueous suspension is in agreement with that of Hawirko (1951). She reported, however, that liquid paraffin gave good results when employed for the collection of tubercle bacilli from sputum digests. Later experiments by the author showed that liquid paraffin was readily emulsified with digested sputa. However, when shaken with saline the oil did not produce a true emulsion, but broke up into globules which were relatively large, whereas coconut oil was readily

TABLE VI

The Inhibitory Effect of Coconut Oil Partition on the Growth of Small Numbers of M. tuberculosis (BCG)

Expt. No.	Number of BCG	Growth# of oil partitioned bacilli		Growth# of untreated bacilli	
		2 weeks	4 weeks	2 weeks	4 weeks
1	5×10^7	+	+++	++	+++
	5×10^5	-	++	+	+++
	5×10^3	-	+	+	++
	5×10^1	-	-	-	+
2	5×10^5	-	+++	+++	+++
	5×10^4	-	++	++	+++
	5×10^3	-	++	-	+++
	5×10^2	-	-	-	++
	5×10^1	-	-	-	+

Bacilli were collected on membranes and cultivated on Löwenstein (Jensen) medium

+++
++ Extent of growth
+
- No growth

emulsified. The limited contact between bacilli and oil is the most probable explanation for the observation that liquid paraffin is not an efficient vehicle for oil partition under the experimental conditions.

Neither was there a true emulsion when petroleum ether was used. In this case it is possible that the bacilli do not migrate into the solvent, but collect in the interface between the layers; earlier workers who used ligroin and other hydrocarbons for the concentration of tubercle bacilli in sputa, customarily found the bacilli in the interface.

It is concluded that, under the experimental conditions, coconut oil is more efficient than either liquid paraffin or petroleum ether for the collection of small numbers of tubercle bacilli by oil partition.

Investigations on Tuberculo-Lipids

In order that the oil partition method may be effectively applied to the collection of tubercle bacilli, it is essential to find a means of preventing or reducing the lethal action of cocomut oil for small numbers. It was suggested by Hawirko and Murray (1954) that the bactericidal effect might be due to the removal of an essential lipid from the cell surface by solution in the oil. That this might possibly be the mode of action was indicated by the finding that addition of cholesterol or cephalin to a bactericidal oil reduced its effect to a considerable degree.

A study was made to determine whether or not lipid fractions extracted from the tubercle bacillus could overcome the inhibitory action of coconut oil, by incorporation in it, or allow the growth of oil partitioned bacilli by incorporation in the culture medium. The principles underlying this approach are, (1) by addition of tuberculo-lipid to coconut oil, (i.e. by saturation of the oil with lipid) the capacity of the oil to dissolve further bacillary lipids may be diminished; (2) addition of lipid to the culture medium may provide an essential lipid which had previously been lost to the oil during the process of partition. In this connection, Marks (1953) has shown that an ethereal tuberculo-lipid extract will allow the growth of small inocula of tubercle bacilli when incorporated in an otherwise deficient medium. In addition, extracts of M. phlei are

commonly used in the preparation of culture media for M. johnei, which appears to be deficient in essential substances necessary for proliferation on artificial media.

Procedure

A large quantity of BCG culture# grown on Sauton's medium was killed by steaming at 100°C. for one hour, washed, dried and extracted with diethyl ether by the method of Anderson (1927). Ultimately, the crude extract was separated into three fractions - (i) an acetone-soluble fat, (ii) an acetone-insoluble fraction obtained as a white powder after purification and (iii) an acetone-insoluble wax. These correspond in method of preparation to Anderson's neutral fat, A-3 (phosphatide) and A-4 (low-melting wax) fractions, respectively. Though not subjected to chemical analysis, they will be referred to as fat, phosphatide and wax, with the understanding that they correspond in method of preparation to Anderson's fractions of the same designation. Full details of the extraction process and separation of the fractions are provided in Appendix B.

Each of these fractions was miscible in all proportions with coconut oil. The crude ether extract and the acetone-soluble fat fractions, though miscible, were not completely soluble; even after thorough mixing and heating the mixtures remained turbid. The

Supplied by L'Institut de Microbiologie et d' Hygiéne, Université de Montréal, through the courtesy of Professor A. Frappier

addition of petroleum ether did not bring about solution of the mixtures, nor were these fractions themselves soluble in petroleum ether. Attempts to pass these turbid suspensions through membrane filters were unsuccessful, due to clogging of the membranes. The phosphatide and wax fractions produced completely clear solutions with coconut oil and with petroleum ether.

To test the effect of the lipid fractions on the growth of the tubercle bacillus, they were incorporated in Löwenstein (Jensen) medium. The method for the preparation of medium containing these extracts is detailed in Appendix B.

Results

The effect of tuberculo-lipid added to coconut oil: Preliminary attempts were made to collect tubercle bacilli from aqueous suspension with mixtures of coconut oil and the phosphatide and wax fractions. Centrifugation of the emulsion did not achieve separation of the two phases; a soapy mass resulted and there was no clear distinction between oil and water layers. As it was impossible to recover the oil, a different procedure was adopted.

Tubercle bacilli of the BCG strain were deposited on membrane filters by the passage of one ml. of 'single cell' suspensions which contained 5×10^3 and 5×10^2 bacilli per ml. respectively. Suction was applied to the membranes for one hour, at the end of which time

the membranes were quite dry. The suction was cut off, and 1 ml. aliquots of coconut oil, coconut oil containing 10% phosphatide and coconut oil containing 10% wax were deposited on each membrane. After one hour of exposure, 10 ml. of sterile petroleum ether was added to each filter and agitated vigorously with a pipette to dissolve the oil. This was drawn through the membrane, which received a final wash of 2-3 ml. of petroleum ether. The membranes were transferred to Löwenstein (Jensen) medium and incubated at 37°C. This method was used instead of depositing the bacilli on filter paper squares, as the latter does not allow reproducible results when the inoculum is small.

The results in Table VII show that the phosphatide fraction incorporated in coconut oil had a slight protective effect on the viability of the tubercle bacillus, when the original inoculum consisted of 5×10^3 bacilli. When the wax fraction was incorporated in oil, there was slightly more profuse growth than in those cultures which had been exposed to the oil alone. With the smaller inoculum there was no significant difference between the tests. It is noteworthy that growth was obtained from an inoculum of 5×10^2 bacilli after exposure to coconut oil under the conditions of this experiment, while growth was never obtained from an inoculum smaller than 5×10^3 bacilli in oil partition experiments.

TABLE VII

The Growth of M. tuberculosis (BCG) After Exposure to Cocomut Oil and Tuberculo-Lipid Fractions

Bacilli exposed for one hour to	Inoculum	Growth after incubation for:		
		2 weeks	3 weeks	4 weeks
Cocomut Oil	5×10^3	-	+	++
	5×10^2	-	-	+
Cocomut Oil plus 10% phosphatide	5×10^3	+	++	+++
	5×10^2	-	-	+
Cocomut Oil plus 10% wax	5×10^3	-	++	+++
	5×10^2	-	-	+

+++
++ Extent of growth
+
- No growth

The effect of tuberculo-lipid incorporated in Löwenstein medium:
'Single cell' saline suspensions of BCG were prepared which contained 5×10^4, 5×10^3 and 5×10^2 bacilli in volumes of 5 ml. These were subjected to coconut oil partition, the bacilli were collected on membranes and planted on media containing the crude lipid extract, and each of its three fractions in a final concentration of 0.5% (see Appendix B). The results in Table VIII show that not only do these lipid fractions fail to assist the growth of oil partitioned tubercle bacilli, but the crude lipid extract and the acetone-soluble fat actually inhibit their growth; no growth was obtained on media containing these fractions even when 5×10^4 bacilli were present in the original suspension.

A second experiment was carried out in order to determine the effect of tuberculo-lipid on the growth of untreated bacilli. The media containing the lipid fractions were dispensed in Petri dishes of 5 cm. diameter. These were seeded with relatively heavy inocula of each of the three strains of BCG, HA and HS; ten day Tween-albumin cultures were adjusted to equal opacity using fresh medium, in order that the inocula be of similar size, and diluted to 1/100 with fresh medium. Calibrated dropping pipettes, which delivered drops of 0.025 ml. volume, were used to seed the media, each of which received one drop of the 1/100 dilution of the culture. The inocula were spread over the surfaces of the media, the plates were sealed with

adhesive tape and incubated at 37°C. The plates were examined at four day intervals and the results in Table IX are expressed as that multiple of 4 days on which growth was first observed. Visible growth of the BCG strain had occurred after eight days on Löwenstein medium alone and on the media containing phosphatide and wax, but did not appear until the sixteenth day of incubation on the media containing the crude extract or the acetone-soluble fat; the HA and HS strains grew more slowly than BCG, but the same feature is evident. The HS strain did not grow at all after 24 days of incubation on the medium containing acetone-soluble fat; it appears to be more sensitive to this fraction than either of the other two strains.

The solubility of acetone-soluble fat in oils: It was noted earlier in this section that the acetone-soluble fat and the crude lipid extract were not completely soluble in coconut oil or in petroleum ether. In contrast, the phosphatide and wax fractions formed perfectly clear solutions with both these materials. Testing of the solubility of each of the tuberculo-lipid fractions in other oils showed that there appeared to be a relation between the inhibitory action of an oil and its ability to form a clear solution with the acetone-soluble fat fraction. The moderately inhibitory sesame and olive oils and the highly inhibitory peanut oil formed clear, faintly opalescent solutions with the fat fraction. Liquid paraffin, which has only slight inhibitory activity formed a turbid suspension, of

TABLE VIII

The Influence of Tuberculo-Lipid on the Growth of Oil Partitioned Tubercle Bacilli (BCG)

Number of BCG	Growth after 5 weeks on Löwenstein medium containing:				
	nil	0.5% crude extract	0.5% fat	0.5% phosphatide	0.5% wax
5×10^4	++	-	-	+++	++
5×10^3	+	-	-	+	+
5×10^2	-	-	-		

TABLE IX

The Influence of Tuberculo-Lipid on the Growth of Untreated Tubercle Bacilli (BCG)

Strain	The number of days required for earliest visible growth on Löwenstein medium containing:				
	nil	0.5% crude extract	0.5% fat	0.5% phosphatide	0.5% wax
BCG	8	16	16	8	8
HA	16	20	20	16	16
HS	12	20	-#	12	12

No growth after 24 days

about the same density as that formed with coconut oil. These observations also extend to organic solvents; the fat fraction was soluble in acetone and in ether, both highly toxic for the tubercle bacillus, but not in petroleum ether, which is innocuous.

Oleic acid, which has a marked lethal action, dissolved the fat fraction completely. This was also achieved by undecylic, undecylenic and caprylic acids. The triglycerides, tricaprylin and tricaproin, on the other hand, formed turbid suspensions with the fat fraction; these were not as dense as the suspensions formed with coconut oil and liquid paraffin. The effects of the triglycerides and the three lower fatty acids on the viability of the tubercle bacillus were not tested.

In order to confirm these observations, small quantities of each of the three tuberculo-lipid fractions were mixed with 5 ml. of each oil, fatty acid or triglyceride and placed in a water bath at 60°C for one hour. At the end of this time the tubes were examined and the results of the test with the acetone-soluble fat fraction are shown in Table X. The phosphatide and wax fractions dissolved in all materials tested, but on cooling to room temperature the mixtures of wax and each of the triglycerides became turbid. All others remained clear, even after a further twenty four hours at room temperature. The insoluble material in the mixtures of fat and coconut oil, liquid paraffin, tricaprylin and tricaproin had settled to the

TABLE X

The Lethal Action of Lipids and their Ability to Dissolve the Acetone-Soluble Fat of the Tubercle Bacillus

Lipid	Lethal action	Acetone-Soluble fat
Coconut oil	+	Insoluble
Sesame "	++	Soluble
Olive "	++	"
Peanut "	+++	"
L.Paraffin	+	Insoluble
Caprylic acid		Soluble
Undecylic acid		"
Undecylenic acid		"
Oleic acid	+++	"
Tricaproin		Insoluble
Tricaprylin		Insoluble

bottoms of the tubes after forty-eight hours at room temperature, and could be resuspended on shaking.

It would appear that a free carboxyl group is one of the features necessary to achieve solution of the acetone-soluble fat; both saturated and unsaturated acids dissolved it, while the two triglycerides did not. As an unsaturated triglyceride was not available, it was not possible to determine to what extent the ability of peanut, olive and sesame oils to dissolve the fraction was due to their content of free fatty acids or of unsaturated triglycerides.

These results would indicate that if vegetable oils exert their lethal action on the tubercle bacillus by dissolving and removing an essential lipid from the surface of the cell, rather than by an inherent toxicity, this essential material is closely associated with the acetone-soluble fat fraction of the bacillus. The finding that the acetone-soluble fat inhibits the growth of the tubercle bacillus when incorporated in egg medium might appear to contradict this, but this fraction contains a considerable amount of free fatty acids in addition to neutral fats (Anderson 1939), and free fatty acid is bacteriostatic for growing tubercle bacilli even in minute concentrations (Drea 1944; Dubos 1950). The inhibitory effect of the crude lipid extract on growth is doubtless due to its content of acetone-soluble fat.

The phosphatide fraction appears able to afford a slight protection to tubercle bacilli exposed to coconut oil. Whether this is a specific effect is doubtful, as Hawirko (1954) showed that cephalin and cholesterol also protected tubercle bacilli exposed to other vegetable oils. It is interesting to note that other phosphatides and sterols, e.g. lecithin and calciferol have been reported to be able to reverse the bacteriostatic effects of small amounts of free fatty acid present in a nutrient medium (Kodicek 1949).

The finding that the Anderson A-3 phosphatide fraction when incorporated in egg medium is without apparent effect on the growth of either untreated or oil partitioned tubercle bacilli is in agreement with that of Boissevain and Schultz (1938); these workers reported that this fraction had no influence on the growth of small inocula in a synthetic medium.

Since the phosphatide and wax fractions were without influence on the growth of oil partitioned bacilli, and oil partition using a solution of tuberculo-phosphatide in coconut oil was not technically feasible, the need for another approach was indicated.

Fluid Media for the Cultivation of Oil Partitioned Tubercle Bacilli

A comparison was made of the relative merits of Löwenstein (Jensen) medium, Tween - albumin fluid and Kirchner's semi-synthetic medium (Reed 1953) for the cultivation of oil partitioned tubercle bacilli. Preliminary trials had shown that the two fluid media were equally effective in initiating the growth of small inocula of normal bacilli. The Kirchner medium contained 10% human serum, as this was available in large quantity; its preparation is described in Appendix A.

Each medium was seeded with membranes on which had been collected tubercle bacilli of the BCG strain partitioned by coconut oil from suspensions which contained 50,000 to 50 bacilli. Table XI shows that the fluid media are superior to Löwenstein medium for the cultivation of oil partitioned bacilli; each allowed growth of 500 bacilli, while growth on the Löwenstein medium did not occur with fewer than 5000 bacilli. Further, growth appeared earlier in the fluid media. Essentially the same results were obtained with the HA strain. It grows more slowly than BCG, but is less susceptible to the lethal action of coconut oil. The superiority of the fluid media is no doubt due, in part, to the fact that nutrients are more readily accessible as a result of more intimate contact between the bacilli and the medium.

TABLE XI

Solid and Fluid Media for Cultivation of Oil Partitioned Tubercle Bacilli (BCG)

Number of BCG	Löwenstein medium		Dubos' medium		Kirchner medium	
	2 weeks	4 weeks	2 weeks	4 weeks	2 weeks	4 weeks
5×10^4	-	+++	+	++	+	++
5×10^3	-	+	+	++	+	++
5×10^2	-	-	-	++	-	++
5×10^1	-	-	-	-	-	-

+++
++ Extent of growth
+

\- No growth

For reproducible results with small inocula in Kirchner medium it was found necessary to clean the culture flasks by immersion for twenty four hours in a mixture of 10% concentrated nitric acid in concentrated sulphuric acid; visible films observed after washing the culture flasks with a detergent were removed by treatment with the acid mixture. By the use of flasks cleaned in this manner, it was possible to obtain growth consistently from an inoculum as small as 20 bacilli (0.2 ml. of a 10^{-5} dilution of a suspension which contained 1×10^{7} bacilli per ml.).

Kirchner's medium was selected for use in all subsequent experiments. It is easier and more economical to prepare than Tween-albumin medium and is as good as the latter for the cultivation of tubercle bacilli. It was preferred to Tween - albumin medium in experiments with growth factors, as it has been shown by a number of workers that Tween 80 may affect the action of substances, present in the medium, on the multiplication of tubercle bacilli (Kirby and Dubos 1947; Youmans and Youmans 1948). Another advantage is that smears need not be prepared in order to check the purity of a culture. Growth occurs in the depth of the medium as characteristic granular clumps and the medium remains clear at all times; any appearance of turbidity indicates the presence of contaminants.

The Influence of Accessory Growth Factors on Oil Partitioned Tubercle Bacilli

As attempts to restore the viability of oil partitioned tubercle bacilli by the use of tuberculo-lipids were unsuccessful, attention was directed toward the possibility of augmenting or assisting their metabolism by the incorporation of accessory growth factors in the culture medium. A number of diverse substances were examined which included B-complex vitamins, α-tocopherol acetate, phthiocol, menadione diphosphate, d(-) arabinose, acetic, oleic and linoleic acids, an acetone extract of M. phlei and the filtrate from a culture of Candida albicans.

Few reports have appeared in the literature concerning the stimulation of growth of the tubercle bacillus by the vitamins B. Indeed, Pope and Smith (1946) and Bird (1947) have shown that vitamins of this group are elaborated by tubercle bacilli grown in synthetic media devoid of preformed vitamins. Uyei (1930) claimed that inositol, in concentrations of 0.01 - 1% stimulated the growth of tubercle bacilli in synthetic media, provided a large inoculum was used. Nagaya (1951) reported that inositol stimulated the growth of M. avium provided asparagine was also present. Leitner (1937) reported stimulation of growth by thiamine and Lutz (1949) demonstrated that pantothenic acid in high concentration (10 - 15 mgms per 50 ml.) stimulated the growth of tubercle bacilli in synthetic

media. A most interesting report was that of Schaefer (1955), who showed that certain strains of the tubercle bacillus do not grow on oleic acid - albumin agar upon primary isolation except in the presence of biotin or in an atmosphere containing 5% carbon dioxide. This finding suggested that the function of biotin in the metabolism of the bacillus is associated with assimilation of carbon dioxide.

Dubos (1947) reported that the water-soluble tocopherol phosphate produced a marked improvement in the growth of tubercle bacilli in Tween - albumin medium in a concentration of 0.005%. Kodicek (1949) showed that α-tocopherol and its acetate reversed the inhibitory effect of long chain fatty acids for Lactobacillus casei. Tocopherol is thought to play a role in lipid metabolism by virtue of its antioxidant action; it apparently spares the oxidative breakdown of essential fatty acids in animal tissues (Gortner 1949). Phthiocol, the pigment isolated by Anderson (1939) from the acetone-soluble fat of the tubercle bacillus, was shown by Wooley and McCarter (1940) to be active in supporting the growth of M. johnei in a snythetic medium. Phthiocol is structurally similar to Vitamin K, but most reports concerning this vitamin and its analogues indicate they are tuberculostatic (Inland 1949; Panisset 1952). Kimler (1950) suggested that the tuberculostatic action of Vitamin K and its analogues is due to their functioning as inhibitory competitive analogues of phthiocol, which he regards as an essential metabolite.

Long chain fatty acids may have an inhibitory or stimulatory effect on the growth of the tubercle bacillus, depending on the experimental conditions. Dubos (1947, 1948) showed that they enhanced growth when added to culture media in admixture with sufficient serum albumin to neutralize their toxicity. Youmans and Youmans(1954) reported that long chain fatty acids can replace glycerol as a carbon source in a completely synthetic medium provided the concentration is not too high and the inoculum is not too small.

The pentose sugar d (-) arabinose is not often found in nature. It is found, however, in the polysaccharide obtained by saponification of the chloroform - soluble wax of the tubercle bacillus (Anderson 1939). It was considered worthy of study despite the finding of Youmans and Youmans (1953) that neither the d(-) nor l(+) isomers could replace glycerol as a carbon source for the tubercle bacillus.

In 1954, Mankiewicz reported that Candida albicans produces a factor which promotes the growth of tubercle bacilli of reduced viability. Growth of two recently isolated strains of tubercle bacilli was obtained in culture filtrates of C. albicans grown in brain heart infusion broth.

Francis et al. (1949, 1953) reported the isolation from M. phlei of a growth factor for M. johnei which was purified and given the name 'mycobactin'. This compound had not been described

heretofore and was shown to have a chemical structure distinctly different from any other known growth factor. Most known growth factors are of widespread occurrence in nature, while mycobactin appears to be distributed only within the genus Mycobacterium. Francis suggested that its production results from some peculiarity in the metabolism of the genus; M. johnei apparently has only a limited ability to synthesize this substance upon primary isolation.

Procedures

The growth factors were incorporated in Kirchner's fluid medium by the addition of 1 ml. of a stock solution, or a dilution thereof, to give the final concentration desired. The sources of these substances are given in Appendix C and the chart below summarizes the mode of preparation of stock solutions.

Compound	Solvent	Stock Solution
Biotin	Phosphate buffer pH 7.2	1 mgm/ml
p-Aminobenzoic acid	Basal medium	1 mgm/ml
Pyridoxine.HCL	"	5 mgms/ml
meso-Inositol	"	100 mgms/ml
Thiamine. HCL	"	10 mgms/ml
Calcium pantothenate	Distilled water	50 mgms/ml
dl-α-Tocopherol Acetate	Ethanol-water (1:10)	5 mgms/ml
Menadione diphosphate	Distilled water	1 mgm/ml

Compound	Solvent	Stock Solution
Phthiocol	Distilled water	0.5 mgm/ml
Sodium acetate	prepared in basal medium	
Oleic acid	N NaOH	10 mgms/ml
Linoleic acid	N NaOH	5 mgms/ml

All materials except Tocopherol acetate and sodium acetate were sterilized by membrane filtration. Tocopherol, being insoluble in water was made up in ethanol, sterilized by Cellafilter filtration and sterile distilled water added to give an emulsion in which the final concentration of tocopherol acetate was 5 mgms/ml. Sodium acetate was sterilzed by autoclaving after incorporation in the basal medium.

d(-)Arabinose: This was prepared in 10% solution in Kirchner basal medium which contained no glycerin and sterilized by membrane filtration. A 10% solution of glycerin in the basal medium was also prepared and these were added, in the proportions shown below, to basal medium without glycerin; 10 ml. of serum was added to each 100 ml. of the various media before use.

ml. Basal medium (no glycerin)	ml. 10% glycerin	ml. 10% arabinose	Final concentration after addition of serum % glycerin	% arabinose
80	20	-	1.8	-
70	20	10	1.8	0.9
70	10	20	0.9	1.8
90	-	10	-	0.9
80	-	20	-	1.8

Acetone extract of M. phlei: This was prepared after the method of Francis (1953); its preparation is described in Appendix D. A 1/8 dilution of the acetone extract was prepared in basal medium and steamed at 100°C until the nitroprusside test for acetone became negative (Hawk, Oser and Summerson 1954). The extract remained evenly dispersed throughout the basal medium after removal of the acetone. Increasing $\frac{1}{4}$ dilutions were prepared in the basal medium and 10% serum was added. The final concentrations of acetone extract in the complete media were 1/35, 1/140, 1/560 and 1/2240.

Candida albicans filtrate: Candida albicans was grown for two weeks in a medium consisting of 2% peptone and 4% cerelose in distilled water#. The fully grown culture was autoclaved and Seitz-filtered to remove the yeast cells. The Seitz filtrate was added to Kirchner medium in concentrations which extended from 1 - 10%. The amount of serum was reduced so that the combined proportions of filtrate and serum did not exceed 10%.

The HA strain was used in these investigations. The bacillary suspensions used for oil partition contained numbers ranging from 1000 to 100 bacilli per 5 ml. The content was determined by the

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extent of dilution of the filtered saline suspension upon which the total count was performed. In the majority of cases, the bacilli were transferred to the culture medium immediately after oil partition. In some cases however, the bacilli were allowed to remain in the oil for varying periods of time in order to determine the effect of added substances on bacilli damaged by prolonged exposure to coconut oil.

In order to obtain a rough quantitative estimate of the efficacy of added materials, the practice was adopted of examining the cultures daily and recording the day on which growth first became visible. As there was some variation, usually not more than two days, in the time taken for duplicate samples to show growth when small inocula were used, five replicates were included in each test, in order to minimize the sampling error and also to prevent contamination from invalidating an experiment. All experiments were terminated after 28 days of incubation.

The effect of these growth factors on normal tubercle bacilli was also examined. As tubercle bacilli grow readily in Kirchner's medium, the inoculum was reduced to about 50 bacilli (0.2 ml. of a suspension diluted to contain 250 bacilli per ml.) in order to render the test the more sensitive.

Results

The results of these experiments are summarized in Tables XII to XVII inclusive and show that only inositol and the Candida albicans filtrate exerted any favourable influence on the growth of oil partitioned tubercle bacilli of the HA strain.

In Table XII, it is shown that inositol in a concentration of 100 micrograms/ml. or greater allowed the growth of all five replicates when a suspension containing 100 bacilli was subjected to oil partition. No cultures grew when the inositol content was 10 micrograms/ml. and only one grew when the medium contained no inositol. Inositol appeared to have little or no influence on the growth rate; there appeared to be a slight increase in the time required for earliest visible growth when the original suspension contained 500 bacilli. Biotin in concentrations from 0.1 micrograms to 10 micrograms allowed more rapid growth of organisms collected from a suspension containing 100 bacilli, but in the higher concentration, not all samples grew. Calcium pantothenate had a slight favourable influence on growth rate; pyridoxine, in a concentration of 50 micrograms per ml. appeared to be somewhat inhibitory for oil partitioned bacilli; the time required for earliest visible growth increased and the proportion of replicate samples showing growth decreased.

TABLE XII

The Influence of B Vitamins on the Growth of Oil Partitioned Tubercle Bacilli (HA)

(The time in days required for first appearance of growth*)

Compound	Concentration γ/ml	Number of bacilli	
		500	100
Biotin	0	-	21
	0.1	-	18.2
	1	-	17.3
	10	-	19.3
Thiamine. HCl	0	14.7	-
	1	15.4	-
	10	15.2	-
	100	14.8	-
p-Aminobenzoic acid	0	16.6	-
	0.1	15.5	-
	1	22.7	-
	10	16	-

TABLE XII (Cont'd)

Compound	Concentration γ/ml	Number of bacilli	
		500	100
Calcium pantothenate	0	16.2	-
	5	16.8	-
	50	14.6	-
	500	14.6	-
Pyridoxine.HCl	0	16	-
	0.5	15.1	-
	5	15.5^{4}	-
	50	18.5^{2}	-
Inositol	0	14	16^{1}
	10	14	X
	100	16	15.6
	1000	15.2	15.6

* The average time required for 5 samples to show visible growth. Growth of less than 5 samples is indicated by the superscript.

- omitted

X growth failure in all samples

Table XIII shows that none of the fatty acids exerted any favourable influence on growth. Oleic acid was slightly inhibitory in a concentration of 0.05 mgms per ml. and 0.1 mgm per ml. was completely inhibitory for both oil partitioned and normal bacilli. Linoleic acid did not inhibit growth in a concentration of 0.05 mgms per ml.; higher concentrations were not tested.

Preliminary tests were carried out to determine the effect of tocopherol acetate, phthiocol and menadione diphosphate on the growth of normal bacilli, in order that a suitable range of concentrations could be selected for testing. Phthiocol and menadione delayed growth in concentrations of greater than 10 micrograms per ml. and 100 micrograms per ml. respectively, while lower concentrations had no inhibitory influence. Tocopherol acetate had no influence on growth in concentrations from 0.1 micrograms to 50 micrograms per ml. provided the concentration of ethanol, which was used to dissolve it, was less than 0.1% in the complete medium; growth was retarded when ethanol was present in 1% concentration. Table XIV shows that none of these substances exerted a favourable influence on the growth of oil partitioned bacilli. Tocopherol acetate delayed growth in a concentration of 50 micrograms per ml.

Table XV shows that the combination of d(-)arabinose and glycerin in concentrations of 1.8% and 0.9% respectively caused a marked delay in the growth rate of oil-partitioned bacilli; the

TABLE XIII

The Influence of Fatty Acids on the Growth of Oil Partitioned Tubercle Bacilli (HA)

(The time in days required for first appearance of growth*)

Compound	Concentration mgm. per ml.	Number of bacilli		
		1000	500	100
Sodium acetate	0	15.8	-	25.6^{3}
	0.1	15.4	-	22
	0.5	15.0	-	22.5^{3}
	1.0	16.8	-	24^{4}
Sodium oleate	0	14.4	-	19^{4}
	0.01	15.2	-	17.4
	0.02	13.4	-	19
	0.05	16.6	-	24.6^{3}
Sodium linoleate	0	-	16	-
	0.002	-	14.7	-
	0.01	-	16.	-
	0.05	-	15	-

* The average time required for 5 samples to show visible growth. Growth of less than 5 samples is indicated by the superscript.

- omitted

TABLE XIV

The Influence of Tocopherol, Phthiocol and, Menadione on the Growth of Oil Partitioned Tubercle Bacilli (HA)

(The time in days required for first appearance of growth*)

Compound	Concentration γ/ml	Number of bacilli	
		500#	250
α-Tocopherol acetate	0	17^{4}	16.2
	5	18.2	18.2
	50	19.4	19.5^{4}
Phthiocol	0	20.5^{4}	17^{4}
	0.5	18.2	18.6^{4}
	5	19.6	19.4
Menadione diphosphate	0	-	20.2^{4}
	1	-	19^{4}
	10	-	18

* The average time required for 5 samples to show visible growth. Growth of less than 5 samples is indicated by the superscript.

\# Exposed to coconut oil for 3 hours

\- omitted

TABLE XV

The Influence of d(-) Arabinose on the Growth of Oil Partitioned Tubercle Bacilli (500 HA)

(The time in days required for first appearance of growth*)

Composition of medium		first appearance of growth (days)*
% Glycerin	% Arabinose	
1.8	0	15
1.8	0.9	17.6^{4}
0.9	1.8	21^{4}
0	0.9	16.5
0	1.8	17^{2}

* The average time required for 5 samples to show visible growth. Growth of less than 5 samples is indicated by the superscript.

same effect was noted with normal bacilli. This effect was not as pronounced when the medium contained only d(-) arabinose.

Table XVI shows that the M.phlei acetone extract has no influence on the growth rate of oil-partitioned bacilli; normal bacilli were not tested. Preliminary tests showed that this extract contained a factor which promoted the growth of M. johnei, however. (See Appendix D).

Table XVII shows that the Candida albicans filtrate contains a factor or factors which stimulated the growth of bacilli which had been exposed to coconut oil for three hours subsequent to oil partition. In a concentration of 5%, growth occurred in each of the five replicates and in only two of five replicates when the medium contained no Candida filtrate. Complete replacement of the serum by Candida filtrate allowed no growth, either of oil partitioned or untreated bacilli. This indicates that a factor is present in serum which is essential for growth of a small inoculum of the tubercle bacillus in synthetic medium, and which is not replaced, or substituted for, by the Candida factor.

Under the experimental conditions, none of the factors which were examined exerted a stimulating effect on the growth of normal bacilli of the HA strain. These results are not included in the tables which summarize the results obtained with oil partitioned bacilli; no direct comparison can be made because a smaller inoculum was used in the tests with normal bacilli.

TABLE XVI

The Influence of *M. phlei* Acetone Extract on the Growth of Oil Partitioned Tubercle Bacilli (HA)

(The time in days required for first appearance of growth*)

Concentration of Extract	Number of bacilli	
	500#	250
0	16.7^{4}	17.2
1/35	20.6^{4}	16.7^{4}
1/140	17	17.4
1/560	17.4^{4}	16.8
1/2240	18^{4}	18.7

* The average time required for 5 samples to show visible growth. Growth of less than 5 samples is indicated by the superscript.

Exposed to cocomut oil for 3 hours.

TABLE XVII

The Influence of C. albicans filtrate on the Growth of Oil Partitioned Tubercle Bacilli (500 HA)#

Composition of Medium		first appearance of growth (days)*
% serum	% Candida filtrate	
10	0	17.5^{2}
9	1	16.7^{4}
7.5	2.5	17.4
5	5	15.5
0	10	X

* The average time required for 5 samples to show visible growth. Growth of less than 5 samples is indicated by the superscript.

X Growth failure in all samples.

\# Exposed to coconut oil for 3 hours.

Further Studies with Inositol and the Candida Albicans Factor

In the preceding section it was shown that inositol and the Candida filtrate exerted a favourable influence on the growth of oil partitioned tubercle bacilli. Accordingly, they were selected for study and additional experiments confirmed the original findings. In order to obtain an accurate estimate of the amount of growth ensuing from inocula of oil partitioned bacilli, the total nitrogen of the bacterial mass in cultures was determined by the micro-Kjeldahl method. Preliminary experiments showed this to be a more sensitive means of estimating relative bacterial mass than dry weight determinations or the measurement of the volume of packed cells.

Procedure

The confirmatory experiments were performed using the same methods described in the previous section.

The micro-Kjeldahl determinations were performed on cultures grown in 30 ml. of Kirchner medium, with and without added growth factor, contained in Erlenmeyer flasks of 125 ml. capacity. The flasks were plugged with gauze-wrapped cotton and sealed with a sheet of polyethylene plastic film to prevent evaporation of the medium during incubation. Tubercle bacilli were oil partitioned, collected on membrane filters and the latter were transferred to the medium. The grown cultures were killed by heating in a water bath at 60 - 62°C for two hours; serum proteins in the medium were

precipitated at temperatures greater than 65°C. The killed cultures were transferred to 50 ml. centrifuge tubes. The membrane and walls of the flask were rinsed with distilled water and the washings were also added to the centrifuge tubes. After centrifugation at 2500 rpm for 30 minutes the supernatant medium was removed and the cell mass was washed twice with ammonia-free distilled water, followed each time by centrifugation. Determination showed that the second wash water contained only insignificant traces of nitrogen. The washed cell mass was transferred to digestion flasks and digested with 3 ml. conc. H_2SO_4 and a trace of catalyst (powdered selenium: potassium sulphate = 1:7). The digest was steam-distilled and the distillate collected in 12 ml. of N/70 H_2SO_4; N/70 NaOH was used for back-titration.

Results

The influence of inositol: Suspensions of the HA strain which contained 1000, 300 and 100 bacilli were subjected to oil partition and seeded in Kirchner medium containing 500 micrograms inositol per ml. and in the same medium without inositol. Table XVIII shows that inositol has a distinctly favourable influence on the growth of 100 oil partitioned bacilli; four of five replicates grew in the medium containing inositol and only one of five grew in the control. Its influence is not so marked when the number of bacilli is greater.

TABLE XVIII

The Influence of Inositol on the Growth of Oil Partitioned Tubercle Bacilli (HA)

Number of bacilli	Time* (days) for first growth in medium containing:	
	500 γ Inositol/ml	No Inositol
1000	15.5	15.2
300	14	15.4
100	17.7^{4}	18^{1}

TABLE XIX

The Influence of Inositol on the Growth of Tubercle Bacilli (500 HA) after Prolonged Exposure to Coconut oil

Exposure (hours)	Time* (days) for first growth in medium containing	
	500 γ Inositol/ml	No Inositol
2	14.8	16
7	16.6	21.5^{2}
24	21^{1}	19^{1}

* The average time required for 5 samples to show visible growth. Growth of less than 5 samples is indicated by the superscript.

A second experiment was performed to determine the effect of inositol on the growth of tubercle bacilli damaged by prolonged exposure to coconut oil. The suspensions subjected to oil partition contained 500 bacilli, and after removal of the aqueous subnatant, the bacilli were kept in contact with the oil for 2, 7 and 24 hours before transfer to the culture medium. Table XIX shows that inositol aids the growth of 500 bacilli which have been exposed to cocomut oil for seven hours. It did not restore the viability of bacilli exposed for 24 hours, however. Bacilli exposed to cocomut oil for two hours appear to suffer little damage.

The combined influence of Candida factor and Inositol: A third experiment tested the influence of inositol and the Candida factor alone and in combination. Media were prepared as follows:

A. Kirchner medium (control)

B. Kirchner medium + 500 micrograms inositol per ml.

C. Kirchner medium + 5% Candida albicans filtrate

D. Kirchner medium + 500 Micrograms inositol/ml. + 5% C. albicans filtrate

Inocula consisted of 500 and 100 oil partitioned bacilli transferred to the medium immediately after oil partition. The results in Table XX show that the combination of Candida factor and inositol exerts a more powerful stimulus on the growth of oil partitioned tubercle bacilli than either alone. The effect of inositol was not so marked

TABLE XX

The Influence of the Candida factor and Inositol on the Growth of Oil Partitioned Tubercle Bacilli

(Time* (days) required for first appearance of growth)

Medium		Number of bacilli	
% Candida filtrate	Inositol γ per ml.	500	100
0	0	16.2	17.3^{3}
-	500	14.6	19^{4}
5	0	14.6	18.2^{4}
5	500	14.2	16.8

* The average time required for 5 samples to show visible growth. Growth of less than 5 samples is indicated by the superscript.

in this experiment, as growth was obtained in three of five samples in the control series which received the smaller inoculum. In tests with normal bacilli, the combination of inositol and Candida factor increased the growth rate to a slight extent, while either factor alone had no apparent influence.

Quantitative estimation of bacterial nitrogen: Media were prepared as above, but in volumes of 30 ml. contained in 125 ml. Erlenmeyer flasks. Suspensions containing 2500 tubercle bacilli were subjected to oil partition and the bacilli were kept in contact with the oil for 8 hours before implantation. The cultures were examined at frequent intervals and growth appeared in all flasks between the 16th and 21st days of incubation. On the 24th day the cultures were removed from the incubator, killed by heat and the total bacillary nitrogen determined by the micro-Kjeldahl method. All tests were done in triplicate.

Table XXI shows the results of two experiments with inositol and the Candida factor, singly and in combination. Candida filtrate in 5% concentration, either alone or with 500 micrograms inositol per ml. medium enhanced the growth of oil partitioned tubercle bacilli to a considerable extent. Inositol alone had no appreciable effect. The ratios between the amounts of bacterial nitrogen found in the various media have been computed from Table XXI and are shown below:

TABLE XXI

The Amount of Bacterial Nitrogen in Cultures of Oil Partitioned Tubercle Bacilli

Experiment number	Medium	Composition of medium		mg. nitrogen found	mg. nitrogen per culture (average)
		γ/ml. Inositol	%Candida filtrate		
1	A	0	0	0.122 0.122 0.098	0.114
	C	0	5	0.140 0.168 0.166	0.158
2	A	0	0	0.058 0.068	0.063
	B	500	0	0.062 0.072 0.066	0.067
	D	500	5	0.134 0.138 0.120	0.131

Ratios of bacterial nitrogen in media A,B,C,D

Experiment	D/A	C/A	B/A	D/B
1	-	1.39	-	-
2	2.08	-	1.06	1.95

The ratio of bacterial nitrogen in the medium containing 5% Candida filtrate to that in the control medium was 1.39/1. When inositol and Candida filtrate were present together, the ratio increased to 2.08/1, and inositol alone showed no significant effect in stimulating the growth of oil partitioned tubercle bacilli under the conditions of this experiment.

Paper chromatography of Candida filtrate: To determine whether or not the Candida filtrate contained inositol, the filtrate was examined by a paper chromatographic method. Specimens of Candida filtrate were spotted on Whatman #1 chromatographic paper, the largest being 0.16 ml. in volume. Aqueous solutions of inositol were prepared and spotted similarly, so that the amounts of inositol deposited on the paper were 1000, 500, 100 and 10 micrograms. The solvent used for development of the chromatogram was 90% ethanol. The colour reagent used was 5% mercuric oxide in dilute nitric acid, followed by treatment of the paper with a mixture of 10% aqueous barium acetate and glacial acetic acid, as described by Fleury et al. (1953). Inositol was detected in

all concentrations except 10 micrograms in the controls but not in the Candida filtrate. If inositol is present in the filtrate, it is not present in a concentration equal to or greater than 100 micrograms per 0.16 ml. i.e., 600 micrograms per ml.

It is concluded that the Candida filtrate contains a factor which stimulates the growth of oil partitioned tubercle bacilli, and probably that of normal bacilli. The influence of inositol appears to be rather complex. It stimulates the development of small numbers (100 - 300) of bacilli damaged by exposure to oil, but has no apparent influence on the amount of growth which results from cultivation of larger numbers of bacilli. However, it enhances the stimulating effect of Candida factor when incorporated in the medium with the latter.

Growth of Oil Partitioned Tubercle Bacilli - In Vivo

In the preceding section it was shown that it is possible to restore the viability of small numbers of tubercle bacilli damaged by coconut oil by the incorporation of inositol or the Candida factor in the culture medium. However, after very prolonged exposure to coconut oil, the bacilli were not assisted by the presence of inositol. Hawirko (1951) suggested that the damage suffered by the organisms was not irreparable, as inoculation of relatively small numbers of oil partitioned bacilli into guinea pigs rendered them tuberculous; no growth was obtained from similarly treated bacilli on artificial media. In order to determine to what extent *in vivo* conditions may restore the viability of small numbers of oil partitioned tubercle bacilli, an experiment was performed to compare the effect of *in vivo* and *in vitro* conditions.

Procedure

A group of 24 young adult guinea pigs, weighing about 350 grams each, were selected for the *in vivo* test. Two groups of 3 guinea pigs received injections of coconut oil containing 1000 and 100 HA bacilli, respectively, immediately after oil partition from aqueous suspensions. The injections were made subcutaneously in the left groin. Two groups of control animals were inoculated in a similar manner with 1 ml. of saline suspensions which contained the same number of organisms. The remaining groups of animals were

injected with oil after 24 and 48 hours, i.e., the bacilli which they contained had been exposed to the action of the oil for these periods, before injection. All pigs were sacrificed after three months and autopsied, as also were pigs which had died before three months had elapsed. A record was made of the gross appearance of the organs of each animal; no cultural tests were performed.

A parallel group of suspensions were subjected to oil partition, the bacilli were collected on membranes in the usual manner, and planted in Kirchner medium without added growth factor. Control suspensions which contained the same numbers of normal bacilli were collected on membranes and planted in Kirchner medium. Five replicates were included in each *in vitro* test.

Results

Table XXII, which summarizes the *in vitro* test, demonstrates very clearly the lethal action of coconut oil for small numbers of tubercle bacilli. Only two of five replicate samples of 100 bacilli showed growth after one hour exposure to coconut oil. No growth was obtained after prolonged exposure. The growth rate of oil partitioned organisms is retarded considerably, as compared to the controls. There is a marked difference between the susceptibility to coconut oil exhibited by 1000 bacilli and 100 bacilli. This has been observed on numerous other occasions.

TABLE XXII

The Growth of Tubercle Bacilli *in Vitro* After Prolonged Exposure to Coconut Oil

(Time* (days) required for first appearance of growth)

Exposure (hours)	Number of bacilli	
	1000	100
1	18.2^{4}	18.5^{2}
24	18.5^{4}	x
48	20.2	x
Controls	10.8	13

* The average time required for 5 samples to show visible growth. Growth of less than 5 samples is indicated by the superscript.

x No growth

Table XXIII shows that prolonged exposure to coconut oil prevents tubercle bacilli from establishing disease in the guinea pig. After 48 hours exposure, the larger inoculum produced slight disease, i.e. a local lesion, in only one of two animals. No animal showed symptoms after receiving the smaller inoculum which had been exposed to oil for 48 hours. However, two of these animals which had received the smaller inoculum after 24 hours exposure to coconut oil developed local lesions i.e., hard swollen caseous lymph glands at the site of inoculation. No growth was obtained with the similar *in vitro* series.

It is noteworthy that after 48 hours exposure to oil, the larger inoculum caused the development of a local lesion in only one of two animals. By contrast, growth was obtained in all five cultures in the parallel *in vitro* series, though the growth rate in the latter was retarded. In the light of this observation, it is suggested that exposure to coconut oil may decrease the virulence of the tubercle bacillus, possibly by a lytic or dissolving action on surface structures of the bacillus which are necessary for virulence.

The progressive diminution in the extent of the disease process in guinea pigs with increasing exposure to coconut oil indicates that the capacity of tubercle bacilli, thus damaged, to regenerate is limited. For the successful demonstration of small numbers of oil partitioned bacilli by animal inoculation, exposure to oil should be minimal.

TABLE XXIII

The Growth of Tubercle Bacilli *in vivo* after Prolonged Exposure to Coconut Oil

Exposure (hours)	The proportion of animals showing:					
	Generalized tuberculosis		Local lesion only		No symptoms of tuberculosis	
	1000*	100	1000	100	1000	100
1	2/2#	2/3	0/2	1/3	0/2	0/3
24	1/3	0/3	0/3	2/3	2/3	1/3
48	0/2#	0/3	1/2	0/3	1/2	3/3
Controls	3/3	3/3	0/3	0/3	0/3	0/3

* Number of bacilli

\# 1 non-specific death

Oil Partition Applied to Tuberculous Sputa

It was demonstrated by Hawirko (1951) that tubercle bacilli could be isolated from sputa by oil partition, but difficulty was encountered in obtaining cultures from oil partitioned organisms on membranes planted on solid media. In this section, the results of an improved method are described which allows the cultural demonstration of small numbers of tubercle bacilli so collected. The essential features of this method are (1) extensive dilution of the specimen before oil partition; (2) the cultivation of membranes on a semi-solid agar medium and (3) the microscopic demonstration of microcolonies on the membrane before they become visible to the naked eye.

Preliminary Experiments

One of the chief difficulties encountered in the application of oil partition to sputum specimens was the incomplete separation of the oil and aqueous layers after centrifugation of the emulsion. This was due, presumably, to reaction between the oil and elements of the digest, especially the alkaline trisodium phosphate which was used for decontamination. After centrifugation of such emulsions the uppermost layer consisted of a soapy mass with only very little free oil. Although it was sometimes possible to demonstrate tubercle bacilli in the little oil which could be collected, the results were not consistent, even when the specimen was positive by direct smear.

In a series of 22 tuberculous sputa#, of which 8 were positive by direct smear, only 6 gave positive results by oil partition and 10 were positive by cultivation of the centrifuged sediment on Löwenstein medium. Microscopic examination of the soap-like mass mentioned above showed that it contained numerous bacilli, which had apparently been trapped.

Eventually it was found that extensive dilution of the sputum digest with physiological saline allowed good separation of the emulsion upon centrifugation; dilution with distilled water did not give as good results. The procedure finally adopted, and which proved successful, was dilution of the digested specimen by 1/10, i.e., 1/40 of the original specimen. It was not considered practical to dilute beyond this point.

In view of the well recognized lethal action of the commonly used digestion agents, an enzymic method was adopted. Decontamination was effected by four hours exposure to an equal volume of 7.5% trisodium phosphate. The enzyme, an α-amylase preparation from cultures of B.mesentericus##, effected good homogenization of sputum within 30 - 60 minutes at 37°C, depending upon the character of the

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Obtained from the Royal Edward Laurentian Hospital, Montreal through the courtesy of Dr. E. Mankiewicz.

Diastafor 'D', Standard Brands, Inc.

specimen. Its lytic action was best when dissolved in N/20 NaOH in a concentration of 1%. The N/20 NaOH was made up in physiological saline as chloride ions were found to enhance its activity further (Meyer 1950). It was necessary to prepare the solution immediately prior to use, as it deteriorated within a few hours.

It was desired to confine growth of the bacilli to the surface of the membrane, in order that it could be the more readily detected; this necessitated the use of a solid medium. Growth was not good on membranes placed on either Löwenstein or oleic acid - albumin agar media. It was found that optimal growth could be obtained on Kirchner medium made semi-solid by the incorporation of 0.3% agar. This was the lowest concentration of agar which would allow the medium to support the membrane. When a smaller concentration was used, the membrane gradually submerged. It has been reported that for optimal growth of the tubercle bacillus on the surface of membranes an atmosphere saturated with water vapour is essential (Wayne 1955); this condition is more easily realized by the use of a semi-solid medium than by the use of a fully solid medium.

Attempts to stain micro-colonies on membranes by the aniline water-fuchsin method of Tietz and Heepe (1950) failed, as decolorization of the membranes proved exceedingly difficult and the extensive washing of the membrane resulted in removal of the micro-colonies. The method described by Wayne (1955) which utilizes a modification of the neutral red reaction of Dubos and Middlebrook (1948)

adapted to the staining of tubercle bacilli on membranes was adopted. This proved successful, as the membranes were readily decolorized by treatment with sodium carbonate solution.

Experiments Using the Improved Method

As the use of tuberculous sputa in experimental work introduces variables, due principally to variation in the bacillary content, experiments were performed with non-tuberculous sputa to which were added known numbers of bacilli of the HA strain. Only one experiment is reported here, and compares centrifugation and oil partition for concentration of bacilli with subsequent cultivation on Löwenstein and Kirchner media, respectively.

In the experiments with tuberculous sputa, which were obtained from individuals with active disease, a conscious selection of microscopically positive specimens was made. This was done in order to achieve a measure of control in the experiment; this is not possible with sputa of unknown bacillary content. The number of bacilli the sputa contained was assessed by performing counts on direct smears stained by the Ziehl-Neelsen method. The sputa were then diluted with non-tuberculous sputa and comparisons of the oil partition and conventional methods were made.

Procedures

Treatment of the specimens: The procedure for treatment of sputa is detailed below:

(i) About 10 c.c. of sputum was collected in 4 oz. round screw-capped glass containers.

(ii) To each specimen was added an equal volume of a fresh solution of 1% α-amylase prepared as follows: 1 gram of amylase powder was suspended in 100 ml. of N/20 NaOH prepared in physiological saline. This was centrifuged and the clear, amber-coloured supernatant was sterilized by Seitz-filtration (centrifugation and filtration caused no loss in activity). The mixture of sputum and enzyme was placed in a 37°C water bath for 30 - 60 minutes and shaken from time to tome. When the specimen was umusually mucoid, shaking with glass beads aided the homogenization process considerably.

(iii) An equal volume of 7.5% Na_3PO_4 (17.4% $Na_3PO_4 \cdot 12H_2O$) was added to the digest which was returned to the water bath for a further four hours.

(iv) One ml. of the decontaminated digest was made up to 10 ml. with sterile physiological saline. This represents a 1/40 dilution of the original specimen.

(v) One ml. of sterile coconut oil was added to each 10 ml. of diluted digest and the mixture was shaken mechanically for 10 minutes.

(vi) The emulsion was transferred aseptically to a centrifuge tube of 15 ml. capacity and centrifuged for 30 minutes to separate the oil and aqueous layers of the emulsion.

(vii) The aqueous subnatant was withdrawn and discarded. The residual oil was dissolved in 10 ml. of sterile petroleum ether and passed through a membrane filter. The membrane was washed with an additional 2 - 3 ml. of petroleum ether to remove vestiges of oil.

(viii) The membrane was removed from the filtration apparatus and transferred aseptically to Kirchner semi-solid medium.

To properly assess the efficiency of the oil partition method as compared to centrifugation, the specimen was divided into two equal parts after stage (iii). One part was diluted and subjected to oil partition, while the other was neutralized with N/I HCL, centrifuged and the sediment was inoculated on Löwenstein medium. Thus, treatment of the specimen was the same in each case prior to oil partition or centrifugation.

Media: In the experiment with synthetic sputa the Kirchner semi-solid

and fluid media contained 500 γ inositol per ml. In the experiments with tuberculous sputa, the media contained 5% Candida filtrate and 500 γ inositol per ml.

Detection of microcolonies on membranes: The membranes were stained according to the method of Wayne (1955) as outlined:

Reagents

(i) A saturated solution of neutral red# in 95% ethanol, filtered and acidified with 1% (v/v) concentrated HCL.

(ii) A 1/10 dilution of a saturated aqueous solution of Na_2CO_3. The dilution was prepared fresh each day.

(iii) 95% ethanol.

Procedure

The membranes were placed, growth surface upwards, on filter paper pads saturated with the reagents indicated, in the following sequence.

(i) - neutral red: 10 minutes

(ii)- Na_2CO_3 : 5 minutes

(iii)- 95% EtOH : 1 minute

The membrane was stained red by the dye and became amber on treatment with sodium carbonate. The ethanol removed excess reagents. The membrane was transferred to filter paper pads and placed in the incubator to dry. When dry, the membrane was mounted on a slide with

National Aniline Division, Allied Chemical and Dye Corp. N.Y.(Lot # N x 18)

Permount fluid (Fisher Scientific Co.) under a cover slip. The preparation became transparent by this treatment and was suitable for microscopic examination under all magnifications.

The bacilli were not killed or fixed before staining; the membranes were transferred directly from the culture flask to the pad saturated with dye.

Results

Synthetic tuberculous sputa: In this experiment, a comparison was made of the relative merits of centrifugation and oil partition and of Kirchner semi-solid and Kirchner fluid media for cultivation of oil partitioned bacilli. A'single cell' suspension of HA in saline was prepared and 2 ml. was added to approximately 8 ml. of non tuberculous sputa#. No total count was performed, but the suspension contained approximately $2 - 5 \times 10^8$ bacilli/ml. by visual estimation of opacity. An equal volume of 1% amylase solution was added to the sputum and digested for thirty minutes. A quantity of non tuberculous sputum which had not been inoculated with tubercle bacilli was digested simultaneously. This digest served as the diluent; it was impossible to make dilutions with undigested sputa. Serial 1/10 dilutions were made to 1/10,000 of the original sputum. When the

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Obtained from the Clinical Bacteriology Laboratory, Royal Victoria Hospital, Montreal

dilutions were made, an equal volume of 7.5% Na_3PO_4 solution was added to each and they were returned to the 37°C water bath for four hours. Following decontamination, the digest was divided into three portions, one of which was used for centrifugation and the remaining two for oil partition. The latter two portions were diluted to 1/20 of the original volume of sputum with physiological saline before oil partition. Membranes from the latter two series were cultivated in Kirchner fluid medium and on Kirchner semi-solid medium. All tests were performed in duplicate and all cultures were examined daily. Membranes on semi-solid media were removed after 10 and 14 days and stained.

Table XXIV shows that oil partition and cultivation of membranes on the semi-solid medium allowed earliest detection of growth. Cultivation of membranes in the fluid medium was also superior to cultivation of centrifuged sediments on Löwenstein medium. The superiority of the oil partition method in allowing early detection of growth is especially pronounced in the higher dilutions of the specimen. The figures in the second column of the table show the extent of dilution of the original saline suspension of HA as prepared for oil partition, and the figures in the first column, the corresponding dilutions of the original sputum. One of two oil partitioned specimens of the 1/1,000,000 dilution showed growth on the membranes in semi-solid medium after 14 days of incubation. It is possible

TABLE XXIV

Comparison of Oil Partition and Centrifugation for Concentration of Tubercle Bacilli in Sputum (Synthetic Sputa)

Sputum dilution	Dilution of HA suspension for oil partition	The average time (days) for first detection of growth		
		Löwenstein medium	Kirchner Media	
			Fluid	Semi-solid
1	1/100	14	12	10
1/10	1/1,000	16	14	14
1/100	1/10,000	20	14.5	14
1/1,000	1/100,000	20.5	16.5	14
1/10,000	1/1,000,000	25.5	17	14*

* only one membrane positive after 14 days.

that the other membrane would have been positive if incubation had been continued beyond this time.

Tuberculous sputa: Three microscopically positive sputa from patients with active tuberculosis were obtained. A direct smear was made, stained by the Ziehl-Neelsen method and the number of bacilli in 20 fields was counted. The results are shown below:

Specimen	Bacilli/20 fields
#1	15
#2	32
#3	13

The sputa were digested and serial 1/10 dilutions were prepared with non tuberculous sputa which had been digested simultaneously. After decontamination for four hours with 7.5% Na_3PO_4 the specimen dilutions were divided into two equal portions, one for concentration by centrifugation and the other for oil partition. The digests were diluted to 1/40 of the original volume of sputum with physiological saline prior to oil partition. The centrifuged sediments were cultivated on Löwenstein medium and the membranes were cultivated on Kirchner semi-solid medium which contained 500 γ inositol per ml. and 5% Candida filtrate. All tests were done in duplicate. The cultures were examined every 2 - 3 days and the time at which growth was first observed was recorded. If one of two duplicate membranes

was negative when first examined, the remaining membrane was examined only after a further period of incubation which was usually one week.

The results obtained with this series of tuberculous sputum specimens are presented in Table XXV. In specimens #2 and #3, oil partition and cultivation on semi-solid medium was superior to centrifugation and cultivation on Löwenstein medium. Unfortunately, all the Löwenstein slopes inoculated with specimen #3 were contaminated, except for the undiluted sample. This was probably due to gross contamination of the non tuberculous sputa used for making the dilutions and indicates that four hours exposure to 7.5% Na_3PO_4 is not sufficient to destroy all contaminants in heavily contaminated sputa. In spite of this, only one of the ten samples subjected to oil partition was contaminated, which indicates that there is a degree of selectivity in the oil partition process. That this is not absolute however, was shown by Hawirko and Murray (1954).

It is likely that the membranes on which had been collected bacilli from the two lower dilutions of specimen #2 would have been positive if examined earlier than the twenty-first day. In each case, colonies approaching 1 mm. in diameter and visible to the naked eye were observed at this time. The observation was confirmed, however, by staining of the membrane in the usual manner.

In specimen #1, oil partition failed beyond 1/10 dilution while centrifugation gave positive results as far as the 1/1,000

TABLE XXV

Comparison of Oil Partition and Centrifugation for Concentration of Tubercle Bacilli in Sputa (Tuberculous Sputa)

Sputum Dilution	Sputum dilution for oil partition	The average time (days) for first detection of growth					
		Specimen 1		Specimen 2		Specimen 3	
		Centrifug-ation*	Oil Partition	Centrifug-ation	Oil Partition	Centrifug-ation	Oil Partition
1	1/40	28	21	16	14	28	21
1/10	1/400	22	21	16	14	C	21
1/100	1/4,000	25	-(21)	24	14	C	-(21)
1/1,000	1/40,000	31	-(28)	23	21	C	-(28)
1/10,000	1/400,000	-(42)	-(28)	30	21	C	28

* Centrifuged sediments cultivated on Löwenstein medium
Oil partitioned specimens cultivated on membranes on Kirchner semi-solid medium
Brackets indicate last day on which examination was made.
\- no growth.
C Contaminated.

dilution. It is unlikely that incubation beyond 28 days would have revealed the presence of microcolonies on these membranes. Only very rarely has it been observed, even when using very small inocula, that microcolonies will develop if incubated for longer than 21 days. One such instance occurred in this series, however. The membrane on which had been collected bacilli from the highest dilution of specimen #3 showed one microcolony after 28 days of incubation, while the duplicate membrane was negative upon examination after 21 days.

Photographs of microcolonies which had been grown for 14 days on membranes after oil partition of the undiluted sample of specimen #2 and stained by the neutral red method are shown in Figures 11 and 111. The colonies stained red and show the corded appearance which is regarded as typical of virulent tubercle bacilli (Bloch 1950).

A comparison of semi-solid medium with and without added inositol and Candida factor for the cultivation of oil partitioned bacilli, showed that the microcolonies were visibly larger, though not greater in number, when the growth factors were included. This would indicate that the growth rate is enhanced. However, as the bacilli were not exposed to the extreme conditions used in the preceding work, it is to be expected that the effect of added growth factor is less apparent.

It is concluded that oil partition can be successfully applied to the cultural demonstration of small numbers of tubercle

bacilli present in sputa. It has also been shown that positive results may be more rapidly obtained by oil partition in combination with a microscopic detection method than by conventional methods.

FIGURE II

Microcolonies of M. tuberculosis Cultivated on Membrane Filter Surface

Stain - Neutral red x 110

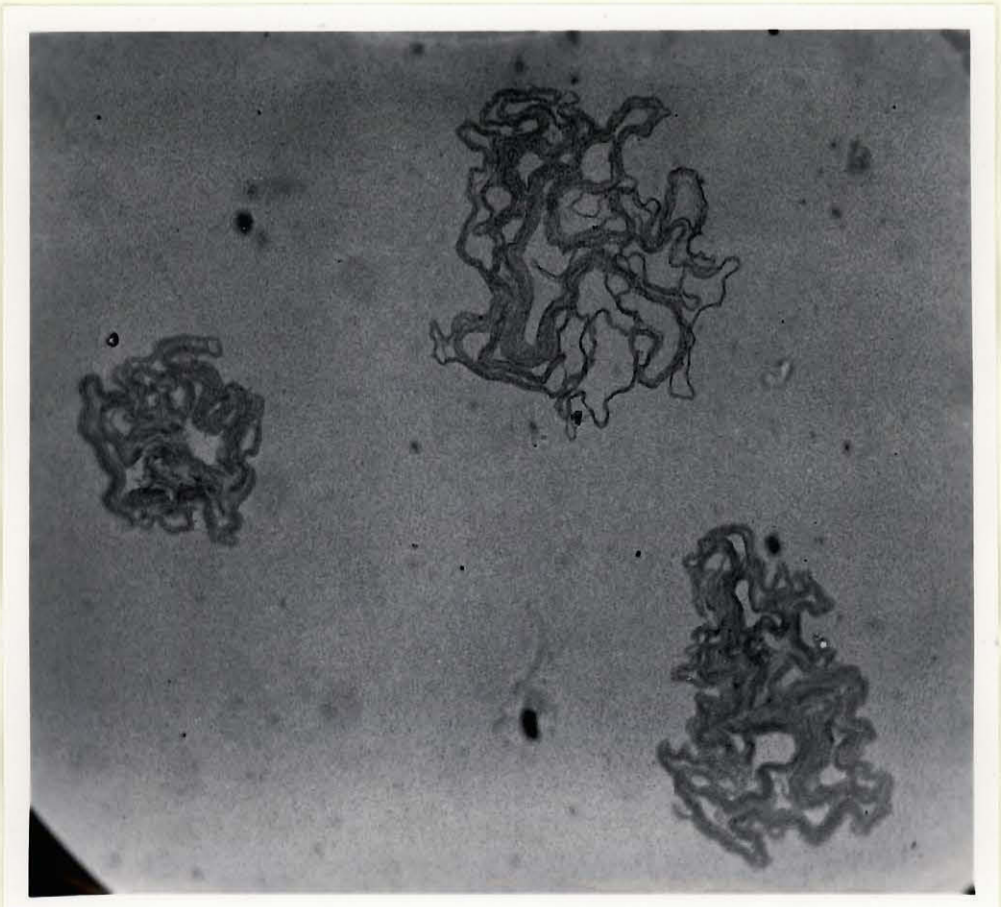
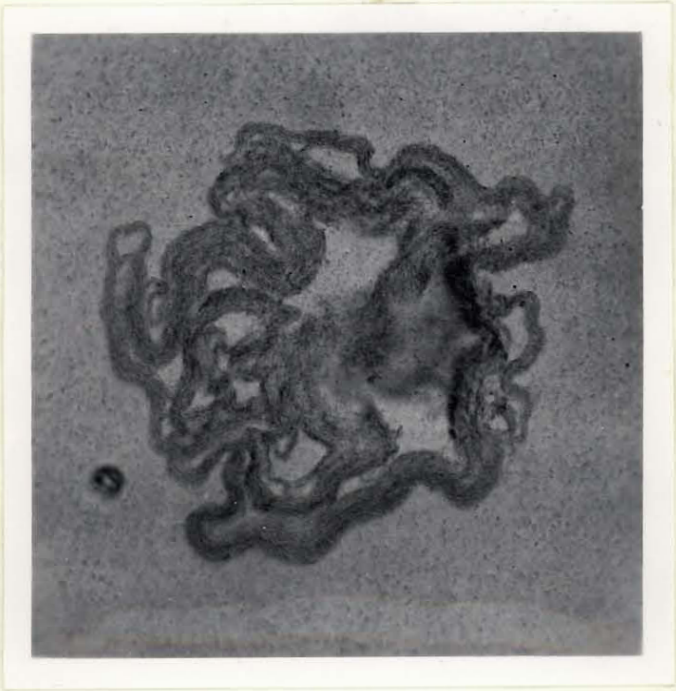


FIGURE III

Microcolony of *M. tuberculosis* Cultivated on Membrane Filter Surface

Stain - Neutral red x 200



Addendum

Toward the latter part of this study a new supply of coconut oil was obtained and was used for oil partition of sputa. This oil showed less lethal action for small numbers of tubercle bacilli than the oil used throughout the earlier work, though both batches of oil were obtained from the same source and carried the same lot number (Brickman and Co., Montreal, Lot #2538 R). In two trials of the newer oil, growth was obtained in all samples of 500 bacilli exposed for seven hours. Inositol in a concentration of 500 micrograms/ml. had no apparent effect on growth while the Candida filtrate, in a concentration of 5%, stimulated the rate of growth to a slight extent. Increasing the inositol concentration to 5 mgms/ml. appeared to stimulate the rate of growth slightly, but this observation was not confirmed. Unfortunately, none of the original oil remained and a direct comparison between the two lots could not be made.

It was noted that the new oil was a very pale yellow colour, in contrast to the dark yellow of the older oil. The latter had been purchased two years previously and it is possible it may have undergone progressive degenerative changes while in use, due to the development of rancidity. Whether this is responsible for its greater lethal action for the tubercle bacilli is not known.

The success in cultivating small numbers of tubercle bacilli,

isolated from tuberculous sputa by oil partition may have been due in part to the use of the less lethal oil.

DISCUSSION

DISCUSSION

A discussion of the influence of vegetable oils on the viability of the tubercle bacillus and the relation between chemical constitution and degree of lethal action is included in the second section of the experimental studies. In the fourth section the relation between chemical constitution, lethal action and the ability to dissolve the acetone-soluble fat fraction of the tubercle bacillus is discussed. To summarize these results briefly, it was found that (1) strains of the tubercle bacillus vary in their susceptibility to the lethal action of oils; this variation is of degree rather than kind; (2) Coconut oil, which contains a predominance of fully saturated triglycerides is less lethal than the other oils examined, which consist principally of unsaturated triglycerides; (3) there appears to be a positive correlation between the degree of lethal action exhibited by an oil and its ability to completely dissolve the acetone-soluble fat of the BCG strain. By the examination of saturated and unsaturated fatty acids and two saturated triglycerides this last observation was extended and it appeared that the ability to dissolve the acetone-soluble fat depended upon the possession of a free carboxyl group and probably also of molecules with unsaturated linkages.

Coconut oil, though relatively less lethal than other oils, nevertheless exhibited a marked inhibitory action on the

growth of small numbers of tubercle bacilli which had been collected from aqueous suspension by oil partition. Fewer than 500 bacilli, as estimated by total count, did not grow consistently in Kirchner fluid medium, while as few as 50 normal bacilli gave first evidence of growth in this medium by the fourteenth day of incubation. Investigations of the effect of added growth factors were carried out using suspensions containing numbers of bacilli of this order. In some instances the period of exposure to coconut oil, after partition, was deliberately prolonged in order to render the test more sensitive. The method used for estimating the efficacy of added growth factor, i.e., measurement of the time required for earliest visible growth, is not without limitations. As turbidimetric measurements were not possible, however, this method was considered adequate for preliminary testing. There was some variation, usually not more than two days, in the time required for visible growth to appear in duplicate samples of oil partitioned bacilli and accordingly, the number of replicates in each test was increased to five. A greater number of replicates would undoubtedly have increased the accuracy of the method, but the design of the experiments made it impracticable to use more than five.

The results obtained are qualified to some extent by the fact that 'single cell' suspensions contained only 50% of viable cells, and also by the fact that the suspensions contained

very small numbers of organisms. The use of suspensions containing greater numbers of bacilli was considered, but there appeared to be a remarkable disparity between the susceptibility of 1000 bacilli and half that number to the action of coconut oil (see Tables XIX and XXII). In spite of the fact that relatively small numbers would possibly introduce a considerable sampling error, the results obtained by the use of five replicates and careful adherence to a standard procedure gave results which were reproducible to a satisfactory degree.

It was found that inositol and the Candida albicans filtrate were able to assist the growth of oil partitioned bacilli, though they manifested their effects in a different manner. Inositol appeared to assist only extremely small numbers of oil partitioned bacilli or slightly larger numbers which had been exposed to oil for a long period. Its effect was manifested by the occurrence of growth in all replicate samples of tests in which the control medium allowed growth of less than two samples. It did not appear to have any effect on the growth rate of the culture; it simply assured growth of small numbers of oil-damaged bacilli. Upon extreme exposure to coconut oil, small numbers of tubercle bacilli are no longer assisted by the presence of inositol in the medium. Later studies with larger numbers of oil partitioned bacilli showed that inositol had no influence on these.

It is impossible to draw conclusions with regard to the action of inositol, but it is suggested on the basis of these findings that it substitutes for a factor which is limiting when the number of bacilli of lowered vitality is small. Inositol very likely plays a part in the metabolism of the bacillus as it has been isolated from the phosphatide and polysaccharide fractions and from culture filtrates of the tubercle bacillus.

Though inositol alone had no influence on the growth of larger numbers of oil partitioned bacilli, as determined by estimation of the total bacterial nitrogen in cultures, it exerted a pronounced synergistic action when combined with the Candida factor. The synergism displayed by inositol with other vitamins of the B group has been described before (Fleury and Balatre, 1947).

That the influence of the Candida filtrate was not due to inositol elaborated by the yeast and released into the medium was indicated by its different action. By paper chromatography, it was confirmed that the Candida filtrate did not contain inositol in a concentration which would be effective in stimulating the growth of oil-damaged bacilli.

The combination of Candida factor and inositol had a slight enhancing effect on the growth of normal tubercle bacilli, but this was not as pronounced as the stimulus provided oil partitioned bacilli. Quantitative experiments should be carried out to confirm this.

Of the other water soluble vitamins, only calcium pantothenate in a concentration of 500 micrograms/ml showed a stimulatory effect on the growth of oil partitioned tubercle bacilli. Pyridoxine in a concentration of 50 micrograms/ml caused a definite inhibition of growth of both normal and oil-damaged bacilli. This probably represents a toxic reaction due to excess of the factor.

The finding that menadione diphosphate allowed growth of oil partitioned tubercle bacilli in a concentration of 10 micrograms/ml and of normal bacilli in concentrations up to 100 micrograms/ml is not in agreement with the findings of others; concentrations of this order in Tween - albumin medium have generally been shown to be tuberculostatic. The discrepancy is most likely caused by the use of a medium in these studies without Tween 80, which has a potentiating effect on the action of bacteriostatic substances.

d(-) Arabinose, which occurs only rarely in nature, is found in a polysaccharide fraction of the tubercle bacillus. Experiments showed that it was not as good a carbon source as glycerin. The presence of glycerin and d(-) arabinose together in the medium delayed growth of both normal and oil partitioned tubercle bacilli. Whether the bacilli were utilizing d(-) arabinose as a carbon source or using some other material present in the serum was not determined. It would be informative to know this, especially as it has

not been reported before that d(-) arabinose can support the growth of tubercle bacilli.

Francis et al. (1953) suggested that mycobactin, which they isolated from acetone extracts of M.phlei, was an essential metabolite for all members of the genus Mycobacterium. This material looked promising as it is one of the few lipid-soluble microbial growth factors which have been described; it has been postulated earlier that the lethal or inhibitory action of oils for the tubercle bacillus is caused by the removal of an essential lipid metabolite. Though the acetone extract of M.phlei as prepared in this laboratory contained a factor which stimulated the growth of M. johnei, it had no influence on the growth of tubercle bacilli damaged by exposure to oil. However, only one test was performed and it is suggested that further studies of this unusual growth factor are needed.

It was possible to produce tuberculous disease in guinea pigs by injection of oil partitioned tubercle bacilli, if the bacilli had not been exposed to oil for too long a time. Exposure for 24 hours or longer caused serious damage as indicated by in vivo testing. In vitro, the larger inoculum was able to proliferate even after 48 hours exposure, though there was a delay in the time required for the first appearance of growth. It has been reported that repeated injections of certain oils retard the development of

the tuberculous process in guinea pigs, presumably by bringing about an alteration in the response of the host tissues to infection. It is unlikely that a single injection of coconut oil, as used in this study for the introduction of oil partitioned tubercle bacilli into the host, would have any influence of this sort.

It is more likely that a material essential for virulence is removed by exposure of the bacilli to the oil. Bloch (1950) has shown that 'cord' factor which is apparently necessary for virulence can be easily extracted from living cells by petroleum ether without impairment to their viability. From the results of the experiments reported in this study, it would appear that components of the tubercle bacillus which are necessary for the expression of virulence are more susceptible to the action of coconut oil than those necessary for viability.

It has been demonstrated that it is possible to isolate in pure culture relatively small numbers of tubercle bacilli which have been collected from tuberculous sputa by oil partition. Further, results may be obtained more rapidly by the oil partition-culture method which is described, than by conventional methods. Essential features of the method are (1) the use of a semi-solid medium to allow optimal development of tubercle bacilli on the surfaces of membrane filters and (2) the microscopic detection of growth before it becomes visible to the naked eye. In this study, the presence on membranes of corded microcolonies which bind neutral

red was taken as evidence that the culture was one of M. tuberculosis. Saprophytic acid-fast bacteria may also show the properties of cord formation or the ability to bind neutral red, or both; hence, these criteria are not absolute for the identification of virulent M. tuberculosis. However, the use of this staining method is justified by the fact that it allows more rapid detection of growth, and confirmatory evidence in doubtful cases may be acquired by further examination of membrane filter cultures.

The inclusion of inositol and the Candida factor in the medium for cultivation of tubercle bacilli isolated from sputa by oil partition did not show any apparent beneficial effect except that it brought about an increase in colony size. However, the results shown in Table XXI justify the use of these factors. Fabrikant (1956), in this laboratory, has reported that stimulation of growth is brought about by the inclusion of Candida factor in the medium used for slide culture of M. tuberculosis. She also concluded that careful examination of the arrangement of bacilli within microcolonies of acid-fast organisms is the most reliable in vitro method of establishing virulence.

Indications were obtained from the earlier experimental studies that the lethal action of vegetable oils for the tubercle bacillus is related to their triglyceride constitution. However, it would be fallacious to draw definite conclusions of this sort, as

vegetable oils contain a multiplicity of other compounds which are most probably not inert. It is suggested that further studies of the factors required for cultivation of oil partitioned tubercle bacilli be undertaken with pure triglycerides, in order to eliminate the possible influence of other materials which vegetable oils contain. Only in this way is it possible to draw definite conclusions relating chemical constitution to effect on viability. The demonstration that inositol and the Candida factor assist the growth of oil partitioned tubercle bacilli indicates that such studies would produce information regarding essential factors which these deficient organisms lack.

SUMMARY

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A method for the preparation of 'single cell' suspensions of tubercle bacilli is described.

An investigation of vegetable and mineral oils revealed that all had a deleterious influence on the viability of the tubercle bacillus, though to different degrees. Coconut oil and liquid paraffin were least inhibitory. Differences existed in the susceptibilities of three strains of tubercle bacilli to the action of oils; these were differences of degree only. All organic solvents exerted a marked lethal action.

Coconut oil was selected for experiments with oil partition as it was shown to be more efficient than either liquid paraffin or petroleum ether. Though coconut oil was relatively less lethal than other oils, it displayed a marked inhibitory action on the viability of small numbers of tubercle bacilli.

Tuberculo-lipid fractions were extracted from dried cells of the BCG strain and incorporated in coconut oil in an attempt to reduce its lethal action. Only the phosphatide fraction had any influence, and that to a slight degree. However, oil partition with coconut oil-phosphatide mixtures was impractical for technical reasons. Incorporation of these fractions in Lowenstein medium had no favourable influence on the growth of oil partitioned or normal tubercle bacilli. On the other hand, the acetone-soluble

fat fraction was shown to inhibit the growth of oil partitioned tubercle bacilli completely and to retard the growth of normal bacilli.

There appeared to be a positive correlation between the lethal action of an oil and its ability to dissolve the acetone-soluble fat fraction of the BCG bacillus.

Kirchner's fluid semi-synthetic medium was superior to Lowenstein medium for the cultivation of oil partitioned tubercle bacilli. The fluid medium was used in all subsequent studies as such, or was made semi-solid by the addition of 0.3% agar.

A survey was made of various recognized microbial growth factors and other substances in a search for a material which could assist the metabolism and growth of small numbers of tubercle bacilli damaged by the process of oil partition. It was found that inositol in a concentration of 500 micrograms/ml of medium and the filtrate of a Candida albicans culture in a concentration of 5% could assist the growth of small numbers of bacilli damaged by oil.

Detailed studies revealed that inositol assisted the growth only of extremely small numbers of oil damaged bacilli and it appeared to be substituting for a limiting factor which these bacilli lack. The Candida factor stimulated the growth rate of oil partitioned bacilli. An interesting finding was that inositol exerted a pronounced synergistic action when combined with the Candida factor. Paper chromatography of the Candida filtrate showed that it did not

contain inositol in a proportion which would be effective in assisting the growth of small numbers of oil partitioned tubercle bacilli.

Animal and culture experiments carried out in parallel indicated that the virulence of the tubercle bacillus for the guinea pig was more severely damaged by prolonged exposure to coconut oil than viability.

An improved method has been developed for the cultural demonstration of M. tuberculosis on membrane filters after collection from tuberculous sputa by oil partition. The essential features of the method include the use of semi-solid Kirchner medium and a microscopic method for detection of growth.

CONTRIBUTION TO KNOWLEDGE

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An improved method has been developed and described for the cultural demonstration of small numbers of tubercle bacilli collected from tuberculous sputa by the oil partition method. The essential features of the method include the use of semi-solid Kirchner medium for the cultivation of the bacilli on the surfaces of molecular filter membranes and microscopic examination of these for more rapid detection of growth.

It has been shown that in Kirchner fluid medium inositol and Candida factor have a synergistic effect on the growth of tubercle bacilli damaged by exposure to coconut oil.

APPENDICES

APPENDIX A

Formulae and Methods for Preparation of Media

1. Löwenstein (Jensen) Medium

Starch-salt solution:

Potassium phosphate (acidic)	2.4 gms
Magnesium sulphate (hydrated)	0.24 gms
Magnesium citrate	0.6 gms
Asparagine	3.6 gms
Glycerin	12.0 cc.
Distilled water	600 cc.
Potato starch	30 gms
Homogenized whole egg	1000 cc.
2% Malachite green solution	20 cc.

The salts are dissolved, the potato starch added and the mixture autoclaved at 120°C for 30 minutes.

Fresh eggs are washed in soap solution and are then placed in running cold water until the water becomes clear. They are broken into a sterile flask, homogenized by shaking and filtered through sterile gauze. The homogenized whole egg is added to the starch-salt solution and the malachite green is added. After thorough mixing, the medium is dispensed by means of a sterile aspirator bottle with a funnel and bell attachment. Five ml. of medium are dispensed in 1 oz. flint glass square bottles with rubber lined screw-caps and inspissated at 85°C for forty minutes on each of two consecutive days. The medium is checked for sterility before use.

2. Dubos Tween-albumin fluid medium

Basal medium:

KH_2PO_4	1.0 gm
$Na_2HPO_4.12H_2O$	6.3 gms
Asparagine	1.0 gm.

This is dissolved by heating and made up to 950 ml. with distilled water. When dissolved, the following ingredients are added:

Bacto-casitone	1.0 gm.
Ferric ammonium citrate	0.05 gm.
$MgSO_4.7H_2O$	0.01 gm. (1 cc of a 1% aqueous solution)
$CaCl_2$	0.0005 gm. (1 cc of a 0.05% aqueous solution)
$ZuSO_4$	0.0001 gm. (1 cc of a 0.01% aqueous solution)
$CuSO_4$	0.0001 gm. (1 cc of a 0.01% aqueous solution)

The pH is adjusted to 6.8 with N/I HCL and brom-cresol purple and the medium is autoclaved at 110°C for 20 minutes in 90cc lots.

Bovine Albumin:

A 5% bovine albumin fraction V solution (Armour Laboratories, Chicago) is prepared in 2% NaCL solution, sterilized by Seitz filtration and heated to 56°C for 30 minutes to inactive lipase. Preparing the solution in 2% NaCL prevents precipitation of the protein on heating.

Tween 80:

This is prepared in 10% aqueous solution and sterilized by autoclaving.

Glucose:

This is prepared in 50% aqueous solution and sterilized by autoclaving.

All four constituents are prepared separately and combined prior to use as follows:

Basal medium	90 cc
5% bovine albumin solution	10 cc
10% Tween 80 solution	0.5 cc
50% glucose solution	1.0 cc

The complete medium is dispensed in 5 cc volumes in screw-capped test tubes 15 mm. in diameter or in 1 oz. screw-capped square bottles.

3. Kirchner Semi-synthetic medium

This is prepared according to the directions provided by Reed (1953).

(a) Fluid Medium:

$Na_2HPO_4.12H_2O$	3 gms
KH_2PO_4	4 gms
$MgSO_4.7H_2O$	0.6 gm
Sodium citrate	2.5 gms
Asparagine	5 gms
Glycerin	20 ml

(a) Fluid Medium:

Distilled water to 1000 ml

The reaction is adjusted to $_{p}$H7.2 with phenol red and N/1 NaOH. The medium is dispensed in 90 ml.lots and autoclaved at 120° for 20 minutes. Prior to use 10 ml of fresh human serum sterilized by Seitz filtration is added to 90 ml. of the basal medium. The complete medium is dispensed in 1 oz. square bottles each container receiving 5 ml.

(b) Semi-solid medium:

To each 90 ml. of basal medium is added 0.33 gm. of Bacto-agar; this is sterilized by autoclaving. Human serum is added in the same concentration as in the fluid medium.

The final concentration of agar is 0.3%.

APPENDIX B

Lipid extracts of BCG

1. Preparation

Two litres of fully grown cultures of the BCG strain in Sauton's medium were killed by steaming at 100°C for one hour. The killed cultures were filtered to separate the bacillary mass from the medium, washed with water, packed in Soxhlet thimbles and dried by lyphilization. The dried material was extracted in a Soxhlet apparatus for two days with diethyl ether, after the method of Anderson (1927). The ethereal extracts were combined, concentrated to small volume and Seitz-filtered to remove bacterial cells. Concentration of the ethereal extract to dryness under reduced pressure left the crude extract. This represented about 15% of the original dry weight of the bacilli. A portion of the crude extract was taken up in ether and mixed with an equal volume of cold acetone, whereupon a white precipitate formed. Further cooling in an ice bath increased the volume of the precipitate which was allowed to settle and the supernatant was decanted. The precipitate was a white gummy material and corresponds to Anderson's fraction A-3 (phosphatide) mixed with low-melting wax. On evaporation of the acetone in the supernatant to dryness the acetone-soluble fat remained. Attempts to separate the gummy precipitate into its fractions by precipitation from cold ethereal solution by acetone failed. However, on

taking the precipitate up in acetone and heating to 40°C, a yellow oil separated and collected at the bottom of the beaker. The layers were separated in a separatory funnel and on cooling to room temperature, the oil hardened to a greyish soft wax. The acetone layer was cooled and the precipitate settled out. This was taken up in ether and reprecipitated several times with acetone in the cold. The final product, representing Anderson's A-3 fraction, was a pure white powder.

2. Löwenstein medium containing lipid fractions

Löwenstein (Jensen) medium was prepared without glycerin. 0.5 gms. of the crude extract and each of its three fractions were mixed with 0.75 c.c. glycerin and dissolved by heating to 45 - 50°C. 4.25 ml. of distilled water, heated to 50°C, was added to each glycerin-lipid mixture, whereupon an emulsion was formed. The whole contents of each tube were transferred to flasks which contained 95 ml. of fluid Löwenstein medium (without glycerin) heated to 45°C. The flasks were agitated vigorously to distribute the material, and the medium was dispensed in square flint-glass bottles of 1 oz. capacity, or in 5 cm. Petri dishes, and inspissated at 85°C on each of two consecutive days.

APPENDIX C

Accessory Growth Factors

Biotin (crystalline) Nutritional Biochemicals Corp.,Lot #8731

Calcium pantothenate (dextrorotatory) Nutritional Biochemical Corp., Lot #9205

Pyridoxine hydrocholoride Nutritional Biochemical Corp.

p-Aminobenzoic acid Eastman Kodak Co.

meso-Inositol Difco Laboratories, Inc. Lot #411582

Thiamin hydrochloride Merck and Co., Ltd., Lot #35044

dl -α- Tocopherol acetate Hoffman-Laroche Ltd., Lot #15

Phthiocol Nutritional Biochemical Corp., Lot #4917

Vitamin K diphosphate Hoffman-Laroche Ltd., Lot #107R

(2-methyl-1,4-naphthohydroquinone diphosphoric acid ester tetrasodium salt)

Sodium acetate (A.R.) British Drug Houses, Ltd.

Oleic acid Nutritional Biochemical Corp. Lot #3653

Linoleic acid Hoffman-Laroche, Ltd., Lot #10205

d(-) Arabinose British Drug Houses, Ltd., Lot #09722

APPENDIX D

The Preparation of an Acetone Extract of M.phlei

M. phlei was grown in 4 litres of beef heart infusion broth which contained 10% glycerin and 4% Bacto-Peptone (Francis et al. 1953). After two weeks incubation at 37°C the cell mass was harvested by centrifugation, washed with distilled water and dried by lyophilization. The yield was 1.8 grams (dry weight). The dried cells were extracted twice with 60 ml. acetone under reflux. The extracts were pooled, Seitz filtered to remove bacterial debris and concentrated to a volume of 15 ml. by vacuum distillation. The resulting clear, dark yellow solution was used as such.

When incorporated in Long's synthetic agar in concentrations of 1/80 - 1/1280 it stimulated more rapid growth of a strain of M. johnei (strain 11, Animal Diseases Research Institute, Hull, P.Q.) than Long's agar with no extract. It had about the same degree of activity in promoting the growth of M. johnei as whole dried M.phlei cells added to the medium in 1% concentration.

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