THE DETECTION OF ACID-FAST MICROORGANISMS BY MEMBRANE FILTER TECHNIQUES

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ABSTRACT

THE DETECTION OF ACID-FAST MICROORGANISMS
BY MEMBRANE FILTER TECHNIQUES

By George L. Wright, Jr.

A new membrane filter (MF) method for the detection of acid-fast microorganisms from skin lesions (cattle) and sputa (human) is described.

Isolations were made by a MF method using a cysteine and disodium versenate activated papain solution, (400 ppm Roccal at pH 5.5). Both digestion and initial decontamination of the specimen was satisfactory. Less contamination occurred when the MF was placed on Middlebrook 7H10 agar which contains 1 ppm malachite green.

A modification of Middlebrook 7H9 agar (semi-solid, double strength) containing Colymycin (20 mcg/ml), Erythromycin (2 mcg/ml), and Mycostatin (100 mcg/ml) was the most effective medium in preventing the growth of contaminants from sputum specimens.

Isolations were more rapid when the cultures were incubated in a 5 per cent CO_2 in air atmosphere than in air alone.

A membrane filter staining technique for acid-fast organisms is also described. Penetration of basic fuchsin into the bacterial cell was enhanced by the addition of wetting agents, Triton X-45 and Tween 80. Glycerol incorporated into

the basic fuchsin solution neutralized the charge on the MF and allowed the excess stain to be washed from the filter with acid alcohol.

The effect of various decontamination procedures and 40 antimicrobial agents on acid-fast and nonacid-fast organisms was investigated.

The MF technique facilitates the deposition of all the tubercle bacilli from the digest on the filter. This is highly desirable when few tubercle bacilli are present in tissue or sputum.

THE DETECTION OF ACID-FAST MICROORGANISMS BY MEMBRANE FILTER TECHNIQUES

by

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TO

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INTRODUCTION

Tuberculosis is still the most deadly microbial disease in the United States, with some 60,000 cases detected each year. While both new tuberculosis cases and deaths have fallen dramatically during the last decade, the rate of new cases reported has been declining more slowly than the death rate.

The case incidence continues at a high level because: (1) increasing numbers of the tubercle bacilli are developing resistance to antibiotics, (2) there is no reliable vaccine, and (3) current techniques for detecting tubercle bacilli are ineffective particularly on low population tissues and body fluids.

The National Tuberculosis Association in 1960 launched an all out drive to eradicate tuberculosis in this country. The campaign, aimed at better detection, better follow up, and wider use of effective drugs, has an ambitious goal: reduction in the number of new tuberculosis cases by two-thirds by 1970. Tuberculin testing of school children is of primary concern in order to obtain a register of known reactors. A positive test in young children may indicate active, closed or a recently acquired infection.

Furthermore, tuberculosis of cattle which was thought to be under control by 1940, again threatens the livestock industry and the general public health of this country. While bovine tuberculosis is at a seemingly low level, every herd

must be regarded as susceptible to infection. A single reactor can infect an entire herd or transmit the bacillus to man. Therefore, the necessity for eradication of both bovine and human tuberculosis is urgent.

Consequently, without a vaccine available, eradication will depend upon detection. The necessary tools to perform this task, namely the X-ray, tuberculin test, bacteriologic and histopathologic procedures exist, but they must be re-evaluated and improved to achieve their maximum efficiency. Recently it has been noticed that it is no longer acceptable to make X-rays the only method of detection, as the radiogram in many instances fails to detect lesions long after the organisms are found in sputum.

The tuberculin test is the most sensitive means of detection. A positive test cannot be used as a measurement of the duration or severity of the infection nor to determine whether the disease is active or arrested. The absence of gross lesions in tuberculin positive cattle presents an equally important problem. Tuberculosis, therefore, can be detected clinically however, the demonstration of the tubercle bacillus in pathological material is the only sure means for diagnosis.

There has been little change in methods used for laboratory diagnosis during the past sixty years. Dubos in 1954 stated, "The primitive bacteriological techniques worked out decades ago for the detection of bacilli in sputum and other pathological materials are still in use today. Yet it is

certain that most bacilli do not survive the barbarious treatment with alkalies or acids to which they are submitted during the preparation of the specimen for the culture tests".

It has been the policy in most laboratories to use methods consisting of digestion followed by centrifugation, and inoculation of the sediment on specific media. Direct smears and animal inoculations are usually run in conjunction with the cultural methods. Culture techniques afford more sensitive determination of the presence of small numbers than do microscopic or pathological demonstration. At present, it takes from 2-6 weeks for diagnosis by cultural methods. During this time, the disease may progress to a point where the infection becomes more difficult to treat.

Although most of the tubercle bacilli do not survive the harsh treatment by strong alkalies or acids used for digestion and decontamination, the procedures still persist. In specimens of high tubercle bacilli population, diminution in viable cells may result but isolation still can be made. On the contrary, in low population specimens, negative results are frequent. Attempts to develop more reliable and accurate methods have failed primarily because contaminants could not be destroyed without harming the acid-fast organisms. In the use of present methods, most of the contaminating organisms are destroyed and at the same time a large proportion of the tubercle bacilli present are destroyed.

The membrane filter (MF)[1] offers the most effective method of concentrating tubercle bacilli from tissues and body fluids, and is especially valuable in situations where the tubercle bacillus population is low. However, if the MF is to become a diagnostic tool in the tuberculosis laboratory, better methods than those now currently used are required for the digestion and decontamination of specimens before filtration.

An attempt has been made in the current study to develop techniques for the use of the membrane filter for the detection of tubercle bacilli from tissues and sputum, with special emphasis on procedures for digestion and decontamination.

[1]A thin, porous sheet material which contains capillary pores of extremely uniform size, small enough to prevent the passage of bacterial organisms.

LITERATURE REVIEW

Homogenizing Agents

There have been numerous reports, since the discovery of the tubercle bacillus, describing the use of various agents for the digestion of pathological specimens: antiformin (Schmid, 1952, Tietz, 1949, 1950, and Uhlenhuth and Xylander, 1909); ammonium hydroxide (Braver, 1918); caustic soda (Gottel, 1947, and Mackie and McCartney, 1953); hydrochloric acid (Corper and Uyei, 1927, Lowenstein, 1924, and Willis and Cummings, 1952); potassium hydroxide (Ditthorn and Schultz, 1917); sodium carbonate (Griffith, 1914); sodium hydroxide (Chu, 1959, Petroff, 1915, and Tison, 1947); sodium hypochlorite (Cameron and Castles, 1946, Collins, 1952, Moreau, 1922, and Preuss, 1949); sulfuric acid (Corper and Uyei, 1927, Lowenstein, 1924, and Weigand, 1953); trisodium phosphate (Corper and Stoner, 1946, Sirsi, 1951, and Vranken, 1947); and urea (Bregmann, 1951). The majority of these agents have been discarded primarily because of their toxicity to the tubercle bacilli.

Nevertheless, the sodium hydroxide method originated by Petroff (1915) is still the most widely accepted method for pretreatment of tuberculous specimens. Several modifications of the original method have been employed. Extreme concentrations as low as 1 per cent (Funk, 1947), and as high as 25 per cent (Ogawa, Saba, and Suzuki, 1949) have

been utilized, with the 4 per cent concentration usually being chosen (Willis and Cummings, 1952).

Sodium hydroxide has been demonstrated to be detrimental to the tubercle bacillus. Yegian and Budd (1952) reported a marked reduction in the number of viable cells after a five minute contact with 2 per cent sodium hydroxide as measured by the colony count. Gerundo and Evangelista (1956), Gray, et al. (1954), and Lipe, et al. (1961) demonstrated that various concentrations of sodium hydroxide had adverse effects on the tubercle bacillus both in a delay in growth and reduction of culturable organisms. The latter two teams reported that up to 80 per cent of the acid-fast organisms were destroyed. Jensen and Bindslev (1946) found that only 1-5 per cent of the tubercle bacilli contained in sputum could be recovered after treatment with sodium hydroxide.

The sodium hydroxide method is still widely used because it does a satisfactory job of digestion as well as decontaminating the specimen if the tubercle bacilli are in high populations. However, the foregoing reports indicate that bacilli in low populations would be missed.

Many investigators claim digestive enzymes are effective digestants and harmless to acid-fast organisms. Spengler (1903) first described the use of enzymes for treatment of sputa. He used pancreatin and decontaminated sputa with formaldehyde.

McNamara and Ducey (1935) and Gerundo (1942) described the successful use of pepsin and hydrochloric acid for the

digestion and decontamination of sputum. Trypsin and oxalic acid was reported by Haynes (1942) as ideal agents for digesting and decontaminating sputum, gastric washings, urine and other body exudates. This combination gave better results than flocculation methods.

A number of enzymes were evaluated by Rice and Rowan (1953). They found pancreatin superior to pepsin, trypsin or papain for digestion of sputum.

Middlebrook, Cohn, and Schaefer (1954) used pangestin activated with magnesium sulfate for the homogenization of sputum. The digest was decontaminated with a mixture of sodium hydroxide and trisodium phosphate. This was followed by the addition of a mixture of albumin and lima bean trypsin inhibitor to stop the action of the enzyme.

Sullivan and Sears (1939), Sedallian and Carraz (1947), and Tison (1952) found more isolations could be obtained using papain as a digestive agent. Scolori (1955) reported the digestion of sputum with hyaluronidase isolated from horse testes.

There are few reports in the literature describing methods for the digestion of tissue specimens from cattle. However, the many techniques reported for isolating the human bacillus from pathological specimens have been considered (Willis and Cummings, 1952).

Digestive enzymes have been reported as being nontoxic to microorganisms contained within tissues, (Northrop, Kunitz, and Herriott, 1948). However, there is little information on

the use of enzymes to digest tissue for the purpose of isolating microorganisms.

Gianforte, Brown, and Burkhart (1959) reported the use of an experimental enzyme X-108, (a powerful proteinase isolated from fermentation beer of Condiobolus brefeldianus), to release the organisms from infected tissue.

Lew and Carpenter (1956) reported the satisfactory use of trypsin for the digestion of tissue in the isolation of Mycobacterium leprae.

When dealing with low populations, such as exist in no-gross-lesion (NGL) reactor cattle, the complete digestion of tissue is necessary for the liberation of the tubercle bacilli in order that the organisms can be concentrated for inoculation onto media.

Palen, Wong, and Leifsen (1957) digested sputa with pepsin and concentrated the acid-fast organisms with pentane. Lipe, et al. (1961) used a similar technique for the concentration of acid-fast bacilli from bovine tissue. They found papain to be more suited for the digestion of tissue and less toxic to the tubercle bacilli than pepsin. The procedure was put into routine operation and found to be more successful than a modified sodium hydroxide method. However, it was noted that small particles of undigested connective tissue were concentrated in the pentane-water interface along with contaminants which tended to hinder the technique. (See Appendix I).

Methods for Decontamination of Specimens

Contamination has plagued every investigator attempting to develop or improve diagnostic techniques. Where alkali or acid methods are used, the agent acts both as a digestant and decontaminant. Agents such as glycerol (Schiller, 1925, and Twort, 1922), urea (Dold, 1924) and potassium hydroxide (Tison, 1951) have been used specifically to decontaminate specimens with little or no success because they failed either to remove contaminants or were toxic to the tubercle bacillus.

Another means of destroying contaminants has been to incorporate dyes into media. Petragnani (1926) and Lowenstein (1931) described egg media which contained various concentrations of malachite green; while Petroff's medium, (Petroff, 1920) contains 1:10,000 crystal violet. However, Dubos (1954) and Lipe, et al. (1961) believed these media do not simulate natural conditions and inhibit not only contaminants but also the tubercle bacilli. Faucher and Hedgecock (1961) used both crystal violet and malachite green successfully in decontaminating sputum. Although the dye method had been developed for the primary isolation of M. tuberculosis, unclassified mycobacteria were isolated in cultures from the dye-processed sputum but not from identical sputum treated with sodium hydroxide.

Wide utilization has been made of penicillin as a selective inhibitory agent in various culture media. Ungar and Muggleton (1946) found the addition of small amounts of

penicillin to media resulted in an increase in growth of the tubercle bacilli. Iland (1946) reported that recently, isolated strains of mycobacteria were inhibited by 20-30 units per ml of penicillin; however, Friedman (1945) reported no inhibition with similar concentrations.

Goldie (1947) used 0.05-2 units per ml of penicillin, while Abbott (1951) found that acid-fast organisms survived in sputum concentrates treated with 100-200 units per ml. Contaminants were reduced by 50 per cent and the number of positive cultures increased; however, the rate of growth decreased.

Penicillin in concentrations of 50-100 units per ml added to both solid and liquid oleic acid-albumin medium was reported as a valuable adjunct in culturing acid-fast organisms from contaminated material, (Kirby and Dubos, 1947). Sula (1948) found penicillin to be superior to malachite green as an inhibitor of contaminants in liquid media due to less toxicity to the tubercle bacillus. However, partial lysis of the tubercle bacilli occurs in Dubos medium in the presence of high concentrations of penicillin. Further evaluation of Dubos solid medium was performed by Smith et al. (1949). Mycobacteria colonies appeared 10 days earlier with this medium than with the medium without penicillin. Of several digestion mixtures studied, Whalen (1951) found a mixture containing 25 units per ml penicillin G, 1.4 per cent "Triton A-20", 10 per cent trisodium phosphate, and 0.03 per cent "Dowicide A" was least toxic to Mycobacterium tuberculosis and most effective in reducing the number of contaminants.

Penicillin has been shown to be an excellent inhibitor of contaminating organisms when present in suitable concentration in culture media. Unfortunately, in the presence of wetting agents which accelerate the growth of tubercle bacilli, penicillin is markedly bactericidal, (Cummings, 1949, Iland and Baines, 1949, McColloch, 1945, and Youmans and Youmans, 1948).

The use of quaternary ammonium compounds (QAC) has been used as a means of ridding pathological materials of contaminants. Hornung (1935) found that contact with 0.5-1.5 per cent Zephiran (Benzalkonium chloride) for 1 hour had no effect on tubercle bacilli in sputum. Freelander (1940) studied various wetting agents and reported that the growth of the tubercle bacillus was inhibited in a 1:80,000 dilution of Zephiran. Growth of the tubercle bacillus occurred after contact with a 1:1,000 dilution of Zephiran for 30 min, followed by two washings prior to inoculating onto media. Therefore, the bacteriostatic action of Zephiran against tubercle bacilli was high but the bactericidal action was low. The resistance of the tubercle bacilli to QAC was reported by Baker et al. (1941) to be due to the high lipid content of the acid-fast organisms.

Numerous QAC were found by Smith et al. (1950) to be weak tuberculocides. Zephiran was reported by Hirsch (1954) to have little germicidal effect on tubercle organisms but showed marked activity on saprophytic mycobacteria and non-acid-fast bacteria. Hirsch stated that specimens treated

with Zephiran should be followed by washing, dilution, etc., to reduce the contamination below the bacteriostatic level in the cultural medium. Patterson (1956) and Patterson et al. (1956) reported that mycobacteria can remain viable 11 days in a 1:1,000 concentration of Zephiran and up to 6 days in a 10 per cent trisodium phosphate-1:1,000 Zephiran mixture.

Mallmann and Lipe (1960) compared the germicidal effect of a number of agents on the tubercle bacilli and found that Roccal (benzalkonium chloride) in a concentration of 10 ppm, destroyed less pathogenic acid-fast organisms than any of the compounds tested. Sodium hypochlorite, sodium lauryl sulfate, sodium azide, potassium dichromate, and various triphenyl methane dyes, were found to be extremely toxic to the tubercle bacilli.

The principle reason the digestive enzymes have not been accepted is their lack of activity to rid the specimen of unwanted microorganisms. Research workers have tried to combine digestion with QAC in an attempt to improve diagnostic results.

Saxholm (1954, 1955a, 1955b, 1956, and 1958) has given considerable attention to the use of QAC for decontaminating tuberculous specimens after treatment with an enzyme. He used a 2 per cent pancreatin with equal amounts of 2-3 per cent Desogen, (methyl phenyl dodecyl trimethyl ammonium methosulfate). Equal amounts of this mixture was added to the specimen, which was left at room temperature 4-24 hours depending on thickness. One to 2 drops of the mixture were inoculated

on both Lowenstein and Tarshis media. There was indication that the digestive power of pancreatin increased simultaneously with a reduction of contaminants. Of the 14 QAC tested, Desogen, Vantoc B, (cetyl pyridinium bromide), and Bradosol, (beta phenoxyethyl dimethyl dodecyl ammonium bromide), were the most effective. This method proved to be very serviceable as it gave larger yield of tubercle bacilli than the sodium hydroxide method, as well as earlier growth. However, one disadvantage was indicated: the Lowenstein-Jensen medium tended to liquefy when using large amounts of inoculum. To partially overcome this softening, the final concentration of pancreatin was reduced to 0.25 per cent. With a low concentration of pancreatin, Saxholm showed a slightly greater yield of mycobacteria could be obtained.

Lend (1956) used a mixture of trypsin and Desogen to digest and decontaminate sputum. Five grams of trypsin and 7.5 grams of Desogen were added to a sputum sample, shaken by hand and the sample was allowed to digest at room temperature for 19 hours. The enzyme-digest procedure was correlated with a sodium hydroxide method, with better results observed when the sputum was digested 15 min with sodium hydroxide.

Trypsin plus a QAC was reported by Stinson and Fahlberg (1957) to be a satisfactory method for the pretreatment of sputum for culturing. They used a 1 per cent solution of trypsin prepared in 0.7 per cent sodium hydroxide, (pH 8.5) to activate the trypsin and added to a 1:1,000 concentration

of a QAC. The QAC's tested were: Zephiran, G-4, and Cetyl chloride. Zephiran was found to be the most effective, as it inhibited growth of all organisms except the acid-fast organisms after 1 hour incubation at 37 C.

Concentration Methods

The most widely used method for concentration of tubercle bacilli is centrifugation. However, some workers feel this method is not selective enough in that all organisms and particles with a specific gravity greater than that of the suspending fluid are precipitated. Hata, Venters, and Cummings (1950) investigated the efficiency of centrifugation as a method of concentrating tubercle bacilli in sputum specimens. Of 130 specimens, 38 per cent were positive by culture after centrifugation, while 35 per cent were positive without centrifugation. In a similar experiment, Klein et al. (1952) found centrifugation at 3,000 rpm for 15 min was not a highly efficient method for concentration.

A flocculation method using alum prior to centrifugation was recommended by Hanks, Clark, and Feldman (1938) to improve the concentration of tubercle bacilli. A solution containing 4 per cent sodium hydroxide, 0.2 per cent potassium alum, and 0.002 per cent brom thymol blue was mixed with equal volumes of the specimen. The mixture was allowed to digest for 1 hour at room temperature, then neutralized with N/1 HCL, shaken 30 sec or until flocculation occurred, and if flocculation did not occur in 5 min, 0.2 ml of a 1 per cent ferric chloride

solution was added. The mixture was shaken again, centrifuged at 3,000 rpm for 10 min, the supernatant liquid decanted, and the sediment used for cultural or animal inoculation. These workers felt this was a unique method in that many of the tubercle bacilli which under centrifugation procedures might not be carried down in the sediment were carried down by the flocculation.

The use of hydrophobic agents has shown some promise as a means of concentrating tubercle bacilli from digests. If a water suspension of the organisms is added to a hydrophobic agent and shaken, the tubercle bacilli are coated with the agent, leave the water phase and enter the intermediate layer. If the specific gravity of the water and the hydrophobic agent are different, a separation will occur and the liquids stratify. If this method is to succeed the hydrophobic agent should be nondetrimental to acid-fast organisms, separate the tubercle bacilli from the aqueous phase, and be selective enough to remove only the acid-fast organisms and leave the contaminants in the aqueous layer, (Mudd and Mudd, 1924).

Andrus and MacMaghan (1929) used chloroform as the hydrophobic agent, and claimed an increase of tubercle bacilli from 90-240 times as compared to only 3-4 times that obtained by the common sedimentation methods. Sodium hydroxide, gasoline or xylol, extensive dilution, mechanical shaking, gravity separation, and picric acid, are the essentials of Pottenger's method (1931). He found 60 times as many tubercle bacilli in flotation smears when compared to direct smears

and was able to detect as few as 1,000 organisms present in a 24 hour specimen. Nagy (1939) evaluated the sodium hydroxide method of Petroff, Pottenger's method, and chemical flocculation. He found Pottenger's dilution-flotation method gave 33 times as many bacilli as could be found in direct smears of routine concentration methods, and stated that this method was twice as efficient as chemical flocculation and 4 times as efficient as Petroff's method.

Palen et al. (1957) found pentane to be an excellent hydrocarbon for the concentration of tubercle bacilli from sputum digests. Of 18 organic fat solvents tested, pentane was the least toxic and a higher concentration of tubercle bacilli occurred at the interface. The sputum specimen was first digested with an equal volume of 0.4 per cent citric acid activated pepsin for 2 hours at 37 C. Approximately 1 ml of pentane was added to 10 ml of this mixture and shaken. They found that tubercle bacilli were concentrated in the pentane interface with few contaminating organisms.

The toxicity of a number of organic fat solvents for tubercle bacilli was examined by Crowle (1958). He reported that saturated hydrocarbons such as pentane, heptane, cyclohexane, and petroleum ether were harmless for M. tuberculosis H37Rv and H37Ra, and for the saprophyte M. smegmatis. Pentane was also used by Lipe et al.(1961) to improve the concentration of tubercle bacilli from low population tissue specimens. (See Appendix I).

Since the first report on the use of membrane filters (MF)

in the United States by Goetz and Tsuneishi (1951) for the bacteriological analysis of water in numerous microbiological techniques, the MF has been used for the recovery of microorganisms from other liquids, (Goldberg et al., 1952, Haas and Fleischman, 1957, and Wayne and Juarex, 1955).

The Germans first devised the MF, (Zsigmondy and Bachmann, 1918) and used it to isolate bacteria from pathological materials. Citron (1919) used Zsigmondy's filters for the recovery of tubercle bacilli from urines. These early MF methods were relatively successful in isolating microorganisms, however, the use of the MF did not gain recognition until the late 1940's.

Tietz (1949 and 1950), Tietz and Heepe (1950), and Tietz and Hohorst (1950) reported the successful use of the MF for concentrating and examining tuberculous specimens. The specimen was digested with freshly standardized antiformin and the mixture filtered. If the filter became plugged, sodium thiosulfate was added and filtration continued. By using this method, it was possible to filter several hundred ml of urine, 100 ml of spinal fluid, 100 ml of gastric washings, and up to 10 ml of sputum through 1 cm^2 filters. No indication was given as to how long it took to filter a given volume of digest or the number of isolations obtained when compared to the number of samples examined.

Pothmann (1952) filtered spinal fluid, and Heinemann (1955) mixed 5-10 ml spinal fluid with 1 to 2 ml of a 4 per cent sodium citrate solution and passed this mixture through

membrane filters. When this method was compared with centrifugation, it was found that 33 per cent were positive by sedimentation and 21 per cent were positive by filtration.

Trompke and Kroger (1952) reported the number of positive results using a membrane filter was twice that obtained by centrifugation for urines and other punctates. The urine was diluted with an equal volume of distilled water, 10-15 per cent Brufasol previously heated to 60 C and 12 per cent sulfuric acid in 10-15 per cent amounts were added, and the mixture passed through a MF. The filtration time depended on tissue cell content, contaminating organisms, and salt and albumin content. The time usually ranged from 30 sec to 25 min. If the rate of filtration decreased, acid alcohol or warm distilled water was added to make the pores free again. A similar procedure for other exudates was also described.

Hawirko and Murray (1954) used an oil partition method to remove acid-fast bacilli from aqueous suspensions in an attempt to develop a more precise method of laboratory diagnosis. Various oils were tested to determine their toxicity on tubercle bacilli. Of the oils tested, coconut, castor, cottonseed, and soybean oils exerted the least bactericidal activity. The method consisted of first adding oil to an aqueous suspension of a known number of tubercle bacilli, the mixture shaken, the oil phase removed and mixed with 10 ml acetone and 10 ml of petroleum ether. This resulted in a clear solution that could be filtered through a MF. The filter was washed with buffered saline, pH 6.5, before

planting on Petragnani's medium. Since the acetone-ether mixture proved somewhat bactericidal to the tubercle bacilli, the problem of dissolving the oil must be solved before the oil-partition method can be applied to the collection of tubercle bacilli from sputum.

Morgante and Murray (1954) reported the successful application of membrane filters in concentrating mycobacteria from cerebrospinal fluid. Cerebrospinal fluid was filtered through MF without any pretreatment and the filter placed on Lowenstein medium. This technique was found to be effective for detecting tubercle bacilli when the meningeal reaction was minimal.

Wayne (1955) described the growth of different mycobacteria on MF placed on Lowenstein-Jensen egg medium. A method for detecting very early growth using oblique lighting was also reported. By means of this procedure, it was possible to recognize microcolonies of virulent tubercle bacilli after as little as three days of incubation.

A mouthwash gargle technique used in conjunction with MF cultures was described by Rogers, Cooke, and Meyers (1955) for the recovery of tubercle bacilli from patients with pulmonary tuberculosis. Three digestion procedures were employed: 4 per cent sodium hydroxide, an enzyme solution consisting of 5 per cent amylase and 20 per cent mycozyme, and 10 per cent trisodium phosphate. The use of sodium hydroxide was found necessary to reduce viscosity and to eliminate normal mouth flora. Colony growth was significantly slower

on the filter surface than it was on the surface of routine plating media. Forty-three specimens were examined: 22 were positive by the MF method and 23 were positive by a routine procedure.

Haley and Arch (1957) reported that acid-fast organisms in sputum were usually detected on MF in 3-7 days, in contrast to the usual 27 days on Lowenstein medium and 18 days on Tarshis blood agar. Digestion was accomplished with 4 per cent sodium hydroxide for all pathological material. Of 100 sputum samples examined, 25 were positive and 75 were negative by membrane culture. However, more isolations were obtained by a routine procedure. Orlando and Bolduan (1953) demonstrated that all microorganisms which can be cultured on a suitable medium can be cultured on the MF when placed on solid media.

In preliminary studies, McKinney (1958) found trypsin, papain, and pangestin equally efficient digestion agents, however, pangestin was chosen because it contained a mixture of amolytic, lipolytic, and proteolytic enzymes. She also tried various decontaminants: 4 per cent sodium hydroxide, 10 per cent trisodium phosphate, added alone and with a 1:1,000 concentration of Zephiran; Bactracin, and polymyxin-B-sulfate. Polymyxin proved most effective especially when added with the enzyme so that digestion and decontamination could proceed concurrently. Zephiran was added later to complete decontamination. The final procedure consisted of adding a 4 per cent solution of pangestin (magnesium activated) to a

10 ml sputum sample. This mixture was shaken by machine or incubated in a 30 C water bath 20-30 min. An equal quantity of a 1:500 dilution of Zephiran was added and either shaken or incubation continued 15-30 min. Next the mixture was centrifuged at high speed, the supernatant liquid discarded, and 5 ml of distilled water and 2-4 ml of n-octane (containing 100 units of Nystatin per ml to stop fungal contamination) were added to the sediment. The tube was shaken vigorously for 5 min, then allowed to stand until the interface formed. The n-octane phase was removed, spread evenly on a MF with vacuum being applied, and then the MF was transferred to Lowenstein medium. A 4 per cent sodium hydroxide method was run as a control. Of 143 sputum specimens examined, 12 per cent were positive, 88 per cent negative, and 17 per cent contaminated using the sodium hydroxide method, as compared to 23 per cent positive, 77 per cent negative, and 4 per cent contaminated using the MF technique.

In a recent report (McKinney, 1961) the MF method described above was used in conjunction with media augmented by cationic resins and chelating agents to initiate early growth of M. tuberculosis. Medium thus prepared and used with filtered tubercle bacilli on membranes produced visible growth in 2.5 to 3 weeks compared with controls which grew in 4 to 5 weeks on Lowenstein-Jensen medium.

Wolochow et al. (1959) evaluated a number of enzymes for treating oropharyngeal washes before membrane filtration. Enzymes studied were: mycozyme, alpha-amylase, beta-amylase,

hyaluronidase, glucoronidase, and trypsin. They found that trypsin had little or no effect on filtration time. The best combination was found to be 5 ml of a 20 per cent solution of mycozyme plus 5 ml of a 5 per cent solution of alpha-amylase. This was added to 25-50 ml of saline rinse and incubated at 37 C for 30 min. They pointed out that the effective use of membrane filtration depends on the activity to hydrolyze salivary mucopolysaccharides.

Techniques for Staining Acid-Fast Bacilli on Membrane Filters

Although a number of procedures have been described for staining microorganisms on membrane filters (Carter et al., 1956, Fifield and Hoff, 1957, and Schaufus and Krabex, 1955), few have been reported for acid-fast organisms.

Wayne (1955) reported the use of colonial stain for staining tubercle bacilli. The membranes were placed, growth surface up, on filter paper pads saturated with the reagents indicated in the following sequence: (1) neutral red, 10 min, (2) Na_2CO_3, 5 min, and (3) 95 per cent alcohol, 1 min. The membrane was stained red and became amber on treatment with sodium carbonate. The ethanol removed excess reagents. The filter was transferred to filter paper pads and placed in the incubator to dry. When dry, the membrane was mounted on a slide with Permount fluid under a cover slip to make the preparation transparent. By means of this technique, it was possible to recognize micro-colonies of virulent mammalian

tubercle bacilli after as little as 3 days incubation.

Tietz (1949) and Tietz and Hohorst (1950) developed an acid-fast staining technique for the examination of tubercle bacilli on membrane filters. After filtration of the specimen, the dried filter was put back on the filtering apparatus and hot carbofuchsin or anilin-water-fuchsin was added. The dye was left in contact with the filter for 3-5 min, vacuum applied and the filter washed with warm water. The filter was then decolorized with acid alcohol, and again washed with filtered distilled water. One of 3 counter stains could be employed: 1:500 methylene blue, 1:10,000 malachite green or 1:600 chrysoidin. If the right contrast did not appear after washing the counterstain with distilled water, a few drops of either alcohol, 1 per cent picric acid or 10 per cent nitric acid could be added. After the filter was dried, it was cleared in xylol before mounting in Canada balsam.

Advantages and Disadvantages of Present Techniques for the Bacteriological Diagnosis of Tuberculosis

The advantages and disadvantages of current laboratory procedures for digestion, concentration, decontamination, and staining, can be summarized as follows:

1. Various concentrations of sodium hydroxide and hydrochloric acid are detrimental to the tubercle bacillus.
2. Digestive enzymes have been shown to be excellent agents for the digestion of body fluids since they

do not damage the viability of the tubercle bacillus.

3. Malachite green of the selective dyes is probably the least toxic to tubercle bacilli yet it exerts some inhibitory action upon their multiplication.
4. Penicillin demonstrated to be an excellent inhibitor of contamination, can be bactericidal for acid-fast organisms when used in the presence of a wetting agent.
5. Quaternary ammonium compounds used in a 1:1,000 concentration are fairly reliable decontaminants if neutralized properly.
6. Centrifugation, flocculation, and the use of hydrocarbons have been the more widely accepted methods of concentrating tubercle bacilli from tissues and other body fluids, however, these methods are complex and time consuming.
7. Membrane filters offer the best means of recovering acid-fast organisms from liquids.
8. Some evidence indicates that MF-cultural methods may detect early growth and eliminate the long period it presently takes before final results can be determined.
9. MF-staining technique (acid-fast) offers a means of eliminating the difficulty in preparing primary smears.
10. Presently, the MF offers little improvement over present procedures for culturing mycobacteria due

to one or more of the following objections:

a. Continued use of toxic agents to digest the specimen

b. Incomplete digestion, and dilution of the specimen; thus only a minimal amount of the specimen is used

c. Agents to destroy contaminants are detrimental to the tubercle bacillus

d. Centrifugation and the use of hydrocarbons prior to filtration, resulting in extremely complicated techniques

e. Acid-fast staining procedures are not always reliable.

MATERIALS AND METHODS

Source of Specimens. Negative sputum samples for preliminary investigations were obtained from the Ingham County Chest Hospital, Lansing, Michigan, and from the Arthur Kimball Sanatorium, Battle Creek, Michigan. All positive sputum specimens were obtained from the Herman Kiefer Hospital, Detroit, Michigan.

Skin lesion digests were obtained from the tuberculosis project[1], Department of Microbiology, Michigan State University. The group on this project is investigating various aspects of bovine tuberculosis in cattle. This provides an excellent opportunity to procure specimens as well as to compare the MF method with the enzyme-pentane and sodium hydroxide procedures.

Collection and Shipment of Sputum Specimens. Twenty-four hour and 3 day pools were collected in 25 ml screw cap glass bottles and immediately refrigerated. The specimens were then packed in a cardboard container containing dry ice, and sent by courier to the laboratory.

Filtering Apparatus. Anodized aluminum filter funnels[2] with a 2 inch base and funnel capacity of 200 ml, were cut in half reducing the funnel capacity to 100 ml. This was done in order to facilitate ease of handling small volumes of materials in a bacteriological hood. A stainless steel filter funnel[2] with a 1 inch base and a funnel capacity of 25 ml was also

[1]Project sponsored by the co-operative efforts of the Animal Disease Eradication (ADE) and Animal Disease and Parasite Research Service (ADP), divisions of the ARS, United States Department of Agriculture.
[2]Gelman Instrument Co., Chelsea, Mich.

used in the membrane filter staining studies (Fig. 1). The all metal apparatus was used in preference to glass funnels because of greater ease in cleaning and sterilizing. This device locks the filters in place and allows vacuum filtration of the sample. The membrane filter funnel consists of an upper liquid holding section, a locking ring and a base. A porous stainless steel disk was used to support the membrane. The tops were covered with small (2 inch) sterile petri dish covers to prevent the escape of aerosols when filtration was in progress. This also acted as a cover to minimize contamination of the sterile filter. A manifold with 6 leads was used, in order that 6 samples could be filtered simultaneously (Fig. 2). This reduced the time considerably when a large number of tissues or sputa were processed.

Millipore filters[1] type HA with a pore size of 0.45 micron and a diameter of 2 inches were used routinely for concentrating the materials. Polypore filters[2] type AM-6 with a pore size of 0.45 micron and a diameter of 1 inch were also used in the staining experiments.

Handling of cultures and filtration were performed in a bacteriological hood.

Standarization of Cultures. All cultures of tubercle bacilli were grown in Dubos broth with albumin and the method for enumeration reported by Mallmann and Lipe (1961), was used to obtain a homogeneous suspension of the test organism. This

[1]Millipore Filter Corp., Bedford, Mass.
[2]Gelman Instrument Co., Chelsea, Mich.

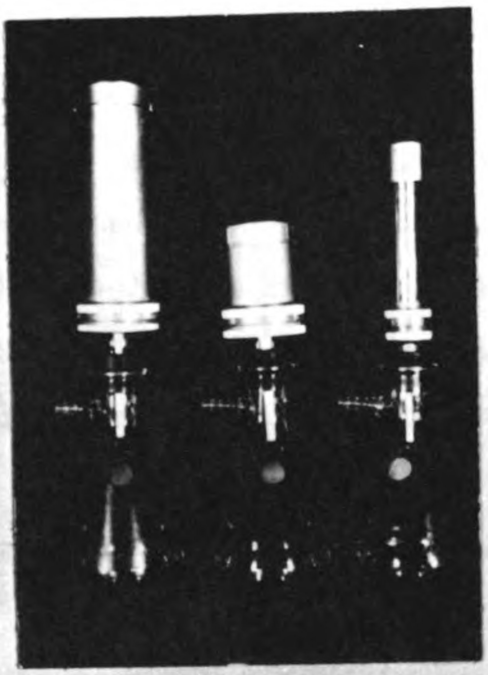

Fig. 1. Filter funnels. (Left) 200 ml, 2" dia., (center) 100 ml, 2" dia., and (right) 25 ml, 1" dia.

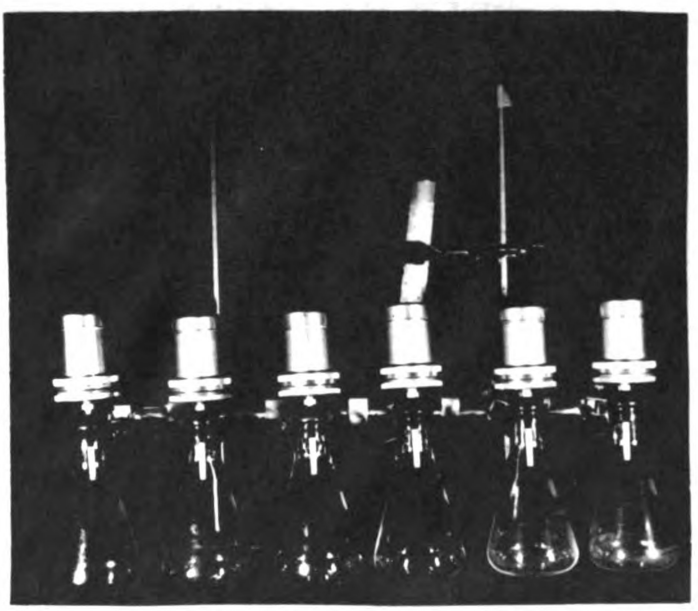

Fig. 2. Manifold for filtering 6 specimens simultaneously

consisted of adding 200 ppm of Triton X-100[1] (a deflocculating and suspending agent isooctylphenylpolyethoxyethanol) to a 2 week old broth suspension and exposing this to sonic oscillation (10 kc at 10 watts/cm^2) for 1 min. This tended to break up the clumps and resulted in single organisms. The suspension was then plated by the drop-plate method (Mallmann and Broitman, 1956) in suitable dilutions on solid media. Using a 0.2 ml pipette, 0.01 ml of inoculum of appropriate dilutions of each culture was discharged onto the medium by touching the tip of the pipette to the agar. The agar plates had been dried previously in a 35 C incubator for 24 hours to produce a dry surface for proper spread of each inoculum. Each dilution was replicated 10 times on each plate and counts were recorded as colonies per ml.

Standard cultures of nonpathogenic mycobacteria were maintained in Dubos medium by transferring every 3 days and pathogenic mycobacteria every 2 weeks, using approximately the same amount of inoculum for each subculture.

All nonacid-fast bacteria were grown in brain heart infusion broth. Counts were determined by drop plating suitable dilutions onto solid media, as described. Cultures were transferred every 24 hours using approximately the same size inoculum for maintaining a standard culture.

Incubation of Cultures. MF were placed on solid media in large petri dishes and incubated filter side up. Tubercle bacillus cultures were incubated in plastic bags at 35 C,

[1] Rohm and Haas Co., Philadelphia, Penn.

unless otherwise indicated. Since the growth of the tubercle bacillus is slow, plastic bags were employed to minimize evaporation of the medium.

Examination of Cultures. Cultures were examined at 2, 4, and 6 weeks. If acid-fast colonies were not isolated within 6 weeks, the plates were discarded. Smears were made of all suspected colonies and stained by the Ziehl-Nielsen acid-fast staining procedure. Thus all isolations are based only on acid-fastness.

Testing of Digestants. Eighteen different enzymes (Table 1) separately and in combination, were examined to find the best possible enzyme to digest the mucopolysaccharide of sputum. To accomplish this, negative sputa (sputa from pulmonary diseases other than tuberculosis) were pooled and homogenized with a magnetic stirrer in order that all sputa treated would have a uniform viscosity. Equal amounts of aqueous solutions of the enzymes were added to 5 ml of the homogenized sputum. The enzymes were varied with respect to concentration, pH, activator, and time of digestion. The enzyme solutions were sterilized by Seitz filtration and stored at 4 C until time of use. The digest mixture was incubated at 37 C, with continuous agitation maintained by magnetic stirrers.

To determine the best possible conditions for enzymatic digestion of sputa for filtration purposes, 2 and 4 per cent aqueous solutions of papain, trypsin, and pangestin, were added in equal amounts to 5 ml of homogenized sputum. The enzyme digestions were made at various pH values, temperature of

digestions, and in the presence or absence of enzyme activators. The temperature was varied from room temperature (22-25 C) to 37 C.

In 1 case, 0.37 mg per cent disodium versenate was added to a 4 per cent papain solution containing 60 mg per cent of l-cysteine. The inclusion of disodium versenate (disodium ethylene diamine tetra acetate), a chelating agent, was recommended to achieve maximum activity of papain, (General Biochemicals, 1961). A water bath with a horizontal shaker (Fig. 3), was employed to obtain a constant temperature of 45 C and continuous agitation of the enzyme-sputum mixture. The mixture was allowed to digest 1 hour. Four per cent sodium hydroxide digests were compared with the enzyme digests.

In all cases, the degree of digestion was determined by observing the time it took to filter 5 ml of the digested material through a membrane filter.

The toxicity of papain, trypsin, and pangestin were compared to the toxicity of sodium hydroxide on various species of mycobacteria. This was determined by placing appropriate dilutions of the test organisms, Mycobacterium phlei, M. bovis (854) and M. tuberculosis (DTA) in contact with the digestive agent at 37 C for 1 hour. These dilutions were also added to sterile buffered distilled water and filtered through MF, which served as controls. After 1 hour, the enzyme solutions were filtered through MF, and the MF placed on Dubos medium.

To determine the toxicity of papain digestion at 45 C, the same test organisms, used in the above experiment, were added

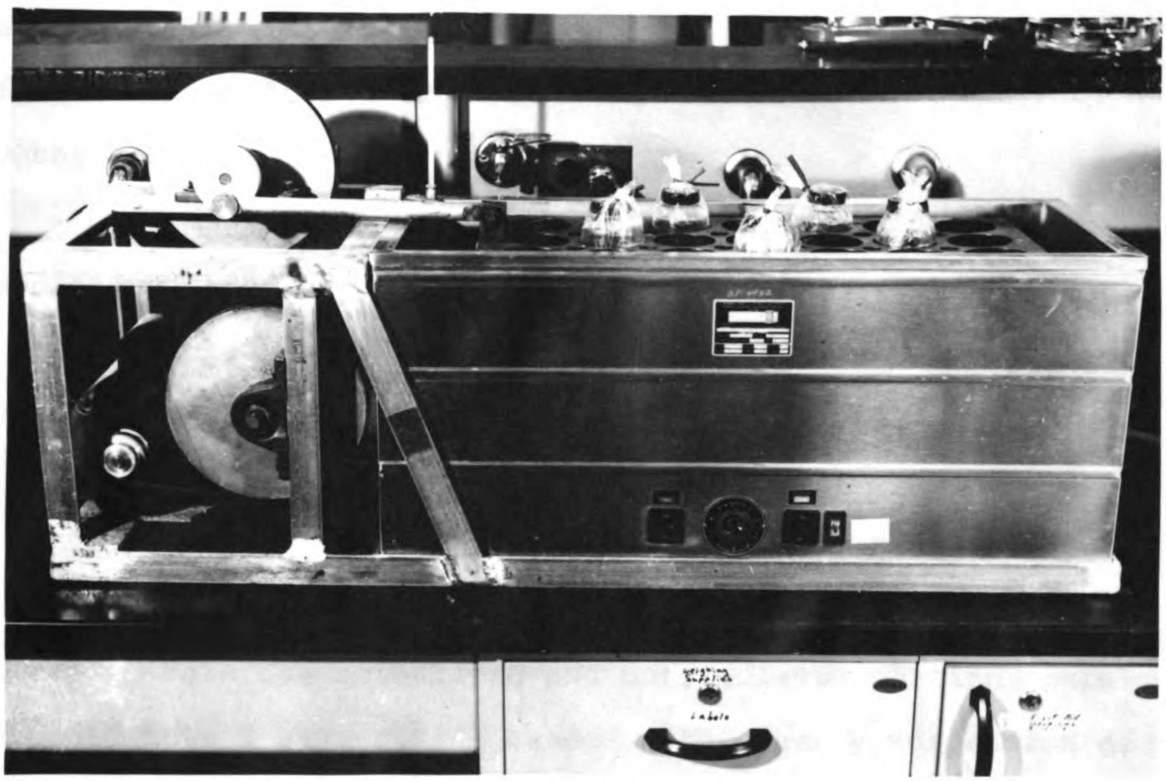

Fig. 3. Water bath with mechanical shaker.

to duplicate tubes containing 9 ml of a 4 per cent aqueous solution of papain, (cysteine and versenate activated, pH 5.5) and to control tubes of buffered distilled water which were filtered immediately. The tubes containing the enzyme plus the test organisms were placed in a water bath with continuous agitation for 2 hours. The contents from one tube from each set was filtered at the end of 1 hour and the contents from a second tube at 2 hours. The filters were placed on Dubos medium.

Determining Toxicity of Inhibitors. A qualitative test employing compounds commonly used to decontaminate specimens was considered necessary to determine the efficiency of these compounds to destroy unwanted organisms and their effect on the tubercle bacillus.

A composite sample of sputum obtained from convalescent patients was divided into 2 portions. One portion was autoclaved. Both the autoclaved and unautoclaved portions were divided into 2 aliquots; 1 seeded with a heavy suspension of M. phlei and the other with a heavy suspension of M. tuberculosis (DTA). A 4 per cent solution at pH 5.5 and containing 60 mg l-cysteine and 0.37 mg versenate per 100 ml was added in equal amounts to each seeded autoclaved portion and to the unautoclaved portion. Digestion was carried out at 45 C with continuous agitation for 1.5 hours. Five ml portions of each were treated with various dilutions of chemical agents, and allowed to remain in contact for 5 and 10 min. The mixtures were then either neutralized before filtration or the MF washed after

filtration. All filters were placed on Middlebrook 7H9 agar B.

To evaluate the quantitative inhibitory effect of various chemical agents on mycobacteria and nonacid-fast bacteria, appropriate dilutions of the test organisms, M. phlei, M. bovis (Rav), Escherichia coli, and Staphylococcus aureus were added to various concentrations of the compounds, (Table 5). E. coli and S. aureus were selected to represent gram negative and gram positive contaminants respectively. M. phlei, a nonpathogenic species was included because Lipe (1960) reported that this organism had less resistance to chemical agents than did the pathogenic tubercle bacilli. The tubes were shaken vigorously and 1 ml samplings were taken at intervals of 10 and 30 min. The 1 ml samples containing quaternary ammonium compounds were introduced into 9 ml of sterile tween "80"[1] (sorbitan mono-oleate)-lecitin[2] (100 mg azolectin[3]-tween "80") to neutralize the QAC, (Weber and Black, 1948). The 1 ml samples containing the dyes and other compounds were introduced into 9 ml of bovine serum to inactivate the effect of these agents. Penicillin was neutralized with an equal volume of 1 per cent penicillinase. The exposed organisms were then inoculated onto Dubos oleic agar (without enrichment and penicillin) by the drop-plate method in suitable dilution to determine the number of surviving organisms.

Evaluation of Antibiotics and Sulfonamides as Inhibitors. To conduct the assay with antibiotics, plates of Middlebrook

[1]Atlas Powder Co., Wilington, Delaware
[2]A mixture of the diglycerides of stearic, palmitic, and oleic acids linked to the choline ester of phosphoric acid.
[3]Associated Concentrates Inc., Atlanta 1, Ga.

7H9 agar B were inoculated by smearing heavily with pure cultures of the test organism in order to obtain confluent growth. Since many organisms produce sulfonamide-like compounds, a diluted culture was employed when testing the sensitivity of sulfonamides in order to avoid inactivation of these compounds in the disk.

Bacto-Sensitivity Disks[1] (Table 9) were removed from the vials using a flamed tweezer. The disks were placed on the inoculated plate leaving 3 to 4 cm between each disk. The plates were then incubated at 35 C. The plates containing the nonacid-fast bacteria and antibiotic disks were examined after 24 hours incubation. Those containing sulfonamide disks were examined after 12-16 hours. The saprophytic mycobacteria were examined after 3 days, and the pathogenic mycobacteria between 1 and 2 weeks, or until sufficient growth appeared to permit detection of zones of inhibition. The test organisms used in this study included the following: (1) acid-fast: M. phlei, M. smegmatis, M. avium, and M. bovis (Rav) and (2) nonacid-fast bacteria (selected to represent contaminants): E. coli, S. aureus, Pseudomonas aeruginosa, Bacillus subtilis, Klebsiella pneumoniae, Serratia sp., Diphtheroid (S-25), Proteus vulgaris, and a gram positive spore former bacillus isolated from a skin lesion.

Since the disk method is only a qualitative test of sensitivity, a more precise quantitative procedure was tested on 2 species of mycobacteria and 4 selected nonacid-fast bacteria.

[1]Difco Laboratories, Detroit, Mich.

A list of these drugs and their potency follows:

DRUG	POTENCY
Colymycin[1]	666 mcg/mg
Erythromycin[2]	870
Spiramycin[3]	900
Ristocitin[2]	1,020
Mycostatin[4]	500,000 units/vial
Elkosin[3]	1,000 mcg/mg

All drugs had to be sterilized before use except Mycostatin and Ristocitin. Stock solutions of the drugs were made as follows: Colymycin was prepared by dissolving an appropriate amount of powder into measured amounts of pH 8 phosphate buffer. Erythromycin and Spiramycin were prepared by dissolving an appropriate amount of powder in a few ml of ethanol and diluting to the desired concentration with pH 8 phosphate buffer. These antibiotics were sterilized by Seitz filtration. Sterile distilled water was added to the vials of Mycostatin and Ristocitin with a sterile syringe and appropriate dilutions were made in 9 ml sterile distilled water blanks. The appropriate amount of Elkosin was dry-heat sterilized at 160 C for 3 hours and sterile pH 8 phosphate buffer added.

[1]Colistin sulfate was supplied by the Warner Lambert Research Institute, Research Affiliate of Warner Chilcott Lab., Morris Plains, N. J.

[2]Erythromycin and Ristocitin were supplied by Abbott Laboratories, North Chicago, Ill.

[3]Spiramycin and Elkosin were supplied by Ciba Pharmaceutical Co., Summit, N. J.

[4]Mycostatin was obtained from the Virology Lab., Dept. of Micro., Mich. State Univ. It can be purchased from E. R. Squibb and Sons, New York.

After suspension, all drugs were stored at 4 C and not used after 2 weeks storage.

Various dilutions of the drugs were added separately and in combinations, under aseptic conditions, to sterile Middlebrook 7H9 Agar DS-ss (described on pages 40 & 41) cooled to between 45-50 C. Suitable dilutions of the test organisms were inoculated onto media by the drop-plate method and the plates incubated at 35 C.

A few spores of Aspergillus sp. and Penicillium sp. were streaked on plates containing Mycostatin.

Preparation and Concentration of Tissue Digest. Skin lesions were digested following the method described by Lipe et al. (1961) (Appendix I). The lesion was first divided into 2 parts, 1 of these was digested with 4 per cent papain, while the other was digested with 2 per cent sodium hydroxide. After enzymatic digestion, the digest mixture was divided into 2 equal portions; 1 portion was used with the enzyme-pentane procedure and the other with the MF method.

The following three experimental methods were employed in preparing the mixture for MF filtration:

1. Two to 5 ml of pentane was added to the digest mixture, depending on the volume of material. The mixture was vigorously shaken by hand to emulsify momentarily the pentane and water, and allowed to stand for 20 min. The pentane and the interface were removed and placed into a centrifuge tube. The tube was centrifuged at 800xG for 20 min. The pentane, interface and sediment were then filtered separately through

membrane filters in the following manner: In order to facilitate filtration, the interface and sediment were diluted with 10 ml of Dubos broth (without albumin). To this was added 2-4 ml of Triton X-100 to make a final concentration of 1:5,000. This concentration was found to be nontoxic to tubercle bacilli, (Lipe et al., 1961). The mixtures were then divided into 4 equal parts and filtered through membrane filter. One filter was placed on each of the following media: Middlebrook 7H9 agar B, Lowenstein egg medium, Tarshis blood agar, and Dubos oleic agar. The pentane phase was filtered directly, the filter washed with 10 ml Dubos broth to remove any trace of pentane left in the filter, and the MF placed on Middlebrook 7H9 agar B.

2. A 40 per cent sugar solution (glucose) was added to the digested mixture resulting in a final sugar concentration of 20 per cent. The mixture was shaken by hand and allowed to stand 20 min. The tubes were centrifuged at 800xG for 30 min. The upper third of the mixture was diluted with Dubos broth, Triton X-100 added to a final concentration of 1:5,000, and filtered as indicated in the first method. The MF were placed on Middlebrook 7H9 agar B.

3. The digest material was divided into 2 to 4 equal parts depending on the viscosity of the liquid. Triton X-100 was added to each part in a final concentration of 1:5,000, and the mixture filtered directly. Any connective tissue was removed with a sterile Pasteur pipette before a vacuum was applied. If the digested material was extremely thick, it was

diluted as described in the above methods. Middlebrook 7H9 agar B again was the medium of choice, since it contains malachite green for inhibition of contaminants.

Growth Studies. Throughout the course of the previous investigations, it was noted that colony size of the tubercle bacillus grown on the surface of the MF on various media, even when incubated 5 weeks, did not appear as large when compared to colonies directly on the surface of the same kind of medium. Therefore, it seemed desirable to study various means to increase growth on the filter surface.

Absorbent pads were soaked with Dubos broth, placed on the surface of the medium, and the MF placed on the absorbent pad.

The charcoal medium described by Whalen and Mallmann (1955), was tried since these workers suggested this medium to be a complete nutrient medium for the cultivation of M. tuberculosis, as well as sufficiently selective to eliminate many of the usual contaminants in sputum specimens.

Since bovine albumin or serum is added to various media for the cultivation of tubercle bacilli, not only as an enrichment but also to remove toxic materials, poor growth on the MF might mean that toxic elements were only partially removed. To determine if this was the case, 4 gm of activated charcoal (Nuchar C-190-N)[1] was added to 1 liter of Middlebrook 7H9 agar B and the medium heated to boiling. The medium was then filtered hot through a Buchner funnel containing 2 sheets

[1]Industrial Chemical Sales Division, West Virginia Pulp and Paper Co., New York 17, N. Y.

of No. 2 Whatman filter paper to remove the charcoal. The filtered medium was then distributed in 180 ml amounts and sterilized for 15 min at 121 C. After the medium cooled, Middlebrook OADC enrichment was added aseptically.

The poor quality of growth might also be due to improper contact of nutrients from the medium into the filter, due to the diluting effect of atmospheric water absorbed by the exposed MF surface. If so, the organisms would have insufficient nutrients for adequate growth.

To evaluate this hypothesis, a semi-solid, double-strength medium was prepared by adding 6.5 gm of Bacto-Agar (Difco) to a double concentration of Middlebrook 7H9 broth in 1 liter portions. The medium was sterilized by the usual procedures (121 C for 15 min). After the medium cooled to 45-50 C, Middlebrook OADC enrichment was added. This medium, Middlebrook 7H9 agar DS-ss (double-strength-semi-solid), was compared to Middlebrook 7H9 agar B by observing the first visible colonies. Microcolonies could readly be made visible by placing a strong beam of light across the surface of the filter. Appropriate dilutions of standarized cultures of the test organisms were filtered through MF and the filter placed on the surface of the medium. Cultures of the test organisms were also drop-plated onto the surface of the medium. The following acid-fast organisms were used in this study: M. phlei, M. smegmatis, M. bovis (Rav), M. avium, M. tuberculosis (H37 Rv), M. bovis (854), and 81 (isolated from swine and typed as M. bovis)[1]. Two atypical

[1]Atypical acid-fast bacteria isolated and identified by ADE and ADP sponsored Tuberculosis project, Mich. State Univ.

acid-fast organisms were also used: $152A_2$-1 (pseudochrome or Runyon Group III, isolated from swine)[1], and P8 (photochromogen or Runyon Group I, of human origin).

Some investigators have suggested that incubation of cultures in 5 per cent CO_2 enhances growth of the tubercle bacillus, (Middlebrook and Cohn, 1958, and Whitcomb, Foster, and Dukes, 1962). Therefore, half of the plates were placed in an air tight container to which approximately 5 per cent CO_2 in air was added. The remaining plates were placed in plastic bags, and all plates were incubated at 35 C. To assure a constant 5 per cent CO_2 atmosphere, the CO_2 was replenished every 2 days.

The formulation of the medium and enrichments used in this investigation are presented in Appendix II.

Some of the detailed techniques will be presented with each experiment along with the results for the convenience of the reader.

Techniques for the Isolation of Acid-fast Bacteria from Pathological Specimens. The following methods were used for the isolation of acid-fast microorganisms from skin lesions of reactor cattle and from sputum specimens collected from human tuberculous patients.

A. MF Method (s)

1. (a) Tissues: 5 gm of tissue were ground in a mortar, and 25 ml of a 4 per cent papain solution, pH 5.5, (containing 60 mg l-cysteine and 0.37 mg versenate

[1]Atypical acid-fast bacteria isolated and identified by ADE and ADP sponsored Tuberculosis project, Mich. State Univ.

per 100 ml of the solution, and a 1:500 concentration of Triton X-100) was mixed with the ground tissue and poured into a 200 ml centrifuge tube.

(b) Sputum: 24 hour and 3 day pools, (containing 2-5 ml sputum were placed into a 200 ml centrifuge tube and equal or double amounts of the enzyme solution added, (see step 3d), depending on the density of the sample.

2. The tubes were then placed in a 45 C water bath (with mechanical shaker), and the material allowed to digest 1 hour with constant agitation.
3. One of the following decontaminating procedures was used at this point:
   a. An equal volume of a 1,000 ppm Roccal was added to the digest (final concentration 500 ppm), and the mixture shaken and allowed to stand 20 min. This was then neutralized with an equal volume of Tween "80"-azolectin.
   b. Same as (a), except 200 ppm Roccal was added to give a final concentration of 100 ppm.
   c. A 1:500,000 concentration of brilliant green, pH 7.2, was added directly to the digest, shaken, and allowed to stand 30 min before being filtered.
   d. Sputum specimens were digested using a 4 per

cent enzyme concentration containing 400 ppm Roccal for 1 hour (as described in steps 1 and 2). This was neutralized with an equal volume of Tween "80"-azolectin, and filtered through a MF. The filters were then placed on Middlebrook 7H10 agar (containing 0.1 ppm malachite green) or Middlebrook 7H9 agar DS-ss containing one of the combination of drugs shown in Table 11.

4. The decontaminated digest mixture was then divided into 4 portions and each portion filtered through a MF.
5. The MF were placed on Middlebrook 7H9 agar B, unless otherwise indicated.

B. The classical methods for the bacteriological examination of sputum, using sodium hydroxide and hydrochloric acid, were performed as follows:

1. Approximately 5 ml of sputum were homogenized with a magnetic stirer until it was possible to pipette the sputum. Each homogenized sample was divided into 2 equal aliquots.
2. To 1 aliquot, an equal volume of 4 per cent (w/v) of sodium hydroxide was added. This was then manually shaken and incubated at 37 C for 20 min. The contents of the tube were centrifuged 800xG for 20 min and the supernate discarded. One drop of phenol red indicator (0.07 per cent) was added

to the sediment and the sediment neutralized with N hydrochloric acid. The sediment was then inoculated onto 4 Lowenstein slants in screw capped glass tubes.

3. To the second aliquot, an equal volume of 3 per cent (v/v) hydrochloric acid was added. This was treated as described for sodium hydroxide, except that after centrifugation the supernatant fluid was discarded and 1 drop of brom cresol purple indicator (0.0015 per cent), was added to the sediment. The sediment was then neutralized with N sodium hydroxide and inoculated onto 4 Lowenstein slants.

C. The enzyme-pentane procedure for isolation of tubercle bacilli from tissues is described in Appendix I. When used with sputum, one modification was made: Triton X-100 was added to the interface in a final concentration of 1:5,000 to aid filtration.

D. The sodium hydroxide procedure used for tissue was as follows: Approximately 30 gm of tissue was blended in 150-200 ml nutrient broth. The blended material was filtered through cheesecloth. Ten ml of the filtrate was added to 10 ml of 4 per cent sodium hydroxide and allowed to stand for 15 min. The mixture was then neutralized with hydrochloric acid and centrifuged. The resulting precipitate was placed in 6 tubes of Middlebrook agar, 6 tubes of Lowenstein and 6 tubes

of Dubos agar.

Both the enzyme-pentane and sodium hydroxide procedures for separation of acid-fast organisms from tissue were performed by the ADE and ADP Tuberculosis Project personel. The techniques are presented in this thesis solely for the purpose of comparing the results obtained by these 2 methods with those by the MF method.

Method for Staining Acid-fast Bacilli on a MF. Numerous formulations of the fuchsin dye solution, various concentrations of a large number of counterstains (both alcoholic and aqueous solutions), and various decolorizing mixtures were run before the final formulations were obtained.

Various strains of mycobacteria were added in pure culture or mixed with nonacid-fast bacteria and filtered, to determine the degree of decoloration of both the filter and nonacid-fast organisms. In later studies, sputa were digested as previously described, filtered and the material stained on the MF.

The formulation and preparation of the staining solutions used are presented as follows:

1. Basic fuchsin:

   Five grams of basic fuchsin (C. I. 677, National Aniline, Cert. No. NF-57, 95 per cent dye content) was added to 100 ml 95 per cent ethyl alcohol and mixed. This solution was then added to a mixture of 94 ml glycerol, 5 ml Triton X-45 (alkyl aryl polyether alcohol)[1], 1 ml Tween "80", and 100 ml of 5

[1]Rohm and Haas, Philadelphia, Penn.

per cent aqueous phenol.

2. Counterstain:

   One-tenth gram of light green SF yellowish (C. I. 42095, National Aniline, Cert. No. NL-18, 99 per cent dye content) was added to 1,000 ml of 95 per cent ethyl alcohol, (1:10,000).

Preparation of decolorizing solution:

Three ml of concentrated hydrochloric acid was added to 97 ml of 95 per cent ethyl alcohol and filtered through a MF. The staining solutions, acid alcohol, and distilled water were filtered through a MF before using.

The staining procedure was as follows:

1. 5-10 ml of material to be stained was filtered.
2. The filter was washed under vacuum with filtered distilled water.
3. The suction was turned off and 1-2 ml basic fuchsin solution was added and left 30 min.
4. The staining solution was filtered and the filter was then rinsed with decolorizing solution to remove most of the stain from the MF. The suction was turned off and 5 ml of the decolorizing solution was added and left for 2 min.
5. Approximately 5 ml of distilled water was added and filtered to remove any trace of acid alcohol.
6. The filter was removed from the filtering apparatus and placed between two filter pads or filter paper. This was placed on a flat surface and weighted down

firmly, and allowed to dry in this manner for 10-15 min, thus preventing distortion of the filter during drying.

7. The dried filter was immersed in a horizontal position in a beaker containing the counterstain (light green) for 5-10 min.
8. The filter was rinsed in distilled water until excess stain no longer was removed.
9. The filter was placed between 2 filter pads and dried as described in step 6.
10. The dried filter was dipped in xylene to clear.
11. The MF was placed on a slide to which a liberal amount of permount had been added previously. More permount was added to the surface of the filter before a No. 1 (24 x 50 ml) cover slip was added. If large (2 inch) filters were used, the filter was cut in half and the halves mounted on separate 1 inch slides. (Fig. 4).
12. The mounted filter was then placed in a 37 C incubator and allowed to dry for 30 min. The filter was then examined by oil immersion microscopy.

RESULTS

Testing of Digestants. Of the various enzymes tested to determine their mucolytic activity, 2 per cent papain, (pH 5.5, cysteine activated), 2 per cent trypsin, (pH 8.1, magnesium sulfate activated) and 2 per cent pangestin, (pH 7.2, magnesium sulfate activated) yielded digests after 2 hours at 37 C, that had similar filtration times, (Table 1).

When papain, trypsin, and pangestin were subjected to various conditions to determine maximal activity, it was demonstrated that similar filtration times were obtained for 4 per cent concentrations of all 3 enzymes and 4 per cent NaOH, (Table 2) although 4 per cent papain (pH 5.5, cysteine and disodium versenate activated, digested at 45 C) yielded a slightly better digest, as determined by the rate it took to filter 5 ml of the digest.

A study of papain, trypsin, and pangestin, was made to determine what affect, if any, these enzymes had on the test organisms as compared to the activity of sodium hydroxide. Papain and pangestin caused only slight inhibition of the test organisms at 37 C (Table 3), whereas marked reduction with sodium hydroxide at both 2 and 4 per cent concentrations, occurred for all 3 species of mycobacteria.

Four per cent papain at 45 C had little toxic effect on the pathogenic bacilli as approximately 92 per cent recovery of M. tuberculosis (DTA) occurred after 1 hour and 86 per cent

TABLE 1. Efficiency of Enzymes in Digesting Sputum

Enzyme	% Conc.	pH	Activator	Time (hr.) Digest	Filtration* Time for 5 ml
Taka-diastase	2	7.0		2	32 min
Elastase	1	6.8		2	20
Ficin	2	4.8	cysteine	2	22
Lysozyme	0.9	5.6		2	45
Hyaluronidase	2	3.5		2	30
Pepsin	2	1.2	citric acid	2	15
Trypsin	2	8.1	$MgSO_4$	2	6
Diastase of Malt	2	4.2	NaCl	2	49
Amylopsin	2	6.1		1	30
Proteinase	2	6.5		1	30
Bromelain	2	6.0		1	30
Pectinase	2	6.3		1	30
Diastase (animal)	2	7.2		1	30
Beta-amylase	2	6.2		1	20
Pangestin	2	7.2	$MgSO_4$	2	6
Papain	2	5.5		2	15
Papain	2	5.5	cysteine	2	5
Buffered Pancreatin Tablets	2	7.8		2	20
X-108**	1M***	7.2		2	23
Papain plus Trypsin	4	6.6	cysteine, $MgSO_4$	2	15
Papain plus Pangestin	4	6.1	cysteine, $MgSO_4$	2	12
Trypsin plus Pangestin	4	7.2	$MgSO_4$	2	18
Auto-digest (control)				2	60

* Based on the average of four runs

** A proteinase isolated from beer of Candiobolus brefeldianus (American Cyanamid Co.)

*** 1,000,000 Azocoll units

TABLE 2. Comparative Efficiency of Papain, Trypsin, Pangestin, and Sodium Hydroxide in Digesting 5 ml of Sputum.

Enzyme	% Conc.	pH	Activator	Temp. Digest	Time (hr.) Digest	Filtration* Time (min)
Papain	2	5.1	cysteine**	37 C	1	3
Papain	2	6.0	cysteine	37	1	8
Papain	2	5.5	cysteine	22	1	6
Papain	4	5.5	cysteine	45	1	3
Papain	4	5.5	cysteine	37	1	4
Papain	4	5.5	cysteine and versenate	45	1	2
Trypsin	2	8.1	$MgSO_4$**	37	1	6
Trypsin	2	7.2	$MgSO_4$	37	1	7
Trypsin	4	8.1	$MgSO_4$	22	1	12
Trypsin	4	8.1	$MgSO_4$	37	1	6
Trypsin	4	8.1	$MgSO_4$	45	1	6
Pangestin	2	7.2	$MgSO_4$	37	1	9
Pangestin	4	6.1	$MgSO_4$	37	1	10
Pangestin	4	7.2	$MgSO_4$	37	1	6
Pangestin	4	7.2	$MgSO_4$	45	1	5
NaOH	4	12.1		37	1	5
NaOH	4	12.1		37	30 min	7

* Based on the average of four runs
** 60 mg/100 ml

TABLE 3. The Toxicity of Papain, Trypsin, Pangestin and Sodium Hydroxide on Three Species of Mycobacteria.

Digest** Agent	% Conc.	pH	Percent reduction in colony count* M. phlei	M. bovis (854)	M. tuberculosis(DTA)
Papain+	2	5.5	2	2	6
Papain+	4	5.5	5	8	13
Trypsin++	4	8.1	45	6	26
Pangestin++	4	7.2	1	1	9
NaOH	2		100	70	88
NaOH	4	12.1	100	81	87

* Based on the average of 3 runs
** Contact with digesting agent one hour at 37 C
\+ Cysteine and versenate activated
++ Magnesium sulfate activated

after 2 hours digestion. Ninety per cent recovery was obtained for M. bovis (854) after 1 hour, and 81 per cent after 2 hours, whereas 83 per cent after 1 hour and 79 per cent after 2 hours were obtained for M. phlei.

Therefore, 4 per cent papain (cysteine and versenate activated, pH 5.5) was found to be the best suited for digestion of sputum.

Determining Toxicity of Inhibitors. The qualitative toxicity testing of various agents used routinely for the bacteriological examination of tuberculous specimens, demonstrated that many compounds were detrimental to the growth of the tubercle bacillus, (Table 4).

The quaternary ammonium compound (QAC) Roccal (alkyl benzal ammonium chloride) at 200 ppm destroyed contaminants but reduced the number of viable acid-fast organisms. Sodium hydroxide (2 per cent) reduced the degree of contamination but also reduced the number of viable acid-fast organisms. Trisodium phosphate (TSP) (23 per cent) reduced the degree of contamination slightly and slightly reduced the number of viable acid-fast organisms. Malachite green in a concentration of 5 ppm was ineffective against contaminants, whereas 5 ppm inhibited the acid-fast organisms. Brilliant green in a concentration of 5 ppm reduced the contaminants materially with little or no reduction of the acid-fast organisms. Thus brilliant green (5 ppm) and Roccal (100 ppm) were the best means of eliminating most of the contaminants and at the same time were the least harmful to the tubercle bacilli.

TABLE 4. The Qualitative Effect of Various Compounds in Decontamination of Sputum and Their Relative Toxicity to Two Species of Mycobacteria.

Compound	Conc.	Amount of Growth*					
		Contaminated sample		*M. phlei*		*M. tuberculosis*	
		Contact time in minutes					
		5	10	5	10	5	10
Roccal[1]	50 ppm	3	3	4	4	4	4
	100	3	2	3	4	4	4
	200	2	1	2	1	3	3
	500	0	0	1	0	2	0
	1000	0	0	0	0	0	0
Penicillin[2]	2 units	4	3	4	3	4	4
	10	4	4	3	3	4	3
	20	3	3	2	1	3	0
	50	2	1	0	0	1	0
NaOH[3]	1 %	3	3	1	0	2	3
	2	1	1	0	0	2	1
	4	0	0	0	0	0	1
TSP[4]	10 %	4	4	4	4	4	4
	23	3	3	4	4	4	4
HCl[5]	1 %	1	1	4	3	4	3
	3	1	0	0	0	0	0
Malachite green	5 ppm	3		3		3	
	100	0		0		0	
Brilliant green	5 ppm	1		4		3	
	10	0		0		0	

* 4=confluent growth, 3=many colonies, 2=few colonies, 1=1 or 2 colonies, 0=no growth

[1]Neutralized with tween-azolectin

[2]Neutralized with 2 ml penicillinase

[3]Neutralized with N HCl

[4]Filter washed with 5 ml Dubos broth to remove alkalinity

[5]Filter washed with 5 ml Dubos broth to remove acidity

The autoclaved specimens were difficult to digest probably due to denaturation of the proteins.

Although Roccal and brilliant green did not appear to be ideal decontaminants without reducing the tubercle bacilli population, various agents, including Roccal and brilliant green, were quantitatively tested to determine their toxicity on acid-fast and nonacid-fast bacteria.

The effects of these compounds are presented in Table 5. Of the compounds tested, quatenary ammonium compounds appeared to be the least toxic to the tubercle bacillus while destroying the majority of the nonacid-fast bacteria. A summary of their effect in a concentration of 100 ppm at 10 min exposure is presented in Table 6.

Roccal was the least inhibitory of the QAC on the tubercle bacilli and reduced the nonacid-fast bacteria considerably. The QAC compounds have been used in sputum samples in a concentration of 500 ppm for extended exposure periods to destroy contaminants with some degree of success. Although data not in keeping with those presented here, it must be remembered that tests were carried out in the absence of organic material. Even though partial kill was obtained for the pathogenic mycobacterium at 100 ppm, it would seem feasible that a higher concentration of QAC would be required to obtain the same effect in the presence of organic material since the organic material would inactivate most of the disinfectant upon contact. However, even with these compounds, complete inhibition of the nonacid-fast bacteria (contaminants) would result in a marked

TABLE 5. Effect of Various Agents on Certain Mycobacteria and Certain Nonacid-fast Bacteria.

Compound	Conc. ppm	*M. phlei*		*M. bovis* (Rav)		*S. aureus*		*E. coli*	
		Contact time in min							
		10	30	10	30	10	30	10	30
Roccal	100	1.7	0	13.5	7.5	5.1	0	3.2	0
	200	0.5	0	7.6	1.2	0	0	0.5	0
	500	0	0	0	0	0	0	0	0
Tetrosan	100	1.5	0	15.8	6.3	8.3	7.1	4.1	0
	200	0	0	8.0	2.7	2.5	1.0	0	0
	500	0	0	0.5	0	0	0	0	0
Lauryl Pyridinium Chloride	100	9.3	8.1	26.6	21.4	10.6	8.3	16.4	12.3
	200	7.3	5.1	22.1	0	4.3	1.0	5.3	0
	500	1.0	0	10.1	0	2.0	0	1.0	0
Hyamine	100	2.8	1.8	12.4	6.5	7.3	2.2	4.3	0.5
	200	0	0	11.3	0	2.2	0	1.2	0.5
	500	0	0	0	0	0	0	0	0
Potassium dichromate	100	0	0	0	0	0	0	0	0
	20	10.6	8.3	24.3	21.0	24.0	20.0	20.1	18.6
	10	12.6	12.0	30.6	31.2	27.0	26.1	24.0	24.1
Potassium tellurite	200	0	0	0	0	0	0	0	0
	100	0	0	0	0	0	0	0	0
	20	0	0	2.1	0	0	0	0	0
	10	4.1	0.5	3.2	0	9.5	0	0	0
Sodium azide	200	0	0	0	0	0	0	0	0
	100	0	0	0	0	6.5	2.2	0	0
	20	0.5	0	4.5	1.2	6.7	3.1	18.6	14.1
	10	1.5	2.0	8.6	5.0	16.5	13.0	25.4	23.0
Sodium acid selenite	100	0	0	0	0	0	0	0	0
	20	0	0	0	0	0	0	0	0
	10	0	0	0	0	0	0	1.5	0.5
	2.5	3.1	1.0	2.6	0	23.2	24.0	4.5	1.5
Sodium Lauryl sulfate	200	0	0	0	0	0	0	19.3	18.6
	100	0	0	3.1	0	2.4	0	23.0	21.0
	20	6.0	4.8	18.5	16.1	10.5	8.6	24.0	24.0
	10	11.9	11.0	26.2	26.8	24.0	20.0	24.0	24.0

TABLE 5. (Continued)

Compound	Conc. ppm	*M. phlei*		*M. bovis* (Rav)		*S. aureus*		*E. coli*	
		Contact time in min							
		10	30	10	30	10	30	10	30
Thallium acetate	200	0	0	0	0	22.1	20.0	23.9	23.0
	100	0	0	0	0	25.6	23.1	24.1	24.3
	20	10.3	8.6	26.4	22.3	26.1	27.0	24.0	23.9
	10	12.0	12.1	31.0	30.9	26.9	26.1	23.6	24.0
Phthalic acid	100	0	0	0	0	0	0	0	0
	20	0	0	9.1	5.0	10.2	0	2.5	0.5
	10	11.9	10.3	15.3	13.0	16.7	13.1	12.5	10.5
	1	12.3	12.0	26.2	21.0	25.8	24.0	23.0	24.0
Sulfanilic acid	100	0	0	0	0	0	0	0	0
	10	2.1	0	0	0	0	0	0	0
Quinoline	100	10.9	8.6	22.1	20.0	22.2	20.0	20.1	19.3
	2	11.2	10.1	30.0	26.0	24.0	26.0	24.0	21.0
Brilliant green	100	2.5	1.0	1.5	0	0	0	0	0
	10	6.1	3.0	6.5	2.0	2.5	0	0.5	0
	2	9.3	5.6	10.0	4.0	4.6	2.5	1.2	1.0
	1	11.2	8.3	12.7	6.5	12.7	4.9	3.0	1.4
Acid fuchsin	2000	9.5	4.6	22.3	18.6	12.3	10.3	4.3	1.2
	1000	11.7	9.3	22.0	19.1	20.0	19.2	15.3	14.6
Penicillin G	100	12.5	12.1	31.1	30.6	0	0	23.6	24.0
	10	12.2	12.0	30.1	29.6	0	0	24.1	23.8

Inoculum used: *M. phlei* = 12.5×10^6, *M. bovis* (Rav) = 31×10^6, *S. aureus* = 26×10^6, and *E. coli* = 24×10^6

TABLE 6. Summary of the Effect of Quaternary Ammonium Compounds on Acid-fast and Nonacid-fast Bacteria.

QAC	Per cent reduction in colony count*			
	M. phlei	M. bovis (Rav)	S. aureus	E. coli
Roccal	86.4	56.5	80.4	86.7
Tetrosan	88.0	49.0	68.1	82.9
Lauryl Pyridinium Chloride	25.5	13.5	59.2	31.7
Hyamine	78.6	60.0	72.0	82.1

* Reduction based on a concentration of 100 ppm at a 10 min exposure

inhibition of the tubercle bacilli.

Some of the anionic wetting agents have been used for inhibiting gram positive organisms in media and for detection of coliform organisms. Such a medium, lauryl tryptose broth, was developed by Mallmann and Darby (1941). The results presented in Table 5 show that lauryl sulfate in concentrations of 1:5,000 to 1:10,000 was markedly inhibitory for all test organisms except E. coli.

Potassium dichromate in a concentration of 1:10,000 also has been shown to inhibit gram negative bacteria. This concentration completely suppressed the growth of all tubercle bacillus and nonacid-fast bacteria tested.

Sodium azide and potassium tellurite have been used successfully in inhibiting the growth of gram negative bacteria in media designed for detection of streptococci, (Mallmann, 1940, and Chapman, 1944). To prevent the growth of gram negative bacteria, a concentration of 1:5,000 sodium azide and 1:100,000 potassium tellurite is used. Sodium azide completely suppressed the growth of all strains of tubercle bacilli and E. coli in a concentration of 1:10,000. Potassium tellurite was markedly inhibitory to the tubercle bacillus and S. aureus, and completely inhibited growth of E. coli in a concentration of 1:100,000.

The antibiotic, penicillin, was bactericidal for S. aureus but not for E. coli and the tubercle bacillus at concentrations tested.

The basic triphenyl methane dye, brilliant green in a

concentration of 1:10,000 exerted a complete bactericidal effect only on the nonacid-fast bacteria for both time intervals tested. However, at this concentration, marked inhibition of the tubercle bacilli resulted. However, as in the case of QAC, organic material interferes with the activity of most dyes and therefore, in a pathological specimen a higher concentration would be required to obtain the same effect that was exhibited here. The acid triphenyl methane dye, acid fuchsin, was less effective against the nonacid-fast organisms.

The following results were obtained by the miscellaneous compounds employed in this study. Quinoline had little or no effect on the test organisms. Phthalic acid was significantly inhibitory at the concentrations employed. Sulfanilic acid was extremely toxic to the test organisms in concentrations tested, and thallium acetate in a concentration of 1:10,000, completely suppressed the tubercle bacilli and exerted little or no effect on the nonacid-fast bacteria at this concentration.

After observing the effect of QAC on tubercle bacilli and nonacid-fast organisms, it seemed desirable to determine what concentration of Roccal would be necessary to overcome the interference of organic material contained in sputum. It also seemed reasonable to add the QAC to the enzyme mixture, in order that digestion and decontamination could be carried out at the same time. The enzyme-QAC mixture was prepared by adding 4 grams of papain, 60 mg of l-cysteine, and 0.37 mg of disodium versenate to 100 ml of Roccal. The concentrations of Roccal ranged from 50 to 500 ppm. The mixture was sterilized

by Seitz filtration. Suitable dilutions of the test organisms were added to 9 ml of the enzyme-QAC mixture. Negative sputa were seeded with the tubercle bacilli and an equal amount of the enzyme-QAC mixture was added. Digestion was carried out as previously described. After filtration, the MF were placed on Middlebrook 7H9 agar B. Tubes of buffered distilled water, containing the test organisms, were filtered and served as controls. The degree of contamination and recovery of the tubercle bacillus was recorded.

In the enzyme-QAC mixture, enough QAC had to be present to exceed the amount neutralized by the enzyme and organic material contained in sputum. Four hundred ppm (Table 7) was necessary to achieve the same results obtained when using 100 ppm free of organic materials, (refer to Table 6).

Evaluation of Antibiotics and Sulfonamides as Inhibitors. The preceding data demonstrated that Roccal and brilliant green were the most efficient decontaminants of those tested. However, in concentrations that would result only in slight diminution of the number of tubercle bacilli, they were not entirely effective against contaminating microorganisms. Therefore, it seemed desirable to survey some of the antibiotics and sulfa drugs as possible inhibitors of contaminating microorganisms.

In Table 8 are presented the results of the effect of various antibiotics and sulfonamides on acid-fast and nonacid-fast bacteria. Erythromycin and Spiramycin, in both low and medium concentrations, were most effective inhibitors of gram positive

TABLE 7. Effect of Roccal when Mixed with Papain and in the Presence of Organic Matter.

QAC* Conc. ppm	Per cent Recovery						
	M. phlei		*M. bovis* (Rav)		*S. aureus*	*E. coli*	Degree of Contamination in sputum
	Enzyme + QAC	Sputum	Enzyme + QAC	Sputum	Enzyme + QAC	Enzyme + QAC	
Controls	11.6		29.6		26.4	22.0	
100	59.2	C	91.0	C	82.0	75.6	C
200	46.8	C	83.6	C	65.3	51.4	C
300	29.8	42.1	71.0	79.3	36.1	17.6	M
400	9.6	15.4	55.6	60.3	18.2	0	M
500	2.5	7.6	29.3	41.6	0	0	F

* Contact time with papain and QAC for 1 hour

C=obscure growth, M=many but isolated contamination, and F=few contaminants

TABLE 8. The Effect of Various Antibiotics and Sulfonamides on Acid-fast and Nonacid-fast Bacteria.

Compound	Conc. per Disk	M. phlei	M. smegmatis	M. avium	M. bovis (Rav)	E. coli	K. pneumoniae	S. aureus	P. aeruginosa	Diphtheroid	P. vulgaris	B. subtilis	Serratia sp.	Spore former*
Vancomycin	5 mcg	S	S	S	S	R	R	S	R	S	R	S	R	S
	10	S	S	S	S	R	R	S	R	S	R	S	R	S
	30	S	S	S	S	S	R	S	R	S	R	S	R	S
Erythromycin	2	R	R	R	R	R	R	S	R	S	R	F	R	F
	5	R	R	R	R	R	R	S	R	S	R	S	R	F
	15	S	R	S	S	R	R	S	R	S	R	S	R	F
Colymycin	2	R	R	R	R	R	S	R	S	R	R	R	R	R
	5	R	R	R	R	R	S	R	S	R	R	R	R	R
	10	R	R	R	R	S	S	R	S	R	R	R	R	R
Declomycin	5	S	S	S	S	S	S	S	S	S	R	S	F	S
	10	S	S	S	S	S	S	S	S	S	R	S	F	S
	30	S	S	S	S	S	S	S	S	S	S	S	F	S
Chloromycetin	5	R	R	S	S	S	S	S	R	S	S	S	F	F
	10	S	S	S	S	S	S	S	R	S	S	S	F	F
	30	S	S	S	S	S	S	S	R	S	S	S	S	S
Ristocetin	5	R	R	R	R	R	R	S	R	S	R	S	R	S
	10	S	S	S	S	R	R	S	R	S	R	S	R	S
	30	S	S	S	S	R	R	S	R	S	R	S	R	S
Kanamycin	5	R	F	R	R	S	S	R	R	S	R	R	R	F
	10	R	F	S	S	S	S	S	F	S	R	S	R	F
	30	F	S	S	S	S	S	S	F	S	S	S	S	S
Aureomycin	5	S	S	S	S	S	S	S	R	S		S	F	
	10	S	S	S	S	S	S	S	R	S		S	S	
	30	S	S	S	S	S	S	S	R	S		S	S	
Terramycin	5	S	S	S	S	S	S	S	R	S		S	S	
	10	S	S	S	S	S	S	S	R	S		S	S	
	30	S	S	S	S	S	S	S	S	S		S	S	

TABLE 8. (Continued)

Compound	Conc. per Disk	M. phlei	M. smegmatis	M. avium	M. bovis (Rav)	E. coli	K. pneumoniae	S. aureus	P. aeruginosa	Diphtheroid	P. vulgaris	B. subtilis	Serratia sp.	Spore former*
Tetracycline	5 mcg	S	R	S	R	S	R	S	R	S		S	S	
	10	S	S	S	S	S	S	S	S	S		S	S	
	30	S	S	S	S	S	S	S	S	S		S	S	
Penicillin	2 units	R	R	R	R	R	R	S	R	S	R	R	R	R
	5	R	R	R	R	R	R	S	R	S	R	S	R	R
	10	R	R	S	R	R	R	S	R	S	R	S	R	R
Streptomycin	2 mcg	S	S			S	S	R	R	S	R	S	R	S
	10	S	S			S	S	S	R	S	R	S	R	S
	100	S	S			S	S	S	S	S	S	S	S	S
Polymyxin B	5	R	R	R	R	R	R	R	R	R	R	R	R	R
	10	R	R	R	R	R	R	R	S	R	R	S	R	R
	30	R	R	R	R	S	S	R	S	S	R	S	R	S
Bacitracin	2	S	S	R	R	R	R	R	R	S	R	R		R
	10	S	S	S	S	R	R	S	R	S	R	R		R
Neomycin	5	R	R			R	R	R						
Elkosin	50	R	R	R	R	S	R	R	R	R	S	R	R	R
	150	R	R	R	R	S	R	R	R	R	S	R	S	F
	300	R	R	R	R	S	R	R	R	R	S	R	S	F
Madribon	50	R	R	R	R	S	R	R	R	R	S	R	R	R
	150	R	R	R	R	S	R	R	R	R	S	R	R	R
	300	R	R	R	R	S	R	R	R	R	S	S	R	R
Sulfamerazine	50	R	R	R	R	S	R	R	R	R	S	R	R	F
	150	R	R	R	R	S	R	R	R	R	S	R	R	F
	300	R	R	R	R	S	R	R	R	R	S	R	R	F
Sulfamethoxy-pyridazine	50	R	R	R	R	S	R	R	R	R	S	R	R	R
	150	S	R	R	R	S	R	R	R	R	S	R	F	F
	300	S	R	S	R	S	R	R	R	R	S	R	F	F

TABLE 8. (Continued)

Compound	Conc. per Disk	M. phlei	M. smegmatis	M. avium	M. bovis (Rav)	E. coli	K. pneumoniae	S. aureus	P. aeruginosa	Diphtheroid	P. vulgaris	B. subtilis	Serratia sp.	Spore former*
Sulfathiazole	50 mcg	S	R	R	R	S	R	R	R	R	S	R	R	R
	150	S	R	R	R	S	R	R	R	R	S	R	R	R
	300	S	R	S	R	S	R	R	S	S	S	S	R	R
Thiosulfil	50	S	R	R	R	S	R	R	R	R	S	R	R	F
	150	S	R	R	R	S	R	R	R	R	S	R	R	F
	300	S	R	R	R	S	R	R	S	R	S	S	R	F
Sulfadiazine	50	R	R	R	R	S	R	R	R	R	S	R	S	R
	150	R	R	R	R	S	R	R	R	R	S	R	S	R
	300	R	R	R	R	S	R	R	R	R	S	R	S	R
Spiramycin	5	R	R	R	R	R	R	S	R	S	R	F	R	F
	10	R	R	R	R	R	R	S	R	S	R	F	R	F
	30	S	S	S	R	R	R	S	R	S	R	F	R	F
Mandelamine	1 mg	R	R	R	R	S	S	S	R	S		S	S	
	2	S	S	S	S	S	S	S	S	S		S	S	
	3	S	S	S	S	S	S	S	S	S		S	S	
Mycostatin	100 units	R	R	R	R	R	R	R	R	R	R	R	R	

* Gram positive spore former isolated from a skin lesion
S=sensitive, R=resistant, and F=few resistant bacteria

bacteria, except the spore formers, but did not inhibit the tubercle bacilli. Similarly, Colymycin was most effective against the gram negative bacteria and not effective against the tubercle bacilli. Ristocitin in low concentrations was the only agent effective against B. subtilis and a gram positive spore former isolated from a skin lesion, while non-inhibitory to tubercle bacilli. The sulfonamides Elkosin, Madribon, Sulfamerazine, Sulfamethoxypyridazine, Thiosulfil, and Sulfadiazine were the only effective agents against P. vulgaris but was not effective against the tubercle bacilli. All organisms tested were resistant to 100 units of Mycostatin.

The quantitative effect of Colymycin, Erythromycin, Spiramycin, Ristocitin, Elkosin, and Mycostatin are presented in Tables 9 and 10. This test was performed in order to determine what concentration of the drugs when placed in a medium would be required to inhibit the contaminating microorganisms and secondly, if combinations of the drugs would exert antagonistic or synergistic activity.

Colymycin, in a concentration of 20 mcg/ml, was most effective in suppressing the growth of K. pneumoniae and at the same time, 100 per cent of the tubercle bacilli were recovered. Colymycin in any concentration was not effective against P. vulgaris. Erythromycin at 2 mcg/ml completely suppressed the growth of S. aureus and was less inhibitory to the tubercle bacilli than Spiramycin at 5 mcg/ml. Ristocitin at 0.5 mcg/ml reduced the growth of B. subtilis 94.9 per cent, M. phlei 9 per cent, and M. bovis (Rav) 6.5 per cent. Approximately

TABLE 9. The Quantitative Effect of Various Antibiotics and Elkosin on Acid-fast and Nonacid-fast Bacteria.

Compound	Conc./ml*	Counts x 10^6							
		B. subtilis	*K. pneumoniae*	*S. aureus*	*P. vulgaris*	*M. smegmatis*	*M. bovis* (Rav)	Aspergillus	Penicillium
	Controls	36.0	21.0	24.0	36.0	22.0	30.0		
Colymycin	10 mcg	36.0	2.4	24.0	37.0	22.0	30.0		
	20	36.0	0	23.1	36.3	20.8	31.0		
	40	35.7	0	22.6	36.0	19.4	29.8		
Erythromycin	2	34.4	21.2	0	36.3	19.5	30.1		
	5	18.6	20.0	0	36.0	5.3	10.0		
	15	4.1	19.3	0	35.7	0	0		
Spiramycin	5	30.6	21.0	0	35.3	17.3	22.0		
	10	25.0	21.3	0	36.0	2.1	13.2		
	30	9.1	20.8	0	37.4	0	1.9		
Ristocitin	0.5	2.8	21.3	0	35.4	20.4	28.3		
	2	0	21.0	0	36.1	3.0	21.0		
	5	0	18.6	0	30.5	0	10.0		
	10	0	6.4	0	23.0	0	0		
Elkosin	50	36.7	21.0	24.6	0	22.4	30.0		
	150	30.3	21.6	23.8	0	20.4	30.2		
	300	10.0	21.5	24.0	0	15.3	23.7		
Mycostatin	50 units	36.0	21.5	23.7	36.0	22.0	30.0	NG	NG
	100	35.3	20.4	24.5	36.0	22.0	30.0	NG	NG

*Appropriate concentrations added to modified Middlebrook 7H9 agar B medium
NG=no growth

TABLE 10. The Effect of Various Combinations of Antibiotics and Elkosin on Acid-fast and Nonacid-fast Bacteria.

Drug Combination	Conc./ml*	Counts x 10^6					
		B. subtilis	K. pneumoniae	S. aureus	P. vulgaris	M. smegmatis	M. bovis(Rav)
	Controls	36.0	21.0	24.0	36.3	22.2	30.0
Erythromycin Colymycin Mycostatin	2 mcg 20 mcg 100 units	32.4	0	0	34.3	21.6	29.8
Ristocitin Colymycin Mycostatin	0.5 mcg 20 mcg 100 units	00.8	0	0	35.2	19.4	25.6
Elkosin Colymycin Mycostatin	150 mcg 20 mcg 100 units	36.0	0	24.6	0	21.6	30.0
Elkosin Erythromycin Colymycin Mycostatin	150 mcg 2 mcg 20 mcg 100 units	30.3	0	0	0	20.4	28.7
Elkosin Ristocitin Colymycin Mycostatin	150 mcg 0.5 mcg 20 mcg 100 units	0	0	0	0	16.7	26.3

* Final concentration per ml of Middlebrook 7H9 agar DS-ss medium

100 per cent of the bacteria tested were resistant to both 50 and 100 units/ml of Mycostatin, whereas species of Aspergillus and Penicillium were completely suppressed at these concentrations. Elkosin at 50 and 150 mcg/ml did not inhibit any of the test organisms except P. vulgaris which was completely inhibited.

The effect of the antibiotics and sulfonamides in various combinations (Table 10) was similar to that found when they were added separately to Middlebrook 7H9 DS-22 medium. No antagonistic or synergistic effects were observed.

Preparation and Concentration of Tissue Digest. Three experimental methods were employed to determine the most efficient procedure in preparing tissue digest for membrane filtration.

In the first method in which pentane was employed for the separation of the acid-fast bacilli from tissue, all three phases which resulted after centrifugation were filtered. This was done since the partial aim of the investigation was to find if the tubercle bacilli were actually concentrated in the interface as claimed by Lipe et al. (1961) for the enzyme-pentane technique. Acid-fast colonies were obtained from all portions, which indicates that the enzyme-pentane digest method did not concentrate all the acid-fast organisms in the interface. However, this does not invalidate the enzyme-pentane method for concentrating acid-fast organisms from tissues but does show that the membrane filter concentrates all the acid-fast organisms on the MF. This method was unsuited for the

MF technique, since all portions would have to be filtered to concentrate all the acid-fast bacilli. Contamination was found to be heaviest in the water phase, however, considerable contamination was also found in the interface. The pentane procedure depends on removal of contaminating organisms by the selective action of the hydrocarbon, and again would not be appropriate for the MF technique since all the contaminating organisms would be planted on the MF. It was also found that Tarshis blood agar and Lowenstein egg medium were unsuited for this technique since they tended to liquefy, due either to the breakdown of the constituents by contaminants or by the enzyme.

In the second method, a high sugar concentration was used to change the specific gravity of the mixture to allow particles and organisms with a greater density than the tubercle bacilli to be sedimented down, while the tubercle bacilli remain in the upper portion. Although a partial separation of acid-fast organisms and contaminants was effected, there was no particular advantage over the filtering of the interface in the enzyme-pentane procedure. It was also noted that the surface of the MF appeared "sticky" due to the sugar, and tended to impair growth of the microorganisms.

In the third method, in which the entire digest was filtered, all the contaminating organisms in the digest were concentrated on the MF as well as any acid-fast organisms. Isolations were made only when the tissues had minimal contamination. Filtering the interface portion would minimize the

contamination, but then the procedure would only be equal to the enzyme-pentane method. When the enzyme digestion was made at 45 C the entire digest could be filtered provided Triton X-100 was added. Therefore, this procedure would be satisfactory provided contamination could be reduced to a low enough level to allow isolation of the acid-fast bacilli.

Growth Studies. Dubos broth in absorbent pads, did not appear to stimulate growth any quicker than when the filter was placed directly on the medium.

Growth on the MF placed on charcoal medium (Whalen and Mallmann, 1955) was not significantly different than that on Middlebrook 7H9 agar B.

No observable difference in growth of acid-fast bacilli was noted with charcoal treated Middlebrook 7H9 agar B.

More rapid growth occurred on the semi-solid Middlebrook 7H9 (double strength) medium than on Middlebrook 7H9 agar B medium, (Table 11) which is in agreement with the work of Knox, Swait, and Woodroffe (1956). Cultures incubated under 5 per cent CO_2 in air resulted in earlier growth for some of the tubercle bacilli on both media. Therefore, the results using CO_2 confirm previous observations that this gas is strikingly stimulating to the multiplication of tubercle bacilli on artificial media.

The amount of growth on the MF agreed favorably with the amount on the drop plates, using Middlebrook 7H9 agar DS-ss.

Isolation of Acid-fast Bacteria from Skin Lesions and Sputum. The results of 122 skin lesions processed by the MF

TABLE 11. Comparison of Middlebrook 7H9 Agar DS-ss with Middlebrook 7H9 Agar B for cultivation of Acid-fast Bacilli.

Media	Time microcolonies observed (in days)								
	M. phlei	M. smegmatis	M. bovis (Rav)	M. bovis (854)	M. avium	M. tuber. (H37Rv)	81	P8	152A2-1
DS-ss	2.0	0.5	2.0	12.0	3.0	14.0	5.0	2.0	0.5
DS-ss + CO_2	2.0	0.5	2.0	10.0	2.0	7.35		0.5	
Agar B	2.5	1.0	7.0		7.0		14.0		2.0
Agar B + CO_2	2.5	1.0	5.0		5.0				

method using various decontaminating procedures are presented in Table 12. Of the 122 samples, 41.8 per cent were positive for acid-fast bacilli, 33.6 per cent were negative, and 24.6 per cent were contaminated. More isolations were obtained from specimens decontaminated with 100 ppm Roccal. This was especially the case when the QAC was added to the specimen along with the enzyme, in order that digestion and decontamination could take place simultaneously, neutralized with Tween "80"-azolethin, pH adjusted to 8.0, the digest filtered, and the MF placed on Middlebrook 7H10 agar, containing 0.1 ppm malachite green. This method of decontamination resulted in 30 per cent more isolations than was obtained when using a combination of QAC, (pretreatment of digest), and a medium containing drugs as inhibitors, (Middlebrook DS-ss medium containing Colymycin, Erythromycin, and Mycostatin). The same amount of contamination (20%) was recorded for both methods; however, in the latter procedure, 30 per cent more specimens were negative.

Extremely heavy contamination appeared on membrane filters placed on media containing drugs as the only means of removing or destroying the unwanted microorganisms.

Middlebrook 7H9 agar DS-ss, containing Elkosin, Ristocitin, Colymycin, and Mycostatin, was extremely toxic, with 92.9 per cent of the specimens tested recorded as negative. The combination of Erythromycin, Colymycin, and Mycostatin, as the only means of decontaminating the specimen, was the least toxic. However, 64.3 per cent of the specimens were contaminated.

TABLE 12. Recovery of Acid-fast Bacilli from Skin Lesions by the Membrane Filter Culture Method with Various Decontamination Procedures.

No. Specimens Exam.	Method of Decont.	P*	N	C	%P	%N	%C
24	A	1	20	3	4.0	83.5	12.5
30	B	21	3	6	70.0	10.0	20.0
23	C	8	10	5	34.8	43.5	21.7
21	D	12	2	7	57.2	9.5	33.3
14**	E-1	4	1	9	28.6	7.1	64.3
	E-2	3	7	4	21.4	50.0	28.6
	E-3	5	2	7	35.6	14.4	50.0
	E-4	1	13	0	7.1	92.9	00.0
10	F	4	4	2	40.0	40.0	20.0
Total 122		51***	41***	30***	41.8	33.6	24.6

* P=positive, N=negative, and C=contaminated

** Each sample was divided into 4 equal portions, and each portion placed on Middlebrook 7H9 agar DS-ss containing different combinations of drugs, (See code E-1 through E-4).

*** Of decontamination procedures E-1 through E-4, only E-3 is included in the totals.

Code: Decontamination procedures:

A....500 ppm Roccal, neutralized with Tween 80-azolecthin

B....Digest mixture containing 400 ppm Roccal, neutralized with Tween 80-azolecthin, MF placed on Middlebrook 7H10 agar

C....1-500,00 brilliant green

D....100 ppm Roccal, neutralized with Tween 80-azolecthin

E-1..Erythromycin (2 mcg/ml), Colymycin (20 mcg/ml), Mycostatin (100 units/ml)

E-2..Ristocitin (0.5 mcg/ml), Colymycin (20 mcg/ml), Mycostatin (100 units/ml)

E-3..Elkosin (150 mcg/ml), Erythromycin (2 mcg/ml), Colymycin (20 mcg/ml), and Mycostatin (100 units/ml)

E-4..Elkosin (150 mcg/ml), Ristocitin (0.5 mcg/ml), Colymycin (20 mcg/ml), and Mycostatin (100 units/ml)

F....Digest mixture containing 400 ppm Roccal, neutralized, MF placed on Middlebrook 7H9 agar DS-ss containing Colymycin (20 mcg/ml), Erythromycin (2 mcg/ml), and Mycostatin (100 units/ml).

In the method using 1:500,000 brilliant green, isolations were possible but this method was less effective than those already described.

Comparative results of isolations obtained by the MF method with those obtained by the enzyme-pentane and sodium hydroxide procedures are presented in Table 13.

More isolations of acid-fast organisms were made by the MF method (41.8%) than by either the sodium hydroxide method, (28.7%) or the enzyme-pentane procedure (32.0%). As might be predicted from previous data, more negative cultures were obtained when using sodium hydroxide, and more contamination occurred using the enzyme-pentane procedure.

The data presented in Table 14 are the results of the processing of 198 sputa samples, decontaminated by various procedures.

Of the 198 specimens, acid-fast organisms were isolated from 47.2 per cent, (includes per cent isolated from contaminated filters) 30.6 per cent were negative, and 39.9 per cent were contaminated. Contamination was reported if 75 per cent of the plates were contaminated (4 plates/specimen). Of the 79 specimens contaminated, isolations in mixed culture were obtained from 59.5 per cent. It was also noted that more contamination occurred in 3 day pool specimens (50%) than in 24 hour specimens (31.2%).

More isolations were obtained when the specimens were pretreated with Roccal, the MF placed on media containing Colymycin, Erythromycin, and Mycostatin, and the plates incubated

TABLE 13. Comparison of Recovery of Acid-fast Bacilli by the MF Culture Method, Enzyme-Pentane Method, and Sodium Hydroxide Method.

	MF		MF ONLY		PENTANE		P ONLY		NaOH		NaOH ONLY		ALL 3		P+MF		S+MF		S+P	
	No.	%*	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
P	51	41.8	36	29.6	39	32.0	31	24.6	35	28.7	44	36.0	21	17.2	14	11.4	6	4.9	7	5.7
N	41	33.6			48	39.3			57	46.7			26	21.3						
C	30	24.6			35	28.7			30	24.6			18	14.7						

* Per cent based on 122 specimens (skin lesions)
P=positive, N=negative, C=contaminated, P ONLY=pentane method only, P+MF=pentane plus membrane filter methods, S+MF=sodium hydroxide plus membrane filter methods, S+P=sodium hydroxide plus pentane method

TABLE 14. Recovery of Acid-fast Bacilli from Sputum by the Membrane Filter Method with Various Decontamination Procedures.

Type of Specimen	No. Specimen Exam.	Method of Decont.*	P+ P-C	N	Contamination INP	Contamination IP	% P**	% N	% C INP	% Total C
	17	A	0	17	0	0	0	100.0	0	0
	20	B	9	3	2	6	75.0	15.0	10.0	40.0
	19	C	7	4	6	2	47.4	21.0	31.6	42.0
24 hour	20***	E-1	4	2	12	2	30.0	10.0	60.0	70.0
Sputum		E-2	4	16	0	0	20.0	80.0	0	0
Specimen		E-3	9	4	6	1	50.0	20.0	30.0	35.0
		E-4	0	18	1	1	5.0	90.0	5.0	10.0
	20	F	12	2	0	6	90.0	10.0	0	30.0
		F+CO_2	16	0	0	4	100.0	0	0	20.0
	10	PE	2	2	6	0	20.0	20.0	60.0	60.0
Sub Total	106		43++	30++	20++	13	52.9	28.3	18.8	31.2
3 day pool	23	A	0	19	2	2	8.7	82.6	8.7	17.4
Sputum	25	B	7	1	7	10	68.0	4.0	28.0	68.0
Specimen	26	C	4	8	10	4	30.0	30.0	38.4	54.0
	18	D	4	3	4	7	61.1	16.7	22.2	61.1
Sub Total	92		15	31	23	23	41.4	33.6	25.0	50.0
Total	198		58++	61++	43++	36	47.2	30.6	21.6	39.9

* All filters placed on Middlebrook 7H9 agar B, unless otherwise indicated

** Percentage includes positive, in pure culture, plus isolations from contaminated filter

*** Each sample was divided into 4 equal portions, and each portion placed on Middlebrook 7H9 agar DS-ss containing different combinations of drugs (See code E-1 through E-4)

\+ P=positive, N=negative, C=contaminated, P-C=pure culture, INP=isolations not posible, IP=isolations posible

++ Of decontamination procedures E-1 through F+CO_2, only E-3 and F+CO_2 are included in the totals

Code: Decontamination procedures (Refer to Table 12)

PE....Enzyme-pentane procedure plus Middlebrook 7H10 agar

F+CO_2.(This set incubated under a 5 per cent CO_2 atmosphere

in a 5 per cent CO_2 atmosphere. Isolations were obtained from 100 per cent of the specimens decontaminated by this method, 30 per cent were contaminated (acid-fast organism recovered), and none of the specimens were negative. Isolations were also obtained from 75 per cent of the 24 hour specimens and 68.0 per cent of the 3 day pool specimens, treated in the same manner, as above, except Middlebrook 7H10 agar was used in place of Middlebrook 7H9 agar DS-ss containing the antibiotics. More contamination resulted by the latter method.

As in the case with skin lesions, Colymycin, Erythromycin, and Mycostatin, was the least toxic of the drug combinations employed, but more specimens were contaminated. Again Ristocitin was extremely toxic, since more negatives were recorded for methods in which this antibiotic was incorporated. Of the drug combinations used as the only means of destroying contaminants, the combination of Elkosin, Erythromycin, Colymycin, and Mycostatin, resulted in more isolations (50%). However, decontamination of sputum specimens was extremely difficult with the best procedure described, and when the inhibitor concentration was increased, more negative filters resulted.

The classical methods using sodium hydroxide and hydrochloric acid were compared with the MF method, using Roccal and Middlebrook 7H9 agar DS-ss media containing Colymycin, Erythromycin, and Mycostatin to decontaminate the specimen, on 21 sputum specimens, (Table 15). The objects of this test were: (1) to determine if the MF technique was as reliable as the

TABLE 15. Comparison of Two Routine Procedures with the MF Method for Isolating Acid-fast Microorganisms from Bacteriological Positive Sputum.

No. Specimens Examined	3% HCl			4% NaOH			100 ppm Roccal (MF)		
	P	N	C	P	N	C	P	N	C
21	14*	3	4	11*	4	6	15*	1	5

* Includes isolations from contaminated plates or tubes
P=positive, N=negative, and C=contaminated

routine procedures and, (2) to learn if it was possible to isolate acid-fast organisms from all the specimens by routine procedures, especially 3 per cent hydrochloric acid, since all 21 specimens were originally recorded as positive by this method at the hospital.

With the MF procedure, one more isolation was made than was obtained with the 3 per cent hydrochloric acid method and four more than was obtained with the sodium hydroxide method. Contamination was approximately the same in all three methods. Only one negative sample resulted with the MF method, as compared to three with the HCL method and four with the NaOH method. This clearly indicated the varability in sampling techniques for the bacteriological examination of sputum specimens.

Method for Staining Acid-fast Bacilli on a MF. The acid-fast bacilli appeared dark red on a light green background. Contaminating microorganisms were lightly stained or not stained by the counterstain. The acid-fast bacilli were easily differentiated from the nonacid-fast bacteria, (Fig. 5 and 6).

The results comparing the MF staining procedure with routine smears for direct examination of the specimens are presented in Table 16. Routine smears were prepared and examined by personnel at the Herman Kiefer Hospital. Small pieces of caseated material from the sputum samples were smeared on a slide and the smear stained by the routine Ziel-Nielson acid-fast staining technique. The results of these examinations were sent with a sample of the patient's sputum.

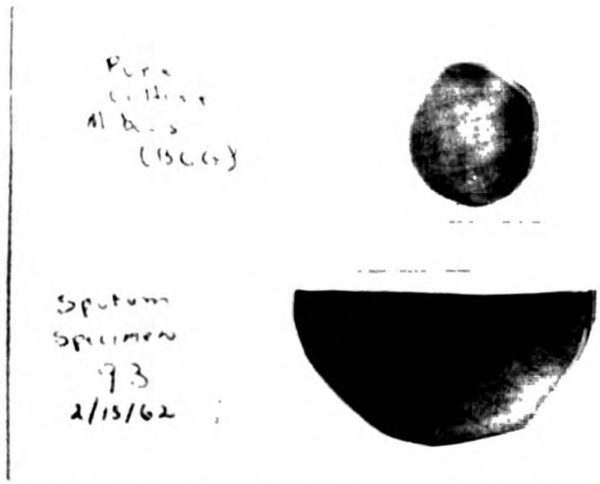

Fig. 4. (Upper), a 1" MF. (Lower), one half of a 2" filter. Both preparations are mounted in permount and a no. 1 (24 x 50mm) cover slip added.

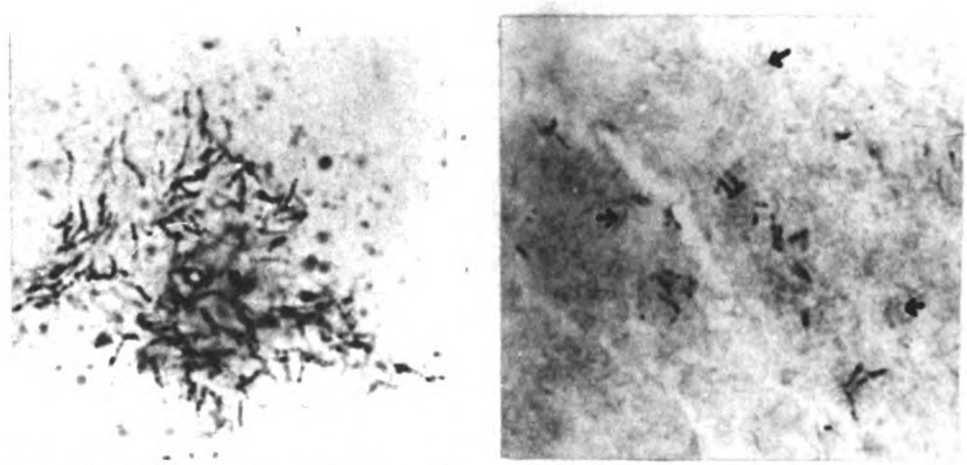

Fig. 5 (Left). Pure culture of M. bovis (BCG) stained on a MF.
Fig. 6 (Right). Acid-fast bacilli (dark staining bodies) from a sputum specimen, concentrated and stained on a MF. Non acid-fast microorganisms are the light staining bodies shown by the arrows. The preparations were stained by the method described. Magnification: approximately 1500X.

TABLE 16. Comparison of the Membrane Filter Staining Method with the Routine Smear Technique for the Direct Examination of Sputum.

Specimen No.	MF Staining Method	Routine Smear
1	P	P
2	N	P
3	P	P
4	P	N
5	N	N
6	N	N
7	P	N
8	N	P
9	P	P
10	P	P
11	P	P
12	N	N
13	P	N
14	P	P
15	N	N
16	P	P
17	P	P
18	P	N
19	N	P
20	N	N

P=positive, N=negative

This sample was digested as described on page 42, and a 5 ml aliquot filtered through a MF, and stained according to the method outlined for staining acid-fast bacteria on membrane filters, (page 46 and 47). The stained filters were examined for acid-fast bacilli for 30 min before reporting as negative.

The MF method agreed with the routine method in 13 cases or 65 per cent of the specimens examined. Four specimens were positive by the MF method and negative by the routine method, and 3 specimens were positive by the routine method and negative by the MF method.

DISCUSSION

A 4 per cent aqueous solution of papain, pH 5.5, and cysteine and versenate activated, was found to be an excellent agent for the digestion of sputum, if the digestion was carried out at 45 C for 1 hour. Besides allowing the specimen to be filtered through a membrane filter, it also was relatively noninjurious to the tubercle bacillus. This demonstrated that enzymatic digestion was more effective than the presently used alkalies and acids.

It should be pointed out, however, that the best liquefying procedure did not always fully digest or homogenize the most viscous specimens. Nevertheless, with the addition of Triton X-100 or QAC (Roccal), 5 ml of digest could be filtered within 10 min. This was also the case when preparing tissue digest for MF filtration. The addition of Triton X-100 in a final concentration of 1:5,000, allowed the entire digest to be filtered. This proved to be an acceptable procedure as long as the undigested strands of connective tissue were removed by pipetting.

Although most of the chemical agents tested had limited value as decontaminants, Roccal in a concentration of 100 ppm and brilliant green, in a concentration of 1:500,000, were the only compounds effective in destroying a majority of contaminants during the pretreatment of sputa. At higher concentrations, the contaminants could be completely suppressed, however, this also resulted in a marked reduction of the acid-fast

bacilli. At these higher concentrations, the compound would compare to the standard procedures used for the isolation of acid-fast organisms from samples containing high populations and apparently would not be applicable to samples containing low populations.

Although Roccal and brilliant green were effective in removing a majority of contaminants from the specimens, some contaminants survived which hindered isolation of acid-fast organisms. In low population samples, such as may be found in tissues from NGL reactor cattle, the loss of microorganisms due to chemicals or technique will result in many bacteriologically negative reports. The use of good technique, to keep contamination at a minimum, is a necessary prerequisite to the success of any technique for the isolation of acid-fast organisms. This is especially the case when working with sputum, since large numbers of normal oral flora are also discharged.

Therefore, subsequent evaluation of various antibiotics and sulfonamides was performed to find an agent or agents which would destroy most of the contaminating organisms. Colymycin was effective against gram negative organisms. Erythromycin against gram positive except gram positive spore formers and Ristocitin against all gram positive microorganisms. The sulfonamide Elkosin, was effective only against P. vulgaris and Mycostatin was effective against molds but not against bacteria. Combinations of these compounds, in keeping with the results, satisfactorily destroyed most of the contaminants. No antagonistic

or synergistic effects occurred with these combinations.

Various attempts were made to improve the growth of acid-fast bacteria on the MF surface. A modification of Middlebrook 7H9 medium (semi-solid, double-strength), was most efficient. Some acid-fast bacilli were able to grow out as much as 5 to 7 days earlier on this medium when exposed to an atmosphere of 5 per cent CO_2 in air. Most of the acid-fast bacilli were laboratory strains that had been subcultured numerous times. However, this method of incubation could be assumed to be effective for the primary isolation of acid-fast bacilli.

After evaluation of the preceeding techniques, a MF method was designed and put into operation for the isolation of mycobacteria from skin lesions and sputum specimens.

It is difficult to evaluate decontaminants in sputum due to variability of the specimen and degree of contamination. Autoclaving the specimen, and seeding with known numbers of bacteria, failed primarily because the material could not readily be filtered. Since the substrates contained in the specimen were denatured, homogenation rather then enzymatic digestion occurred. Therefore, evaluation of these compounds, with the exception of Roccal, was performed in the absence of organic materials. It was also demonstrated that organic matter interfered with the activity of Roccal and this might be expected with other compounds. Therefore, Roccal and brilliant green were also used to decontaminate the specimens in order to determine what affect they might have in actual practice.

The best method for decontaminating skin lesion digest was the procedure in which 400 ppm Roccal was included with the enzyme. This was done so that digestion and decontamination could occur simultaneously. The nonacid-fast organisms that survived this treatment were inhibited in most cases by the concentration of malachite green contained in Middlebrook 7H10 agar.

Triton X-100 was not necessary for filtration when this procedure was employed. The QAC either increased enzymatic digestion or it acted as a wetting agent.

This method of decontamination resulted in 30 per cent more isolations than was obtained when pretreating the digest, as in the former method, and final decontamination with Colymycin, Erythromycin, and Mycostatin contained in Middlebrook 7H9 agar DS-ss was used.

When comparing the results of 122 skin lesions, the MF method accounted for more isolations than either the enzyme-pentane or sodium hydroxide procedures.

All skin lesions were processed by the 3 methods without any attempt to select the specimens. Some of the lesions were received under adverse conditions and no attempt was made to prevent contamination. In a situation such as this, a method which isolates all organisms also exagerates contaminants. For this reason, it is important to remove contamination in order that the specific organisms can be isolated. This is especially the case in isolating acid-fast microorganisms, since the majority of these have a slower generation rate than non-

acid-fast organisms. As a result, the acid-fast organisms are overgrown with contaminating organisms making isolation extremely difficult.

Therefore, in keeping with the results, the sodium hydroxide procedure missed in isolating acid-fast organisms from many of the specimens due to the injurious effect of the NaOH. The removal of contaminants with the enzyme-pentane procedure depends entirely on the selective action of the hydrocarbon, which was shown to be nonselective, and as a result did not sufficiently remove contaminants in some of the specimens, so isolations could not be made. Loss of acid-fast organisms occurred with both these procedures, due either to the toxic effect of the digestive agent or ineffective concentration of the organisms. Whereas in the MF method a decontamination procedure was employed successfully which removed most of the contaminating organisms resulting in a greater percentage of isolations.

It should be pointed out that, in a strict sense, these three procedures were not made on the same identical tissue but on different portions of each skin lesion processed. In other words, the enzyme-pentane and MF procedures used parts of the same digest, the other half of the lesion was processed by the sodium hydroxide procedure. This may account for the isolation of organisms by one technique but not by the other in some cases.

Contamination made isolations from some tissues impossible, which also accounted for the small number of correlations.

It might be expected, however, that more isolations could have been obtained by the MF method if all 122 specimens had been decontaminated by the best decontaminating procedure, (400 ppm Roccal plus Middlebrook 7H10 medium). Seventy per cent of 30 lesions processed in this manner were positive, 10 per cent negative, and only 20 per cent of the specimens were contaminated. Further testing should be done to determine the reliability of this method.

As was demonstrated in the case for tissue digest, pretreatment with Roccal was found to be equally successful for decontamination of sputum. But in this case, the Middlebrook 7H9 agar DS-ss medium containing Colymycin, Erythromycin, and Mycostatin, used in conjunction with the pretreatment technique and incubated in 5 per cent CO_2 in air, resulted in more isolations.

Isolations were obtained from 100 per cent of the specimens decontaminated by this method. However, 30 per cent of the isolations were recovered from contaminated filters.

Since the specimens were processed at different times, using different decontaminating procedures, the results of the other procedures were observed before this procedure was used. Although other combinations of the drugs were demonstrated to be more effective in preliminary studies, the opposite effect occurred in actual practice. This procedure, effective in removing most of the contaminants, nevertheless, failed to inhibit gram positive spore formers and gram negative rods which spread over the entire filter and media. Therefore, the problem

of contamination in some cases still poses a problem in isolating acid-fast organisms in pure culture.

The second best decontamination procedure, in which Roccal was used as above and the filter placed on Middlebrook 7H10 agar, resulted in isolations from 75 per cent of the 24 hour specimens. However, more contamination resulted from the overgrowth of molds, then in the other method.

The enzyme-pentane procedure was not an effective means for isolating acid-fast organisms or destroying contaminates in sputum.

More isolations could be obtained from both skin lesions and sputa when the specimens were treated with Roccal to remove the initial population of unwanted organisms. Using in conjunction with this a medium containing suitable inhibitors, the decontamination process was completed.

Much less contamination occurred in skin lesions digests than in sputa digests. This is probably explained by rinsing the lesions in 1,000 ppm hypochlorite or sterile normal saline. Most of the external contaminants were destroyed or removed. When plates were contaminated, the contamination was almost always due to molds or a gram positive spore former. Middlebrook 7H10 agar does not contain a mold inhibitor. It was demonstrated by microscopic examination that Mycostatin was effective, therefore, the addition of Mycostatin to the medium would be effective.

Contamination in sputa was more serious, since members of the oral flora are always present in the specimen. Contamination

was most troublesome in 3 day pooled sputum specimens. In this type of sputum collection, the specimen remains at room temperature 3 consecutive days. This might be an explanation for the high number of contaminating organisms. The 24 hour samples containing 2-5 ml of sputum, refrigerated immediately after collection, shipped in dry ice to the laboratory, helped reduce the degree of contamination.

A new staining procedure for staining acid-fast microorganisms on MF was developed. Other MF methods have failed primarily because the organisms could not be differentiated on the MF. By the addition of various components to the basic fuchsin solution, differentiation was possible.

The addition of glycerol to the basic fuchsin solution neutralized the charge on the MF and allowed the excess stain to be washed from the filter. The Tween "80" and Triton X-45 incorporated in this solution acted both as a wetting agent and penetrant. This allowed the dye to penetrate the bacterial cell without application of heat.

The MF staining procedure compared favorably with the routine slide method for direct examination of the specimen. Since digestion of the specimen is necessary in any technique for isolation of acid-fast organisms, a portion of the digest could be put aside for the MF staining technique. This would save time and eliminate the hazard caused when making smears directly from the specimen.

This investigation has demonstrated that the MF method can be used successfully in the routine isolation and examination

of acid-fast organisms in both tissue and sputum specimens. However, further work is needed to determine if the MF method, for processing sputa specimens, is reliable. This investigation could best be done by comparing the MF method and an accepted method, on the same sample, in a laboratory where specimens are readily available.

It is the author's opinion, that the membrane filter will be a useful or perhaps the ultimate tool for the recovery of the tubercle bacilli from low population tissues and sputa because all of the acid-fast organisms in the tissue or body fluid digest are deposited on the culture media. Assuming that the accompanying contaminants in the body tissue and fluid samples can be destroyed or inhibited and a suitable medium used, all culturable acid-fast organisms can be isolated.

SUMMARY

A 4 per cent aqueous solution of papain, pH 5.5 (containing 60 mg of l-cysteine and 0.37 mg disodium versenate) was demonstrated to be an excellent agent for digestion of sputum and skin lesions. Digestion at 45 C for 1 hour was not detrimental to the tubercle bacillus.

Triton X-100, added to skin lesion digests in a final concentration of 1:5,000, increased the filtration rate.

Various chemical agents used routinely as selective inhibitory agents were found to be toxic to the tubercle bacillus, (sodium hydroxide, sodium azide, and potassium tellurite).

Roccal (100 ppm) and brilliant green (1:500,000), in preliminary studies, were found to be effective in destroying the majority of contaminants contained in sputa. Roccal (400 ppm) added to the enzyme mixture so that digestion and decontamination could be carried out simultaniously, was found to be effective.

A number of antibiotics and sulfonamides were tested on acid-fast and nonacid-fast bacteria. Colymycin (20 mcg/ml) was effective against gram negative bacteria. Erythromycin (2 mcg/ml) was the least inhibitory to the tubercle bacillus of the antibiotics effective against gram positive bacteria. Ristocitin (0.5 mcg/ml) was the only drug effective against gram positive spore forming bacteria, while noninhibitory to the tubercle bacillus, and Elkosin (150 mcg/ml) was the only drug effective against P. vulgaris. Mycostatin, in concentrations

of 50 and 100 units/ml, was effective against mold contaminants and noninhibitory for all of the bacteria tested.

A modification of Middlebrook 7H9 medium (semi-solid, double strength) was found to be more effective for the cultivation of acid-fast bacteria on membrane filters than routine media. Some acid-fast bacilli were able to grow out as much as 5 to 7 days earlier on this medium when exposed to an atmosphere of 5 per cent CO_2 in air.

Membrane filter methods for the isolation of acid-fast microorganisms from skin lesions and sputa are described. Pretreatment with Roccal was found to inhibit effectively most of the contaminating organisms contained in sputum and tissue digests. In the case of skin lesions, final decontamination was obtained by placing the MF on Middlebrook 7H10 agar, after the pretreatment technique. Middlebrook 7H9 agar DS-ss, containing Colymycin, Erythromycin, and Mycostatin, was found most effective for final decontamination of sputum digests.

More contamination occurred in sputum digests than in skin lesion digests. Furthermore, contaminants were markedly greater in 3 day pool sputum specimens than in 24 hour specimens.

More isolations of acid-fast organisms were made from tissue using the MF technique than were made using sodium hydroxide and enzyme-pentane procedures.

It was also demonstrated that varability exists among procedures for the bacteriological examination of the same specimen due to sampling technique and low acid-fast organism population.

A new procedure for staining acid-fast microorganisms on a MF is described. This method compared favorably with the routine slide method for the direct examination of the specimen, although positives were obtained by one method and not by the other on the same specimen in some instances.

APPENDIX I

An Outline Of The Enzyme-Pentane Procedure For The Concentration Of Mycobacteria Contained In Low Populations From Tissues Of Cattle, (Lipe, et al. 1961)

1. Wash tissue 5 times, 5 min each wash, in 1:1,000 sodium hypochlorite solution or 5 times, 5 min each wash, in sterile saline.

2. Blend approximately 30 gm of tissue in enzyme mixture for 2 min in a Waring blender. The enzyme mixture consists of an aqueous solution of 5 per cent papain containing 60 mg/ml cysteine.

3. Adjust the pH to 8 and place in water-bath shaker at 45 C for 1.5 to 2 hours. The shaker used was a horizontal shaker placed in a water bath. The shaker held 21 two-hundred ml bottles and the shaking rate was 155 times/min.

4. Cool material from shaker, add 5 ml pentane per 100 ml tissue suspension, vigorously shake by hand and allow to stand for 30 min.

5. Place interface and pentane layer in centrifuge tube and centrifuge at 800xG for 20 min.

6. Discard top pentane layer. Seed media with interface and precipitate.

APPENDIX II

The following media were used for the maintenance and isolation of the tubercle bacillus.

Bacto-Middlebrook 7H10 Agar (Difco)

Ammonium sulfate	0.5 gm
l-Glutamic acid	0.5
Sodium citrate	0.4
Disodium phosphate	1.5
Monopotassium phosphate	1.5
Ferric Ammonium citrate	0.04
Magnesium sulfate	0.05
Pyridoxine	0.001
Biotin	0.0005
Bacto-Malachite green	0.001
Bacto-agar	15.0

To rehydrate the medium, suspend 20 grams in 1,000 ml cold distilled water containing 0.5 per cent Glycerol. Heat to boiling to dissolve completely. Distribute in 180 ml amounts in flasks and sterilize in the autoclave for 15 min at 15 pounds pressure (121 C). To each 180 ml sterile medium cooled to 45-50 C, add 20 ml Bacto-Middlebrook OADC Enrichment under aseptic conditions.

Bacto-Middlebrook 7H9 Agar B (Difco)

This medium has the same composition as Middlebrook 7H10 agar, except that it contains 0.00025 gm of malachite green per liter. It is rehydrated and enrichment added as described for 7H10 medium.

Bacto-Middlebrook OADC Enrichment

Oleic acid	00.5 gm
Albumin fraction V, bovine	50.0
Dextrose	20.0
Catalase (Beef)	0.04
Sodium chloride	8.5
Distilled water	1,000 ml

Middlebrook 7H9 Agar DS-ss

This medium has the same composition as Middlebrook 7H9 agar B, except the constituents were doubled and 6.5 grams of agar was used in place of 15 grams per liter. Enrichment was also added.

Bacto-Dubos Oleic Agar Base (Difco)

Bacto-casitone	0.5 gm
Bacto-asparagine	1.0
Disodium phosphate	2.5
Monopotassium phosphate	1.0
Ferric ammonium citrate	50.0 mg
Magnesium sulfate	10.0
Calcium chloride	0.5
Zinc sulfate	0.1
Copper sulfate	0.1
Bacto-agar	15.0 gm

To rehydrate the medium 4.2 gm suspended in 180 ml cold distilled water. Heat to boiling to dissolve the medium completely. Sterilize for 15 min at 121 C. Cool to 50-55 C and add the contents of one tube of Bacto-Dubos Oleic Albumin Complex (20 ml) and 5,000 to 10,000 units of penicillin under aseptic conditions.

Bacto-Dubos Oleic Albumin Complex (Difco)

This enrichment consists essentially of a 0.05 per cent solution of alkalinized oleic acid in a 5 per cent solution of albumin fraction V in normal saline.

Bacto-Dubos Broth Base (Difco)

Bacto-asparagine	2.0 gm
Bacto-casitone	0.5
Disodium phosphate	2.5
Monopotassium phosphate	1.0
Ferric ammonium citrate	50.0
Magnesium sulfate	10.0
Calcium chloride	0.5
Zinc sulfate	0.1
Copper sulfate	0.1
Tween "80"	0.2

This medium was used for the rapid cultivation of pure cultures of mycobacteria and as a suspending medium. To rehydrate the medium 1.3 grams is dissolved in 180 ml distilled water. Cool to 45 C and the contents of one tube of Bacto-Dubos Medium Albumin (20 ml) is added under aseptic conditions. The medium was distributed in 20 ml screw cap tubes in 9 ml amounts. The medium was incubated at 37 C for 24 hours to test sterility. The enriched broth gives a medium meeting all the cultural requirements of the tubercle bacilli. The addition of glycerol enhances the growth of human strains but is not required for bovine or avian strains. Glycerol may be added to the distilled water at the time of rehydrating the medium.

Bacto-Dubos Medium Albumin (Difco)

This enrichment consists of a filter sterilized 5 per cent solution of albumin fraction V from bovine plasma in normal saline and contains 7.5 per cent dextrose.

Bacto-Lowenstein Medium Base (Difco)

Bacto-asparagine	3.6 gm
Monopotassium phosphate	2.4
Magnesium sulfate	0.24
Magnesium citrate	0.6
Potato flour	30.0
Malachite green	0.4

To prepare this medium, 37.2 grams is dissolved in 600 ml of distilled water containing 12 ml glycerol. Heat to boiling with constant agitation and sterilize in the autoclave for 15 min at 15 pounds pressure (121 C). Prepare 1,000 ml of a uniform suspension of fresh eggs under aseptic conditions and thoroughly mix with the sterile base cooled at 45 C, avoiding the formation of air bubbles. Distribute in sterile petri dishes or tubes and coagulate at 85 C for 45 min.

Tarshis Blood Medium

Bacto-Blood Agar Base (Difco)

Beef heart, infusion from,	500.0 gm
Bacto-tryptose	10.0
Sodium chloride	5.0
Bacto-agar	15.0

Completed medium:

Bacto-blood agar base	3.0
Human bank blood	25.0 ml
Glycerol	1.0
Distilled water	73.0
Penicillin	1.0

To prepare the medium, 3 grams of blood agar base is added to 73 ml distilled water containing the glycerol and heat to boiling to dissolve the medium completely. Sterilize in the autoclave for 15 min at 121 C. Cool to 45-50 C in a water bath and add human bank blood containing "ACD solution": citric acid 0.5 gm, sodium citrate 1.37 gm, dextrose 1.53 gm per 100 ml of transfusion solution, and penicillin in 1 ml amounts to give a final concentration of 50 or 100 units. Distribute in sterile petri dishes.

Bacto-TB Charcoal Agar
(Whalen and Mallmann, 1955)

Bacto-yeast extract	2.0 gm
Bacto-proteose peptone No. 3	2.0
Asparagine	1.0
Disodium phosphate	1.0
Monopotassium phosphate	1.0
Magnesium phosphate	0.5
Ferric ammonium citrate	0.5
Sodium citrate	0.5
Bacto-dextrose	7.5
Cobaltous sulfate	0.1 mg
Bacto-ethyl violet	3.3
Oleic acid	80.0 mg
Sodium hydroxide	160.0
Nuchar C-190-N	4.0
Bacto-agar	15.0 gm

This medium is a complete nutrient medium for the cultivation of Mycobacterium tuberculosis and other acid-fast bacilli. It is prepared according to the Whalen C-4 modification (1955). It has the advantage over many other TB media in that it can be sterilized in the autoclave and requires no further nutrient enrichment. To rehydrate the medium, suspend 35.6 gm in 1,000 ml cold distilled water and heat to boiling to dissolve completely. Sterilize in the autoclave for 15 min at 121 C.

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