A COMPARTIVE STUDY OF VARIOUS DIGESTION AGENTS USED IN THE ISOLATION OF MYCOBACTERIUM TUBERCULOSIS FROM SPUTA

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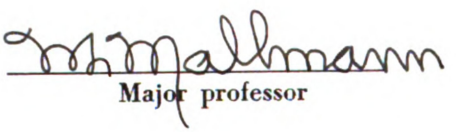

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A COMPARATIVE STUDY OF VARIOUS DIGESTION AGENTS USED IN THE ISOLATION OF *MYCOBACTERIUM TUBERCULOSIS* FROM SPUTA

By

Joseph Wilson Whalen

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Introduction

The clinical manifestations of infection with Mycobacterium tuberculosis have been described in vague fashion since the advent of recorded history. Such references to it may be noted in the Bible and other literature of even greater antiquity. Mummies unearthed in the ruins of ancient Egypt have presented pathological evidence of the ravages of the disease during the period of that nation's zenith of power. The Middle Ages saw many weird remedies advanced for the treatment of "consumption", practically all of which were detrimental to the patient's chances of recovery.

However, despite the pronounced antiquity of tuberculosis, it was not until the relatively modern time of 1819 that Laennec (49) described the clinical aspects of the infection in humans. Villemin, in 1865 (87), produced tuberculosis in the rabbit by means of injection of tissue from human tuberculous lungs and thus demonstrated not only the probable presence of an undiscovered causative agent, but also its apparent communicability.

The actual discovery of the etiological agent was made by Koch (46) in 1882, who also proved by a series of experiments that it gained entry to the body by means of the naso-respiratory tract. He further demonstrated

the extreme refractibility of the organism to ordinary staining techniques and to the then known methods of cultivation. Ehrlich (27) in 1882, was the first to note the unique acid-fast character of M. tuberculosis. His observations led him to propose a staining method of identification which was subsequently modified by Ziehl (93) and later by Neelsen (63).

This acid-fast property is also common to the other members of the genus Mycobacterium, which includes many other species, the majority of which are non-pathogenic soil organisms. Some authorities have expressed the belief that at an early period of animal inhabitancy of the earth, one or several of these saprophytes gained entry into the body of hosts and through mutation and adaptation became pathogenic. Jensen (43) presents an excellent review of the indications in favor of such adjustment by these bacteria. Their function in soil is somewhat superfluous economically since the best known forms seem to be primarily concerned with decomposition of hydrocarbons, a process, which for the most part, is not important in ordinary agricultural soils.

I The Necessity of Cultural Examination for M. tuberculosis in Diagnostic Bacteriology

In the immediate period following the acceptance of the tubercle bacillus as the causative agent of tubercu-

losis, medical science was to a great extent dependent upon microscopic examination of sputa or other pathological body fluids for the presence of this organism as a means of diagnosis. The adaptation of chest x-ray films in the years following 1907 proved to be a great aid to detecting the insidious presence of this invader before the disease had progressed to the exuditive stage, and thus made it possible to arrest it at a much earlier time when cure was relatively simpler (4).

However, it has been repeatedly observed that in numerous instances the radiologist has been unable to detect tuberculous infection long after the bacilli are present in the sputa (59). Also there are many cases wherein the questionable evidence of disease is present, but apparently not to the point of cavitation, when actually such lesions have been formed but are masked by other anatomical structures.

Therefore, it is still essential that the radiographic diagnosis be supplemented by several laboratory examinations. Likewise in the control and arresting of tuberculosis, these laboratory findings prove valuable in determining the lessening or increasing of the organism's ascendancy over bodily defenses.

Microscopic examinations of stained smears of sputa or other fluids during such periods of the disease are

laborious and require the expenditure of a great deal of time in order to be reliable. Smears must be carefully made, properly stained, and observed through the microscope for a considerable length of time. Various concentration methods have proved to be an improvement over the direct smear technique, but nevertheless a large amount of sputa must often be studied to find a single acid-fast rod.

There has been in the past a great deal of controversy as to the actual location of the majority of tubercle bacilli in sputa. It is the contention of some investigators that the direct smear will often detect these organisms present in the surface film while the sediment, upon concentration, will prove to be devoid of them. Because of this, concentration methods have been developed which are designed to recover all the material present in the surface film as well as the sediment, through use of flotation techniques.

In many instances the sputum specimen itself will prove to be extremely mucoid, thus making it much more difficult to prepare representative smears. Some chemicals used for digestion of sputa have been shown to exert a detrimental effect on the acid-fast nature of the tubercle bacillus, while other agents act to precipitate certain constituents, consequently decreasing the accuracy of the slide examination.

During periods of hemoptysis the presence of M. tuberculosis will often be masked by the predominance of erythrocytes in the sputum smears, making it impossible to determine the extent of infection.

Another obstacle far too prevalent in clinical laboratory examinations for tubercle bacilli is improper staining technique. Precipitated stain or improper decolorization can often lead to many errors of varied nature.

In the actual microscopic examination of smears, several pitfalls exist. The presence of artifacts may mislead the inexperienced laboratorian into mistaking them for tubercle bacilli. Likewise scratches in old slides will often simulate acid-fast rods due to the failure of acid alcohol to penetrate into their recesses and remove the retained stain.(73). Also it is possible for tubercle bacilli to be rubbed off highly positive slides and become lodged within the film of oil covering the immersion objective unless care is taken to wipe the latter following examination of each slide. Spores, many of which are acid-fast, will sometimes appear remarkably like a rod when they are grouped together in the presence of other organisms and cellular debris. Even the most experienced observer may sometimes mistake species of mycobacteria for the tubercle bacillus, since they often bear a very close morphological resemblance. Special

staining techniques for differentiation cannot be relied upon to rule out these saprophytes in most instances.

Finally there exists the ever-present factor of chance in slide examination. When but 1 or 2 acid-fast rods are present in a smear, it is very improbable the microscopist will discover them unless he happens, by chance, to observe the fields in which they are present. Corper has estimated that there must be approximately 100,000 organisms per ml. of sputum present before the probability of finding them in smears becomes significantly large (11). To some extent this is very likely due to loss of acid-fastness of the organisms present as the result of physical trauma, dissociation and/or senility (92).

Inoculation of guinea pigs with pathological material is advantageous in diagnosis of tuberculosis since it offers a means of determining the pathogenicity and virulence of the organism, as well as indicating the presence of tubercle bacilli when they occur in numbers so small that it is almost impossible to detect them microscopically.

However, this method is slow. Most laboratories allow a period of 4 to 8 weeks before killing one of these animals for histological studies.

Disadvantageous also is the expenditure of the time and labor inoculating, grossly examining for lesions, performing tuberculin tests, and preparing tissue sections for histological examination.

The guinea pig is unfortunately extremely susceptible to respiratory and intestinal epidemics. One such infection will often wipe out an entire colony in a matter of hours, thus destroying valuable clinical data.

Therefore, for the reasons listed above as well as several others, it is evident that neither the microscopic slide examination nor the guinea pig inoculation is very satisfactory when used alone as a means of detecting M. tuberculosis in pathological body fluids. The advantages of a satisfactory cultural method are numerous: Differentiation of the tubercle bacillus from other Mycobacteria may be made through colony morphology in many instances. Non-staining bacilli will frequently proliferate upon a suitable medium, and specimens containing but one or two of these organisms may give positive cultures. Although they may be masked by vast amounts of blood, growth will be initiated. A reliable medium which would give rapid cultural results would also reduce the time expended in slide examination. The errors inherent in the latter technique would be eliminated and the factor of chance reduced. Likewise, a great deal of time would be conserved

if the routine inoculation of guinea pigs could be rendered unnecessary in this fashion.

In an endeavor to achieve more satisfactory cultural techniques for the isolation of M. tuberculosis, the writer has studied and extensively modified media and digestive agents which may be used in the cultivation of this microorganism. This paper is concerned with one phase of this work--the experimental investigation of means of removing or retarding contaminants which overgrow culture media or otherwise restrict the value of cultural methods.

The Evolution of Inhibitory Agents in Cultured Media and Digestion Techniques for the Isolation of M. tuberculosis-- their Inadequacies

The cultural methods and media in use at present fall short of the ideal goals as presented. Tubercle bacilli have been recognized as being difficult to cultivate since their discovery and original cultivation by Koch. Insolation and identification of this organism were hindered until quite recently by the inability of investigators to develop media which would grow it more rapidly.

For his first isolation Koch utilized bovine blood serum, coagulated by inspissation. He found that it required 4 to 8 weeks for colony formation upon this medium. He solved the problem of dehydration because of this prolonged incubation by sealing the cotton plugs of the tubed slants with paraffin. For removal of other organisms present in sputum specimens, this pioneer recommended concentration and digestion with 4 percent sodium hydroxide, since he had observed that tubercle bacilli appeared to tolerate quite alkaline environments if they were not exposed for a prolonged period. Twelve to 24 tubes were inoculated to insure obtaining slants which were not contaminated or overgrown with saprophytes present in the specimens.

Immediately following Koch's successful cultivation

of these organisms, other investigators attacked the problem of isolating tubercle bacilli from sputa while still retarding the growth of contaminants. Nocard and Roux (64), Smith (78), Raskin (75), Proskauer and Beck (74), Wurtz (90), Dorset (21), Phisalix (71), Lubenau (54), and Hesse (36), all subsequently formulated cultural media for the cultivation of M. tuberculosis. Although this organism could be successfully cultivated on all of these media, some difficulty was experienced in primary isolation from sputa even when 4 percent sodium hydroxide was used as an inhibitory agent for saprophytic bacteria. In spite of this treatment, contaminants often overgrew the medium, or, in other instances, microscopically positive sputa failed to yield positive cultures. It was believed by many investigators that sufficient exposure to the harsh alkaline effects of sodium hydroxide to destroy the saprophytes often detrimentally affected the tubercle bacillus to the extent that it was unable to resume multiplication in the artificial environment of the medium (7).

The introduction of "Antiformin" by Uhlenhuth and coworkers (86, 28) represented a substantial improvement in the search for an agent which would digest the mucoid portions of sputa and also act to inhibit the growth of unwanted organisms. A commercial product of varying composition consisting for the most part of sodium hypochlorite,

"Antiformin" breaks down the undesired tissue and cellular debris as well as destroying the majority of bacteria, but does not disturb too seriously the morphology of acid-fast organisms. Thus it was frequently used exclusively in digestion and concentration of sputum specimens for microscopic examination without an attempt at cultivation of tubercle bacilli which may have been present in the specimens being made. In such instances it was highly satisfactory, compared to the relatively slow digestion of extraneous material encountered with the 4 percent sodium hydroxide.

Griffith (4), experimenting with "Antiformin", found a time period at which uncontaminated cultures of tubercle bacilli could be obtained without destroying all the viable tubercle bacilli. Studies by Patterson (8) and by Brown and Smith (9) indicated that it was considerably less toxic to the tubercle bacillus than the high alkalinity of sodium hydroxide. It was recognized, however, that this agent still left much to be desired when utilized in cultural work. Smith (80) demonstrated that it required at least 1500 times as many tubercle bacilli to seed the then known artificial media after these organisms had been exposed to antiformin as it did to infect a guinea pig with untreated bacilli.

The literature indicates that much of the sodium hypochlorite contained in commercial "Antiformin" is

susceptible to deterioration. Thus the solution may lose over 50 per cent of its activity within 1 year (84). "Antiformin" can, however, be tested for activity by a simple method which yields a close approximation both of the amount of sodium hypochlorite and of available chlorine (6). In routine cultural examinations by digestion and concentration methods where specific amounts of "Antiformin" should be employed, frequent standardization of this reagent is imperative.

Several investigators, aware of these limitations of antiformin, added various so-called stabilizing agents (10), and experimented with shorter exposure times of sputa to this digesting agent, only to find that these measures resulted in a marked increase of contaminated culture (8). Thus it was apparent that some other means of inhibition of saphrophytes was urgently needed.

At least a partial answer to the problem was provided by Petroff's introduction of a medium containing a 1 to 10,000 concentration of gentian violet, in addition to glycerol, beef, and egg (70). This marked the first published utilization of a dye in a medium devoted to the isolation of tubercle bacilli from pathological material. Studies conducted by Petroff and his associates (71, 90), and by Corper (13), revealed that a shorter exposure time

to antiformin was now possible if the resulting digested material were planted on this medium. Other digesting agents of a less toxic nature could now be feasibly utilized with less chance of overgrown cultures.

The extraordinary resistance of acid-fast bacteria in general to gentian and crystal violet had been investigated by Churchman (57). These dyes inhibit the growth of Gram-positive bacteria, but, although mycobacteria retain the Gram stain, most strains will tolerate relatively large concentrations before their growth is seriously retarded.

Following the successful utilization of the Petroff medium, several workers reported formulation of new cultural media containing gentian violet as an inhibitory agent (11, 55, 17, 50, 5, 85).

Corper reported that higher percentages of positive cultures had been obtained using a medium which actually consisted of Petroff's enriched with peptone. It was subsequently found by Twort (83) to be less toxic to tubercle bacilli than was that of Petroff. He attributed this to the richer content of nitrogenous nutrients available to the organism which might act to partially overcome the toxicity of the gentian violet. In the light of recent discoveries by Dubos and Davis (23) and by Youmans (93) of the protective effect of bovine serum albumin against agents which might be toxic to the tubercle bacillus, it is prob-

able that the added peptone modified the detrimental effects of this dye on all bacteria cultured with the medium.

Meanwhile Petroff (71) and Koch (47) had studied the effect of various other concentrations of sodium hydroxide as digesting agents.

Numerous other agents for sputum digestion were reported subsequently; including the digestive enzymes (30), pyridine (31), potassium hydroxide (20), sodium carbonate at moderately elevated temperatures (34), ammonium hydroxide (8), 3 per cent hydrochloric acid (52), 6 per cent sulfuric acid (52), sodium hydroxide-alum mixture (35), and even acetic acid and distilled water. In some instances it was recommended that digestion be followed by precipitation or flocculation with aluminum, zinc, or iron salts. Light benzene or gasoline products were used after hypochlorite digestion to suspend tubercle bacilli in an intermediate surface layer. Likewise chloroform (51), which settles to the bottom, was used in an attempt to obtain zonal separation. A few of these techniques remain in use at the present time, but the majority were discarded due to their complexity or because they were found unsatisfactory either from the standpoint of toxicity to the tubercle bacillus or since they failed to remove a large enough amount of contaminants.

Possibly, also, these methods failed to win widespread acceptance due to the success of Lowenstein's gentian violet medium (53) which was also introduced during this period (1924). When used in conjunction with 3 per cent hydrochloric acid or 4 per cent sodium hydroxide, Lowenstein found that this medium gave a considerably higher percentage of positive cultures and shortened the period of colony development for many strains of bacilli.

Shortly thereafter, Petragnani (69) reported regarding a glycerol-egg medium which contained malachite green as an inhibitory agent. This dye has been demonstrated to be considerably less toxic to M. tuberculosis than gentian violet, yet it also possesses a strong inhibitory effect on the majority of Gram-positive organisms. It was some years following the publication of Petragani's paper before his medium became widely known in this country, but at the present time it, along with Lowenstein's (Jensen's Modification), is probably the mostpopular of those utilized by clinical laboratories. These are the only media, out of 20 studied, recommended by the American Trudeau Society.

Subsequently, Corper and Uryei in a series of papers (14, 13, 12, 15) studied the effect of numerous chemical agents on tubercle bacilli and reported that 5 per cent oxalic acid appeared to slow the growth of this microorganism the least. In their procedure a volume of the oxalic acid reagent equal to that of the sputum specimen

(or sediment resulting from centrifugation) is added to the latter and the resulting mixture is incubated at 37°C for 30 minutes before inoculation of Corper's egg-yolk medium. Van Vranken (89) reported that this technique proved superior to treatment with oxalic acid and culture on Petragnani's medium, or to treatment with many of the previously discussed agents and subsequent inoculation of Corper's and Petragnani's medium. Since the Corper medium contains no dye, the accelerated and more luxurient growth observed was attributed to lack of dye toxicity. Other reports have attested the value of oxalic acid in sputum digestion, not only from the standpoint of saprophytic inhibition but also by virtue of its rapid action in dissolving mucus.

However, as with various concentrations of other acids and with sodium hydroxide and "Antiformin", it is necessary to limit the period of contact of oxalic acid with a specimen. Van Vranken found that if the preparation incubates over two hours, the specimen is lost, since all tubercle bacilli are usually either destroyed or inhibited. This, obviously, is an extreme disadvantage, particularly in public health work where only one sputum per patient is customarily available.

In 1939, "Tergitol" (sodium octyl sulphate) an organic wetting agent, was introduced by Petroff and Schain (73). It was claimed to possess a more rapid and complete action

than any other digestant previously utilized. The procedure, however, rapidly underwent changes as it was studied by other investigators (66). One part of "Tergitol penetrant 08", 1 part of 4 per cent sodium hydroxide and 1 part of water was used for a time, but was subsequently replaced by equal portions of "Tergitol 08" and Javelle water. Since the latter solution contains sodium hypochlorite, it was unstable and required frequent preparation. Actually no controlled data have been presented to substantiate the value of "Tergitol" in sodium hypochlorite mixtures (42). Gradually its use in digestion reagents has diminished, so that at present its place in clinical laboratories is largely as a means of intensifying the Ziehl-Neelsen staining of smears without use of heat.

Another commercial alkaline sodium hypochlorite solution, "Clorox", was studied by several workers (63, 18, 83). In this concentration-digestion technique, 5 ml. of "Chlorox" (active ingredient 5.25 per cent sodium hypochlorite) is added to each of 5-50 ml. screw-capped tubes containing 5 m. of a single sputum specimen and the tube capped and shaken for 5 minutes. It was found that this reagent is satisfactory only for microscopic examination of concentrate smears, since it was shown to be highly toxic, relatively, to the tubercle bacillus. "Clorox" successfully eliminates such extraneous material

as: blood cells, fibrous tissue, and other bacteria which might intensify the difficulty of detecting acid-fast bacilli microscopically. If it is allowed to act on a sputum specimen longer than 2 minutes, attempted acid-fast staining of any tubercle bacilli present usually fails (66). It has been claimed that 15 per cent more positive results were obtained with this reagent than by the smear method (66).

Meanwhile, in an effort to produce an effective digesting agent which kills or inhibits contaminating microorganisms with relatively little effect on the acid-fast bacilli, Corper and Stoner (16) reported great success with trisodium phosphate ($Na_3.PO_4.12H_2O$). These investigators stated that a 23 per cent solution added in equal volume to sputum specimens could remain in contact for as long as 7 days at room temperature without perceivable harm to the viability of any tubercle bacilli present, and this amount and time, in most cases, destroyed all potential contaminants contained in the specimens. Thus the danger of loss of individual specimens is eliminated, since sufficient time is allowed to render unnecessary the usual cautious watch period necessitated by all reagents previously described where neutralization must be performed within 1/2 to 2 hours at least.

Corper and Stoner recommended that the equal volume of 23 per cent trisodium phosphate solution and the sputum

specimen be incubated for 24 hours at 37°C. before centrifugation of the mixture and inoculation of a culture medium with the sediment. Van Vranken (89) found this technique to be superior to those previously described for the destruction of contaminants, while yielding relatively high percentage of positive cultures. Tarshus and Lewis (83), who added a period of agitation of the reagent-sputum mixture before incubation, also reported a greater number of positive cultures using this technique, and confirmed Corper's claim that neutralization of the digested specimen is unnecessary before seeding cultures.

However, subsequent papers by other workers have expressed varying opinions in regard to the value of the trisodium phosphate technique. Mitchell and Jeffries (60), following Corper's suggestion, placed the reagent in the receptacles used for collecting the pathological specimens. They reported that this prevents further development of molds and contaminants, so that when the specimen was delivered to the laboratory a day of incubation at 37°C. destroyed the remaining viable contaminating microorganisms. This method was also used by workers at the Florida State Health Department Laboratory (44), where it was found that the extra day of incubation following receipt of the specimen was usually unnecessary, and that a greater number of positive cultures were obtained than with 4 per cent sodium hydroxide digestion.

In a study of the most commonly used digestive agents, Spendlove, Cummings, and Patnode (78) concluded that sodium hydroxide and trisodium phosphate are most suitable.

On the other hand, Beattie (7) stated that the latter reagent may adversely affect growth of tubercle bacilli. Mullahy (62) observed in a study with trisodium phosphate that nearly twice the number of contaminated cultures resulted with this method than was obtained with the 4 per cent sodium hydroxide solution, but reported that the former is superior in securing positive results by smear from concentrated specimens. Hunter (40) also observed greater contamination with trisodium phosphate digestion than with sodium hydroxide, but to a much lesser extent. In addition, he found that the former reagent yielded a significantly larger number of positive cultures.

Kurung (48) reported that trisodium phosphate, along with the majority of other digestion reagents, lacked the ability to inhibit most varieties of fungi common to the respiratory system and oral cavities of many long-term chronic tuberculosis patients.

These and further studies of digestion-concentration solutions were stimulated by the promise of more rapid cultivation of M. tuberculosis offered through the announcement by Dubos of a liquid synthetic medium containing a polyoxyalkylene derivative of sorbitan monostearate

("Tween 60") in which he obtained rapid and submerged growth of the H37Rv strain of this organism. Subsequently Dubos and Davis (22) described a liquid medium including a polyoxyethylene ester of oleic acid ("Tween 80") as wetting agent, and bovine serum albumin , fraction V as enrichment and protective agent against substances toxic to the tubercle bacillus. Foley (29) reported that use of this medium in diagnostic work had made possible isolation of human strains from pathological material, following 4 per cent sodium hydroxide treatment, in an average of 6 days, with 88 per cent correlation with guinea pig inoculation.

Dubos and Davis (24), however, after using it to cultivate tubercle bacilli from sodium hydroxide-treated sputa, cautioned against use of the medium in routine diagnostic work, due to frequent contamination.

Dubos and Middlebrook (23) introduced a solid form of this medium containing an oleic acid-albumin complex which eliminated the toxicity of "Tween 80". Although growth of M. tuberculosis on this medium was somewhat slower than with the liquid form, Goldie (32) reported successful isolation of this organism from sputa treated with ammonium carbonate and penicillin. Middlebrook (59) obtained microscopic growth on the solid medium, using penicillin as an inhibitory ingredient, in an average of 13 days. Subsequently he recommended only the solid Dubos medium for diagnostic work.

Mollov, Hill, and Oshinsky, (61) successfully utilized the liquid form to isolate tubercle bacilli from pathological material. They treated sputa with warm 4 per cent sodium hydroxide (temperature not specified) in quantities equal to the volume of each specimen, and agitated the resulting mixture in a shaking machine for 10 minutes before concentration by centrifugation. Fifty units of penicillin was added to each 10 ml. of the medium as a further protection against contamination. These workers reported some difficulty in determination of growth in the medium in the presence of cloudy or granular inoculum, and thus recommended its use primarily in laboratories where only a few cultures for tubercle bacilli are made, since, frequent smears of the liquid are essential to confirm multiplication of these microorganisms.

Numerous reports on the growth of mycobacteria in the presence of penicillin (3, 76, 81) have stimulated wide utilization of it as a selective inhibitory agent in various culture media. Abbott (1) found it effective in this respect when incorporated into Lowenstein-Jensen medium in a concentration of 100 units per ml. He reported a marked reduction in the total number of contaminated cultures by alpha, beta, and non-hemolytic streptococci, as well as by Gram-positive bacilli. Sula (82) claimed penicillin to be superior to malachite green as an inhibitor of contamination

in liquid media due to less toxicity for the tubercle bacillus. Unger and Muggleton (87) observed that penicillin in low concentrations stimulates the growth of this organism. Pramer and Heukelekian (74) added 5 units of penicillin per ml. of solidified Dubos medium in a study of survival of tubercle bacilli following sewage treatment.

However, McCulloch (57) had noted that the presence of wetting agents in a liquid suspension of tubercle bacilli caused these organisms to be much more susceptible to various disinfectants. Youmans and Youmans (93) subsequently warned against the use of antibiotics in culture media containing the wetting agent, "Tween 80", since the dispersive effect of the latter permits a more intimate contact between the antibiotic and the surface of the bacterial cell. Other investigators concurred with this observation (2, 41, 19). It was claimed that partial lysis of M. tuberculosis occurs in Dubos medium in the presence of high concentrations of penicillin (45).

I Advantages and Inadequacies of Contemporary Digestants

Thus, the advantages and inadequacies of current concentration-digestion techniques and inhibitory agents can be summarized as follows:

1. Various concentrations of sodium hydroxide are

relatively rapid in their digestive action. However, the period of activity must be carefully controlled, since the high alkalinity of sodium hydroxide will detrimentally affect the tubercle bacillus.

2. Antiformin is rapid and efficient in destroying not only contaminants, but also extraneous material present in the sputum specimen. It has been shown to be more toxic to M. tuberculosis than sodium hydroxide. It deteriorates rapidly, and thus must be standardized frequently.

3. Crystal violet added to a medium in suitable concentrations, exhibits a selective bacteriostasis of nearly all Gram-positive bacteria excepting the tubercle bacillus. It, too, is somewhat toxic to the metabolism of the latter microorganism.

4. Hydrochloric acid, sulfuric acid, potassium hydroxide, and sodium carbonate are rapid and efficient but they must be precisely timed, and properly neutralized. Their toxicity is also relatively great.

5. Flocculation and emulsifying techniques are complex and time consuming, and their value has never been fully substantiated.

6. Malachite green is probably the least toxic to

tubercle bacilli of the selective dyes, yet it exerts some inhibitory action upon their multiplication.

7. Oxalic acid in a concentration of 5 per cent efficiently removes contaminants with little effect on M. tuberculosis, but its period of action must also be carefully limited and neutralization before culture is essential.

8. "Tergitol" and "Clorox" are satisfactory only for concentration preliminary to preparation of smears, since they are too toxic to permit subsequently reliable cultures.

9. Twenty-three per cent trisodium phosphate may remain in contact with the tubercle bacillus for up to seven days without serious toxicity being manufested. Neutralization is unnecessary. It may be added to sputum receptacles before use to speed digestion. However it has not proved very effective in the destruction of saprophytes when used by several investigators. It does not inhibit fungi, and one worker has expressed the belief that it adversely affects multiplication of tubercle bacilli.

10. Penicillin has been shown to be an excellent inhibitor of contamination when present in suitable concentrations in culture media. Unfortunately, in the presence of wetting agents which accelerate the growth of tubercle bacilli through dispersal, it is markedly bacteriocidal for this microorganism.

Experimental

I Use and Comparative Study of Sodium Hydroxide and Other Chemical Agents as Digestants of Sputa

During the course of investigating the possibility of combining the attributes of Dubos' media (liquid and solid) with those of a liquid medium developed some years previously by Mallmann (56), it was noted that the resulting modifications were seriously hampered in the isolation of M. tuberculosis, when sputa of tuberculosis patients were cultured, by the problem of contamination resulting from multiplication of the normal oral bacterial flora. It was therefore concluded that before these media could be utilized clinically, it would be necessary either to treat sputa chemically or to introduce into the medium some inhibiting ingredient which would prevent development of these contaminants.

Since much of this preliminary study was performed in the laboratories of the Calhoun County Health Department where 6 per cent sodium hydroxide was in routine use before concentrates were inoculated on Petragnani slants, this technique was adopted.

However, it soon became evident that several factors were responsible for the wide variation in the growth-times of different strains of tubercle bacilli isolated from sputa.

Thus an attempt was made to standardize all conditions under which the experimental work was done, including the digestion-concentration process.

Further, it was believed that the relatively long growth period of freshly isolated tubercle bacilli on experimental media compared with that of small inocula of stock strains was due to toxicity of the sodium hydroxide digestant. Therefore a comparative study of sodium hydroxide in concentrations of 6 and 4 per cent, 5 per cent oxalic acid, "Antiformin", and 5 per cent potassium hydroxide was made.

A. Materials and Methods

The technique followed at the Calhoun County Health Department was to permit spontaneous digestion of the sputum specimen (customarily a seven-day pool) for from 1 to 2 days. A direct smear was made in the meantime and examined. Then the sputum was centrifuged at 2500 r.p.m. for 10 minutes to obtain a concentrated sediment, and smears for microscopic examination were prepared from it. The remaining sediment was then diluted with an equal volume of 6 per cent sodium hydroxide, and the mixture stirred. At intervals of 10, 20, and 30 minutes after this dilution, a tube of medium was inoculated with 6 loopfuls of the mixture.

A similar procedure was followed with 4 per cent sodium hydroxide, excepting that the intervals of treatment prior to inoculation of the medium were extended to 20, 30, and 50 minutes.

With "Antiformin", approximately 20 ml. of the sputum was mixed with 15 ml. of this reagent and 65 ml of distilled water. The resulting mixture was incubated at room temperature for one hour, following which it was centrifuged at 2500 r.p.m. for 10 minutes. The sediment was washed 3 times with physiological saline. A portion of it was then used for preparation of smears, following which 6 loopfuls of the remainder were used to seed each tube of cultural medium.

Oxalic acid in a concentration of 5 per cent was added in equal volume to the sputum specimen and the container was incubated at 37°C. for 30 minutes. The liquid was then diluted with 10 ml. of 0.01 N sodium hydroxide and centrifuged. The sediment was used for preparation of smears, then 6 loopfuls of it were introduced into each tube of medium.

The 5 per cent sodium hydroxide reagent was utilized in exactly the same fashion as was the 6 per cent sodium hydroxide.

Twenty sputum specimens were obtained from patients

at the Arthur S. Kimball Sanatorium and pooled. The homogeneous pooled specimen was then divided into 20 equal portions. Patients were selected whose sputa had been negative culturally for at least 6 months, and whose radiographic history indicated that they were nearly arrested cases. Each of the 20 resulting specimens were seeded with an approximately uniform amount of tubercle bacilli from 1 pure culture and then divided into 5 equal portions for treatment with the various reagents.

All cultures were incubated at 37°C. for a total of 6 weeks. The media used in this comparative study were Petragnani's, and liquid and solid experimental preparations, media I, II, and III, respectively (appendix). Three tubes of each medium were inoculated from each sputum specimen. The cultures were examined microscopically frequently, and if growth was detected smears were prepared of the colonies and stained with the Ziehl-Neelsen technique for microscopic examination.

B. Results

It was found that 6 per cent sodium hydroxide treated specimens were largely free of contamination when cultured. In the few instances in which contaminants were found, the microorganisms responsible were found to be molds or extremely active proteolytic-type bacteria which partially liquified the medium.

The potassium hydroxide treatment yielded a slightly greater degree of saprophytic growth, as did 4 per cent sodium hydroxide digestion. The contaminants present were widely varied in species and nature.

On the other hand, the oxalic acid reagent permitted frequent saprophytic overgrowth of the liquid and solid experimental media which, unlike Petragnani's contained no selective inhibitary ingredients. In these instances it was impossible to detect the presence of M. tuberculosis by smear from the cultures.

"Antiformin", however, proved to be the most efficient in destroying contaminants. Only 4 tubes of media yielded saprophytic organisms, three of these being liquid media.

From the standpoint of recovery of M. tuberculosis, it was found that 4 per cent sodium hydroxide treatment gave the largest number of positive cultures, with 5 per cent potassium hydroxide digestion yielding a slightly smaller number. The 6 per cent sodium hydroxide reagent was next in efficiency in this respect, with 5 per cent oxalic acid treatment producing a relatively poor yield of tubercle bacilli, probably due to the excessive saprophytic growth encountered.

The "Antiformin" technique, however, apparently destroyed most of the viable tubercle bacilli along with the contaminants, since only 8 tubes of this liquid medium

contained these microorganisms at the conclusion of the 6 weeks incubation period.

An analysis of the relative time required for development of macroscopic colonies on the various media utilized reveals that most rapid growth was obtained with oxalic acid digestion. Potassium hydroxide treatment was second in this respect, with 4 per cent sodium hydroxide, 6 per cent sodium hydroxide, and "Antiformin" third, fourth, and last, respectively. The latter reagent was extremely poor from this standpoint, as 38 days were required for colony development in the liquid medium, compared to 29 days for 6 per cent sodium hydroxide and 14 days for oxalic acid digestion.

Detailed results of this study are presented in Table 1.

II Determination of the Relative Efficiency of Trisodium Phosphate Digestion as Contrasted with Other Agents

Van Vranken's report regarding the superiority of 23 per cent trisodium phosphate over any of the other digestive agents which she studied, served to vastly increase its popularity and bring it to the attention of many investigators. Prior to this time, use of it as a digestant was almost unknown in hospital and public health laboratories, although Corper's introduction of it predated Van Vranken's report by nearly two years.

Since sodium hydroxide, as well as the other reagents previously studied had proved far from ideal, a comparative survey of the efficiency of trisodium phosphate, to 4 to 6 per cent sodium hydroxide, 5 per cent oxalic acid, and 5 per cent potassium hydroxide was undertaken.

A. Materials and Methods

Trisodium phosphate ($Na_3PO_4.12\ H_2O$) was prepared in a concentration of 23 per cent by weight (equivalent to 10 per cent anhydrous salt). One portion of this solution was utilized without further alteration as a digesting agent, while to another portion, 3 per cent by weight of "Triton X-100"(an alkylated aryl-poly-ether alcohol of high molecular weight which is a non-ionic surface active agent, compatible with alkaline conditions--manufactured by Rohm and Haas, Philadelphia, Pa.) was added. The pur-

pose of this addition was to endeavor to more rapidly eliminate contaminants in sputa sediments by reducing the thickness of the mucoid film found in many specimens which envelopes and serves to protect these undesirable bacteria.

It was realized that prolonged exposure of the tubercle bacillus to a powerful wetting agent such as "Triton X-100" would be detrimental, as indicated by the work of Youmans and Youmans which was previously reviewed. However, by conducting a timed study of the digestion process, it was hoped that an average period of time could be ascertained during which destruction of saprophytes would at least partially disperse the latter bacteria so that more rapid growth would result upon inoculation of media with the digested sediment.

The 23 per cent trisodium phosphate and the trisodium phosphate--"Triton X-100" reagents were utilized in identical fashion: Equal volumes of the sputum sediment and the digestant were mixed and incubated at 37°C.

Treatment of sputum specimens with 4 and 6 per cent sodium hydroxide, 5 per cent oxalic acid, and 5 per cent potassium hydroxide was performed in the same manner as in the previous experimental study.

At intervals of 0.16, 0.34, 0.50, 1.5, 2.5, 6.0, 12, 24, and 26 hours following the start of the respective

treatments, one tube each of Petragnani's, and the liquid and solid experimental media were inoculated with 6 loopfuls of the digesting sediment.

Seeded, pooled, and subsequently divided sputum specimens were again utilized as in the previous experimental study of digestive agents.

All cultures were incubated at 37°C. The media were contained in screw-capped culture tubes. Cultures were examined frequently for macroscopic evidence of growth. All apparent colonies were checked microscopically. Tubes of liquid medium were slanted during incubation, since previous cultural studies had indicated that more rapid and luxuriant growth is obtained under this condition. These tubes were also shaken frequently to aerate the medium.

B. Results

Twenty-three per cent trisodium phosphate treatment partially destroyed saprophytes in 24 hours, as evidenced by uncontaminated tubes of solid experimental and Petragnani media which were inoculated after this period of digestion. However, the liquid medium planted at this time was contaminated. Twenty-six hour treatment apparently destroyed all contaminants, since all 3 media produced pure cultures of M. tuberculosis.

The trisodium phosphate--"Triton X-100" reagent eliminated saprophytes sufficiently in 6 hours at 37°C. to produce uncontaminated Petragnani and solid media. Complete inhibition was not recorded until after 12 hours treatment.

Sodium hydroxide in a concentration of 6 per cent partially removed contaminants in 0,50 hours and completely in 1.5 hours. The 4 per cent concentration of this reagent completely destroyed extraneous bacteria in 1.5 hours, with no partial destruction evidenced.

Oxalic acid treatment brought about partial in 1.5 and complete destruction in 2.5 hours, while 5 per cent potassium hydroxide produced the same effects in 2.5 and 6.0 hours respectively.

Trisodium phosphate reagent treatment for 24 hours yielded growth of tubercle bacilli in the liquid medium in 12 days, trisodium phosphate phosphate--"Triton X-100" for 6 hours in 11 days, 6 per cent sodium hydroxide for 0.5 hours in 20, 4 per cent sodium hydroxide for 1.5 hours in 16, oxalic acid for 1.5 hours in 18, and potassium hydroxide for 2.5 hours produce growth in 18 days.

A complete summary of the findings of this study is to be found in Table 2.

III Utilization of Trisodium Phosphate in Culture of Routine Sputum Specimens

For a period of 4 months, all routine sputum specimens from patients at the A. S. Kimball Sanatorium, as well as those coming into the laboratory at that institution from out-patients, was divided into 2 portions. One of these was digested with 23 per cent trisodium phosphate for 24 hours, while the other was treated with the trisodium phosphate--"Triton X-100" reagent for 12 hours at this temperature. Total of 345 specimens were handled in this manner. All were cultured on Petragnani and the experimental solid media (3 tubes of each for each specimen). Of these, 126 were positive for tubercle bacilli following trisodium phosphate digestion, while 144 were positive after the combination reagent treatment.

In relation to amount of contamination, it was noted at the conclusion of the period that neither digestant proved effective in inhibiting the growth of certain fungi which are present either in a parasitic or saprophtic state in the oral and respiratory tracts of many tuberculous individuals. Some of these contaminants may also possibly enter the sputum specimens at intervals when the patient has removed the top of the container. Since the above sputa were, for the most part, collected over a period of seven days, these molds had ample opportunity

to enter in this fashion and even, in some instances, to multiply within the specimen.

Twenty-three per cent trisodium phosphate with 0.1 per cent by weight "Tween 80" added was also used experimentally as a digestant during this period. A comparative study with the trisodium phosphate--"Triton X-100" reagent indicated, however, that a greater toxicity to the tubercle bacillus was occasioned, since a much lower number of cultures of sputa with the former were positive.

Slower and more sparse growth of positive cultures was also noted. At the same time, contamination by various microorganisms was greater, particularly on the solid experimental medium.

Experiments were conducted also in the addition of various reagents in 5 ml. quantities to sputum jars before submitting them to sanatorium patients for collection of specimens. Twenty-three per cent trisodium phosphate, trisodium phosphate--"Triton X-100", and trisodium phosphate--"Tween 80" were utilized in this fashion.

Upon receipt of the sputum specimens in the laboratory, they were immediately placed in a 37°C. incubator. Jars containing trisodium phosphate were incubated for 6 hours, trisodium phosphate--"Triton X-100" for 4, and trisodium phosphate--"Tween 80" for 8 hours.

It was found that the trisodium phosphate--"Triton X-100" digestant procedure was very successful in digesting the mucoid substance of the sputa and destroying contaminants when incubated for 4 hours. A longer incubation time, however, was responsible for several negative cultures from specimens which were positive microscopically by concentrate smear. Likewise sputa which were not brought immediately to the laboratory upon completion of the seven day collection period, also yielded similar falsely negative cultural results. A more rapid rate of growth and more luxuriant colonies were obtained from specimens of patients who customarily raised sputa of a mucoid character, but not necessarily more highly positive.

On the other hand, no advantage to this addition was observed in the case of 23 per cent trisodium phosphate, contrary to claims of Corper, Van Vranken, and others. In fact, in most instances the 6 hour digestion period proved to be inadequate, so it was subsequently deemed advisable to incubate these jars for 12 hours, and even following this interval of treatment, considerable contamination of cultures was encountered.

With the trisodium phosphate--"Tween 80" reagent this technique was completely unsuccessful. The majority of cultures were contaminated, and many were falsely negative.

All three reagents permitted the multiplication of fungus contaminants upon the culture media, despite variation of incubation periods. However, if the trisodium phosphate--"Triton X-100" jars were incubated sufficiently long to render all subsequent cultures for M. tuberculosis negative, fungi, for the most part, were destroyed.

Due to much confusion on the part of patients in regard to the liquid present in the specimen containers, and the added hazard of more frequently spilled specimens resulting from the increased volume, this method of digesting the specimen while collecting, was abandoned.

Sputa containing tubercle bacilli were heated to 56°C. for one-half hour in an effort to destroy all contaminants. It was found, however, that many strains of staphylococci and molds are resistant to this treatment. Likewise, heat treatment in conjunction with chemical digestion was found to be unreliable, as the wide variation in character of individual specimens apparently made it impossible to devise a uniform technique which would destroy extraneous microorganisms without seriously harming the tubercle bacillus.

IV Study of Fungicidal Agents

In an endeavor to incorporate into the trisodium phosphate--Triton X-100" digestion mixture an agent which

would prove non-toxic to M. tuberculosis, but would eliminate, largely, the problem of fungus contaminants, a study was made of the relative efficiency of fungicides and mycostats.

A. Materials and Methods

Cultures of nine species of fungi were obtained (from Dr. A. S, Kelner of the Westover Field Regional Station Hospital, Mass.). Following consideration of various chemical agents, "Dowicide A" (orthophenylphenol, sodium salt), "Dowicide B" (2,4,5-trichlorophenol, sodium salt) and"Dowicide F" (2,3,4,6-tetrachlorophenol, sodium salt) were added, in concentrations of 0.001, 0.002, 0.003, 0.004, 0.005, 0.006, 0.007, 0.008, 0.009, 0.01, 0.02, 0.03, 0.04, 0.05, 0.06, 0.07, 0.08, 0.09, and 0.1 per cent by weight to approximately uniform suspensions of each of the fungi in 10 ml. of physiological saline solution.

The mixtures were incubated at 37°C. for 12 hours, then centrifuged for 10 minutes at 2500 r.p.m. The supernatant fluid was decanted and the sediment washed 3 times with physiological saline, after which 2 Sabouraud dextrose agar slants (Difco) were inoculated from each centrifuge tube, 6 loopfuls being utilized as the standard inoculum.

The Sabouraud slants were incubated at 37°C. for a total period of 12 days. At the end of this time, presence or absence of typical colonial morphology was recorded.

B. Results

All three agents were found to be fungicidal in relatively low concentrations (Table 3). "Dowicide F" appeared to be the most effective against the common fungus residents of sputa with "B" next in efficiency, and "A" least.

Since these apparently efficient concentrations were sufficiently low to be satisfactorily utilized in digestion mixtures, efforts were subsequently made to develop suitable solutions in which they could be used in a study of their toxicity for the tubercle bacillus. In the meantime they were individually tested for toxicity to this microorganism.

V Determination of the Relative Toxicity of Dowicides for M. tuberculosis

A. Materials and Methods

Fourteen saline suspensions of an approximately uniform amount of tubercle bacilli obtained from the same pure culture, were treated respectively, with the percentages listed in Table 4 of "Dowicides" "A", "B", and "F". The method utilized was identical to that employed in the study of these agents against fungi as given above, excepting that 1 tube of Petragnani and 1 tube each of experimental solid and liquid media were used for culturing the sediment in each centrifuge tube. Cultures were observed

daily. All colonies which developed on the media were smeared, stained, and examined microscopically.

B. Results

It was found that "Dowicide A" permitted growth of M. tuberculosis in eleven days on Petragnani's medium in concentrations up to and including 0.05 per cent, while 0.005 per cent "Dowicide B" was sufficient to prevent colony development by this organism, as was also the case with "Dowicide F".

Table 4 records the relative degrees of inhibition of each agent, and the variation in rate of growth which resulted, both phases being influenced by the medium upon which the sediment was cultured. "Dowicide A" allowed multiplication of tubercle bacilli on the liquid medium through all percentages tested, the highest being 0.15. "Dowicide B" yielded results with the liquid medium identical with those obtained with Petragnani's. "Dowicide C" permitted growth of M. tuberculosis up to a concentration of 0.01 per cent in the case of the liquid medium.

Since 0.05 per cent "Dowicide A" failed to inhibit this organism on any of the media utilized, and this concentration had previously been demonstrated to be fungicidal for all species of molds studied, it was selected as the proper proportion of the agent suitable for use in

a digestion mixture.

Moreover, the tubercle bacilli treated with the 0.05 per cent concentration compared very favorably in growth time with those which were untreated. These times were identical for Petragnani's and the liquid medium, and differed by only 3 days with the solid experimental medium.

The 0.001 per cent concentration which permitted growth of M. tuberculosis with "Dowicides" B and F was shown by the previous study to be insufficient to inhibit the majority of the fungi tested. Therefore these compounds were not added to various digestion mixtures for further study, as was "Dowicide A".

VI Comparative Studies of Various Formulated Digestion Mixtures

A. Materials and Methods

The search for more rapid and efficient sputum digestants was continued through the preparation and testing of several solutions, some of which had previously been utilized, and others which were new mixtures.

The agents tested were: 23 per cent trisodium phosphate, 23 per cent trisodium phosphate with 3 per cent "Triton X-100", 23 per cent trisodium phosphate with 1 per cent "Triton X-100", 23 per cent trisodium phosphate with 0.1 per cent "Triton X-100", 6 per cent sodium hydroxide,

4 per cent sodium hydroxide, 5 per cent oxalic acid, 7 per cent ammonium carbonate with 10 units of penicillin-G per ml., 17 per cent sodium tripolyphosphate with 3 per cent "Triton X-100", 23 per cent sodium phrophosphate with 3 per cent "Triton X-100", 10 per cent trisodium phosphate with 1 per cent "Triton X-100", 7 per cent trisodium phosphate with 1 per cent "Triton X-100", 10 per cent trisodium phosphate with 1 per cent "Triton X-100", and 25 units of penicillin per ml., 7 per cent trisodium phosphate with 1 per cent "Triton X-100" and 25 units of penicillin per ml.

Twenty culturally negative sputum specimens were obtained and pooled. This large multiple specimen was then inoculated with a phosphate buffer solution containing dispersed tubercle bacilli. To achieve this, these organisms were removed in the colonial state from the same pure culture and gently ground with a mortar and pestle in the presence of 0.001 per cent "Triton X-100". When a watery, apparently homogeneous paste was achieved, it was transferred to 100 ml. of the phosphate buffer solution. During 2 days observation at room temperature, only a small fraction of these organisms settled to the bottom of the buffer container.

Following the addition of approximately 30 ml. of this buffer suspension to the pooled specimen, the latter was agitated vigorously for 2 minutes and then divided into 14 approximately equal portions.

Direct smears from each portion were examined microscopically and it was found that the number of tubercle bacilli in each of the seeded specimens corresponded roughly to IV on the Gaffkey scale.

Each of these was then transferred to a centrifuge tube and centrifuged at 2500 r.p.m. for ten minutes. The supernatant liquid was decanted, and an approximately equal volume of each of the reagents was added--1 reagent to 1 specific sediment.

During treatment of the sediment, the individual tubes were incubated at 37°C. Two tubes of the cultural medium were incubated at the end of the following periods: 0.16, 0.34, 0.50, 1.5, 2.5, 6.0, 12, 24, and 26 hours following addition of the respective reagents.

Medium III was utilized for cultivation. It was incubated at 37°C., and examined frequently for macroscopic evidence of growth through a period of 42 days.

B. Results

The findings for 23 per cent trisodium phosphate, tri-sodium phosphate--3 per cent "Triton X-100", 4 and 6 per cent sodium hydroxide, and 5 per cent oxalic acid were very similar to results obtained with these agents in the previous study recorded in Table 2. Comparison of the latter data with Table 5, which presents the findings

of the work under discussion, shows that the inhibitory effects for saprophytes and relative toxicity for M. tuberculosis proved to be almost identical. Since 5 per cent potassium hydroxide was not used in the present study, comparison cannot be made with the previous findings for this reagent.

Twenty-three per cent trisodium phosphate--1 per cent "Triton X-100" proved to be as effective in all respects as 23 per cent trisodium phosphate--3 per cent "Triton X-100", while 23 per cent trisodium phosphate--0.1 per cent "Triton X-100" appeared nearly as efficient. Since 24 hour digestion effected complete inhibition of saprophytes with the latter reagent, it was considered to be very promising as a digestion mixture ingredient, as it had proved to be less toxic to the tubercle bacillus, probably due to the lower content of wetting agent.

The ammonium carbonate--penicillin reagent was as effective in removing contaminants as 23 per cent trisodium phosphate with 3 and 1 per cent "Triton X-100". However it increased the incubation time of M. tuberculosis by 2 to 3 days, thus indicating a greater toxicity for this microorganism.

The sodium tripolyphosphate and sodium pyrophosphate reagents proved ineffective as digestants in this study. Since the seeded sputa utilized were considered to be

representative of the characteristics of the great majority of specimens encountered in routine cultural work, it was concluded that these two chemicals are unsatisfactory as digestion agent components. However, a further trial was planned, using routine sputa from sanatorium patients.

Ten per cent trisodium phosphate with 1 per cent "Triton X-100" and 7 per cent trisodium phosphate plus 1 per cent "Triton X-100" gave identical results in time required for colony development, and in speed of removal of saprophytes. In the latter respect, they were less effective by 2 hours, than 23 per cent trisodium phosphate with this concentration of the wetting agent. However, they proved to be less toxic to tubercle bacilli.

The 2 trisodium phosphate mixtures containing penicillin were almost as rapid in their removal of contaminants as were the 2 concentrations of sodium hydroxide. Toxicity to M. tuberculosis was at a minimum, thus indicating that these agents were promising as digestion mixture ingredients.

VII Use of Formulated Digestion Mixtures in Routine Culture of M. tuberculosis

The same digestive agents utilized in the comparative study of speed and efficiency of digestion of seeded sputa (section VI) were used to treat 100 specimens from sanatorium patients. These sputum specimens were not selected on the

basis of presence or absence of tubercle bacilli, but instead were those received in the laboratory in the course of several weeks. This afforded a better opportunity to study the effect of the agents under routine hospital laboratory conditions.

Each of the 100 specimens (7 day pools) was divided into 14 approximately equal portions. Each portion was then treated with 1 of the digestion solutions, using the individual techniques previously described for each reagent. Three tubes of medium II were then inoculated from each digested portion. These cultures were incubated at 37°C. with frequent subsequent examination for macroscopic growth. The number of days which elapsed before the appearance of colonies was recorded for each culture.

Results of this study are to be found in Table 6. Twenty-three per cent trisodium phosphate plus 1 per cent "Triton X-100", 23 per cent trisodium phosphate with 0.1 per cent "Triton X-100", 10 per cent trisodium phosphate and 7 per cent trisodium phosphate each with 1 per cent of the wetting agent, and 10 and 7 per cent trisodium phosphate with the "Triton" and penicillin again proved to be the most promising in their action. Of these, the 2 tri-sodium phosphate--penicillin solutions were most effective.

The irregularity of results encountered in this experiment, as compared with that of previous studies is

probably due to the difficulty encountered in preparing homogeneous portions of each sputum specimen. The mucoid character of some of these made it almost impossible to divide them into equal fractions of identical properties.

A large percentage of the comtaminants encountered were fungi, thus indicating that none of these agents is universally effective against these microorganisms.

VIII Preparation and Study of Digestion Mixtures Containing "Dowicide A"

Therefore, now that favorable results had been obtained with augmented trisodium phosphate solutions, an effort was made further to develop digestion mixtures which would inhibit fungi, as well as other saprophytes. "Dowicide A" was added in varying concentrations to the more successful digestion agents.

A. Study of Fungicidal Activity--Materials and Methods

Liquid mixtures of the following composition were formulated and prepared: I -- 23 per cent trisodium phosphate, 3 per cent "Triton X-100", IV -- 23 per cent trisodium phosphate, 1 per cent "Triton X-100", 0.03 per cent "Dowicide A", VII -- 10 per cent trisodium phosphate, 1 per cent "Triton X-100", 25 units/ml. of penicillin G, VIII -- 10 per cent trisodium phosphate, 1.4 per cent "Triton A-20", 25 units/ml. of penicillin G, 0.03 per cent "Dowicide A", IX -- 7 per cent trisodium phosphate,

1.4 per cent "Triton A-20", 100 units/ml. of penicillin G, 0.03 per cent "Dowicide A".

"Triton A-20" is a 25 per cent aqueous solution of an alkyl aryl polyether alcohol with non-ionic surface activity. It is at least as chemically stable under alkaline conditions as is "Triton X-100". Although not as satisfactory a wetting agent as the latter it was shown by Dubos to disperse and subsequently accelerate growth of tubercle bacilli in liquid media (26). It does not possess the toxicity of "Tween 80" for this microorganism. Thus, after a series of experiments observing its action on suspensions of M. tuberculosis, its efficiency as a digestion mixture ingredient with that of "Triton X-100", all of which were very favorable, "Triton A-20" was selected as a more satisfactory component to provide wetting activity in digestion than "Triton X-100". Since it was found that it reduces the lag phase of tubercle bacilli encountered when they are transferred from the digestion agent--sediment mixture to the culture medium, it was utilized to obtain this stimulatory effect.

Twenty high Gaffkey sputum specimens were obtained from sanatorium patients and pooled, This composite specimen was then divided into 63 approximately equal portions. A small inoculum of each of the fungi used in the study of fungicidal agents (section IV) was then added to portions of the pooled specimen--1 fungus to each group of

7 portions, thus making 9 groups of 7 sputa, each seeded with a different fungus.

Each of 5 fractions in each group was then treated with 1 of the digestion mixtures. Mixures I, IV, VII, VIII, and IX were added to the sediment of sputa obtained as the result of centrifugation at 2500 r.p.m. for 10 minutes, mixed and incubated at 37°C. for 24 hours. The treated sediments were then cultured on medium III at 40°C. Cultures were examined frequently for macroscopic growth.

In addition the 6 per cent sodium hydroxide digestion technique was performed with 1 portion of sputum from each group as a control, since it had been observed previously that molds are not particularly susceptible to its action within the limits of the time employed for treatment (30 minutes). As a further control, another portion from each group was left untreated, although it was realized that contamination from growth of sputum saprophytes would make recovery of the seeded fungi very difficult. These sediments were also cultured on medium III at 40°C.

B. Study of Fungicidal Activity--Results

From the results of the study, reported in detail in Table 7, it may be concluded that of those studied, mixtures VIII and IX were most efficient in destruction of fungi without appreciably harming M. tuberculosis, with digestion mixture IV proving nearly as effective.

Digestion mixture I proved almost completely ineffective in checking the growth of fungi, since 6 of the 9 seeded molds were recovered by culture. Mixture VII also permitted 6 of these organisms to multiply, but inhibited their growth sufficiently to allow recovery of tubercle bacilli in 7 instances, 4 of these in mixed culture with the fungus.

The sodium hydroxide reagent proved to be a poor control, since it inhibited or destroyed only 3 of the fungi. Its extreme toxicity for viable tubercle bacilli is again in evidence, as it prevented growth of 3 of the cultures.

The untreated group of sputum portions seeded with fungi were largely overgrown by saprophytic bacteria when cultured. The fungus and tubercle bacilli grew together on the same slant in only 2 instances, and these cultures were highly contaminated by bacteria.

C. Study of Digestion Mixtures Containing "Dowicide A" in Cultivation of M. tuberculosis from Sanatorium Sputa--Materials and Methods

Eighty-five specimens of sputa from sanatorium patients, as they were received in the laboratory were concentrated by centrifugation and a portion of the resulting sediments examined microscopically for the presence of tubercle bacilli. The remaining sediments were then divided into thirds, 1 portion of each treated with digestion mixture IV, the second with mixture VIII, and the third

portion with IX.

All sediments were cultured on medium III; incubation was at 40°C.

D. Results

Thirty-eight per cent of the 85 sputum specimens were found to be positive for M. tuberculosis microscopically. Forty-four per cent were positive by culture following treatment with digestion mixture VIII, 40 per cent following treatment with digestion mixture IX, but only 20 per cent of these exposed to mixture IV. Cultures treated with digestion mixture IV were contaminated by saprophytes in 14 instances, VIII treated cultures in 4 cases, and IX in only 1 instance.

From these results it was concluded that mixture IV is much more toxic to tubercle bacilli than VIII, and IX is moderately more toxic than mixture VIII. However, VIII treatment is more conducive to contamination of cultures than IX, but only slightly so in comparison with treatment by IV, which resulted in 10 more contaminated cultures than did mixture VIII.

IX Formulation of Digestion Mixtures Containing Terramycin and Determination of their Relative Efficiency

Although digestion mixture VIII and, to a slightly lesser degree, mixture IX were found to be more satis-

factory for digestion of sputa than any of the other agents tested, the slight toxicity of penicillin to the tubercle bacillus when used in conjunction with a wetting agent under alkaline conditions, made desirable further efforts toward preparing better mixtures. Therefore, a survey of literature was undertaken, attempting to find a satisfactory substitute for penicillin which could be used in digestion mixtures. At this time terryamycin hydrochloride was suggested by Dr. S. A. Yannitelli, of the A. S. Kimball Sanatorium.

Available literature revealed that this antibiotic is ineffective against the tubercle bacillus in vivo and in vitro, and that it is fairly stable under acid and aklaline conditions (38). It is, however, active against most of the Gram-positive bacteria susceptible to penicillin, as well as several Gram-negative microorganisms resistant to the action of the pioneer antibiotic. It had been noted by Hobby (38) that terramycin is excreted in high concentration in the sputum and appears to exert a marked effect on the nature and consistency of the sputum, as well as killing most of the normal resident bacteria of the mouth and naso-respiratory tract (39). In fact, it was found by this investigator that when terramycin hydrochloride was administered to far advanced tuberculous patients, their sputa were found to yield on culture, in most cases, only tubercle bacilli, other bacteria having been inhibited or killed.

Terramycin also had been found effective against bacterial species which had acquired resistance to penicillin (37). Therefore, since, in the course of routine use of digestion mixture VIII, a slightly greater number of cultures contaminated with staphylococci had been encountered, it was believed that terramycin might prove more effective than penicillin as an inhibitant of some of the more troublesome saprophytes which reside in and on the human body and thus may have recently been exposed to penicillin therapy in any of several ways.

The following digestion mixtures were subsequently formulated: X -- 0.1 per cent terramycin hydrochloride, 1.4 per cent "Triton A-20, 0.03 per cent "Dowicide A"; XI -- 0.1 per cent terramycin hydrochloride; XII -- 0.1 per cent terramycin hydrochloride, 1.4 per cent "Triton A-20", 0.03 per cent "Dowicide A", 7 per cent trisodium phosphate; XIII -- 100 units/ml. of penicillin G, 1.4 per cent "Triton A-20", 0.03 per cent "Dowicide A"; XIV -- 100 units/ml. of penicillin G; XV -- 0.1 per cent terramycin hydrochloride, 0.03 per cent "Dowicide A"; XVII -- 100 units/ml. of penicillin G, 0.03 per cent "Dowicide A".

Digestion mixtures X and XIII were designed to compare the action of penicillin and terramycin. The remaining constituents of each are identical. Mixtures XI and XIV were formulated to compare the efficiency of the 2 antibiotics when used alone in supposedly equivalent amounts.

Mixture XII duplicates the ingredients of IX with the exception of substitution of terramycin for penicillin, and XV differs from XVII also only in these two antibiotics.

Before utilizing these digestion mixtures with sputum specimens from sanatorium patients, cultures were made from several buffered saline suspensions of recently isolated tubercle bacilli, to each of which one of the mixtures had been added. The number of bacilli in these suspensions was roughly standardized by direct smear microscopic counts made after the suspension had been shaken thoroughly. Tubercle bacilli were recovered on culture from all the treated suspensions. Four other digestion mixtures, also studied in this fashion, inhibited M. tuberculosis, so they were discarded.

Digestion mixtures X, XI, XII, XIII, XIV, XV, and XVII where then used to digest sputa in the following manner: Nine microscopically negative sputum specimens were pooled, allowed to stand for 24 hours, then equal amounts were transferred to 9 containers. These were individually seeded with tubercle bacilli from 1 stock culture, concentrated by centrifugation, and each sediment treated with 1 of the given mixtures for 24 hours. Then the sediments were cultured on medium III (2 tubes inoculated/ sediment) at 40°C.

Tubercle bacilli multiplied on cultures from each of the sediments, but cultures of sediments treated with

mixtures XI, XIV, XV, and XVII were contaminated by Saprophytic bacteria and, in 1 instance, by molds.

Treatment with digestion mixtures XI and XVII resulted in macroscopically visible colonies after 10 days incubation, while digestion with XII and XIII produced colonies in 11 days. Mixtures XIV and XV treated material cultured tubercle bacilli only after a much longer period of incubation (28 and 19 days, respectively) probably due to almost complete overgrowth of the medium by contaminants.

It thus appeared that terramycin was at least as effective as penicillin as an ingredient of digestion mixtures, although neither was satisfactory when used alone, the former proving slightly better in this respect.

A. Materials and Methods

The degrees of efficiency revealed by each antibiotic in this limited study, as digestive agents and as components of mixtures, was then checked on a larger scale, using 50 sanatorium sputa. These specimens were pooled and seeded with M. tuberculosis, and then divided into 50 approximately equal portions.

Digestion mixtures IV, VIII, IX, X, XI, XII, XIII, XIV, XV, and XVII were added to the sediments of these portions--1 mixture to each of 5 portions of sediment. One tube of medium III was inoculated from each treated portion at the end of the following periods of digestion

at 37°C.: 1, 1½, 2, 2½, 5, 8, 12, 24, and 48 hours. The cultures were incubated at 40°C., and were examined frequently for evidence of macroscopic growth.

B. Results

It was found, as shown in Table 8, that digestion mixtures IX, XII, and XIII exhibited the most rapid inhibitory action for contaminants, each proving effective after only 8 hours digestion. Mixture VIII was next in efficiency in this respect, as it yielded uncontaminated cultures after 12 hours treatment of the sediments. The poorest mixtures were found to be XI, XIV, and XV, as some cultures were still contaminated which had been planted after the inoculum had undergone a 48 hour digestion period. The remaining digestants all proved effective when permitted to act for this extended time interval.

As indicated by the relative time in days required for visible colony formation on the cultures, digestion mixture VIII manifested the least toxicity for M. tuberculosis. Mixtures IX, XI, IV, and XVII were next in this respect, and X, XII, and XIII treated sediments required only 1 day more of incubation on medium III to produce macroscopic growth than did the former group. Material digested with XV required 16 days, while that exposed to XIV failed to grow tubercle bacilli after 42 days incubation. Since both of these groups of cultures were con-

taminated, the long incubation period in one instance and the failure to grow tubercle bacilli in the other does not necessarily indicate high toxicity of these mixtures to M. tuberculosis. One may conclude, however, that the mixtures are unsatisfactory as inhibitants of saprophytes.

Again it appeared that terramycin and penicillin were nearly equivalent in value as ingredients of digestion mixtures. Mixtures X and XIII both satisfactorily removed contaminants after 24 hours treatment of sediment, and growth of tubercle bacilli was obtained from these sputum sediments after 10 days incubation of their cultures. Digestion mixtures XI and XIV, in which terramycin and penicillin, respectively, were the only active ingredients present, both failed inhibit saprophytes completely. However, XI, seemed more satisfactory of the 2, since growth of tubercle bacilli took place in 9 days, while XIV yielded no growth on culture of the treated sediment, due to marked contamination of all of the tubes planted.

Mixtures IX and XII also were very similar in their action, since saprophytes were inhibited after 8 hours digestion of sputum sediments, in each instance. Sediments treated with IX (containing penicillin) subsequently cultured M. tuberculosis in 8 days. Those treated with XII required a 10 days incubation period to produce visible colonies. Thus, it appears that, in association with

trisodium phosphate, "Dowicide A", and "Triton A-20", penicillin is slightly more effective as a digestive mixture ingredient than terramycin.

This was also the case when XV and XVII were compared. Mixture XVII, containing penicillin, gave uncontaminated cultures after 48 hours digestion, while the terramycin mixture, XV, failed to do so. Growth times of tubercle bacilli exposed to the 2 cannot be compared, since the cultures from material treated by XV were contaminated.

X. Comparison of Efficiency in Routine Hospital Work of Terramycin Digestion Mixtures with those Containing Penicillin

These digestion mixtures were used to treat routine sanatorium and out-patient sputum specimens, which were divided into fractions for separate addition of the individual mixtures. However, it was impossible to divide the majority of specimens into 10 equal portions. In some instances the volume of the sputum was so small that only 1 digestion mixture could be used. Generally, however, each specimen was treated with at least 2 digestion mixtures.

A. Materials and Methods

The sputum specimens were centrifuged at 2500 r.p.m. for 10 minutes, and treated with the digestion mixtures for a period of 24 hours at 37°C. Four loopfuls of each

sediment were then used to inoculate each of 2 tubes of medium III and 2 tubes of Petragnani medium. The latter medium was utilized because it is the policy of the Kimball Sanatorium laboratory to use it to culture all routine specimens examined for tubercle bacilli. All cultures were incubated at 40°C. for a total period of 6 weeks, and were examined macroscopically frequently throughout this time.

Considerably larger numbers of sediment were digested with mixtures VIII and IX, as the majority of the specimens were received before the study reported in Section IX was completed. Other sputa were received at later dates when preliminary results indicated that these 2 mixtures were more advantageous for routine hospital utilization.

As was customary with all cultures, colonies developing on the media used were checked microscopically. The time in days required for development of macroscopically visible colonies was recorded for all sediments cultured on medium III, as was the number of tubes of medium found to be contaminated.

B. Results

Table 9 reports the time required for macroscopically visible colony formation of specimens which were positive microscopically for M. tuberculosis. The microscopic findings are divided into three classes: high and low Gaffky, and positive only by concentrate. The percentage of contamination cultures for all sputa treated with each mixture

is also recorded, as well as the total number of specimens treated. The average elapsed time before macroscopic colonies were noted is recorded for each class of the positive sputa.

Of the digestion mixtures studied, VIII, IX, X, XVII, XIII, and XII, (in the order of their efficiency) were found satisfactory for treatment of sputa sediments. The remainder of the mixtures failed to inhibit saprophytes sufficiently and also exerted a more toxic effect on the tubercle bacillus. Although 2 per cent of the cultures from VIII digestion were contaminated, while none of the mixture IX cultures were, the former mixture was considered more effective since it did not manifest the high degree of toxicity for M. tuberculosis that was displayed by the latter, as shown by the difference in growth times for this organism.

Digestion mixture X, which is the same in composition asXIII except for the substitution of penicillin for terramycin, proved more efficient than mixture XIII, XI and XIV each consisting of 1 of the 2 antibiotics as the sole ingredient were unsatisfactory. Mixture IX, identical with XII except for the use of penicillin the former and terramycin in the latter, was found to be more effective than mixture XII, especially with respect to toxicity for tubercle bacilli in sputa positive microscopically by concentration.

Table 10 records the per cent correlation between concentrate microscopic examination and culture of the sediment following treatment with one of the digestion mixtures. The following values in terms of per cent of total sputa are given: specimens with both positive concentrate and culture, specimens with negative concentrate but positive culture, those with positive concentrate but negative culture, sediments with positive concentrate but contaminated culture, those with negative concentrate and culture, and specimens with negative concentrate and Petragnani culture, but positive culture with medium III.

The last named category was included to further indicate the lower toxicity for M. tuberculosis of digestion mixture VIII, as compared with mixture IX. As the malachite green present in the Petragnani medium is slightly inhibitory for this organism, tubercle bacilli which previously have been subjected to adverse conditions during digestion will be less likely to grow on the medium. Mixture VIII treatment resulted in 4.14 per cent cultures positive only on medium III, as compared with 1.72 per cent for mixture IX.

Mixture VIII proved superior to the other digestants in all respects. A greater recovery of tubercle bacilli from microscopically negative sediments was achieved, and sediments containing tubercle bacilli treated with this solution subsequently gave positive cultures. Contamination was very low--mostly due to bacteria with only rare

recovery of fungi.

Mixture IX, as discussed previously, was more toxic to M. tuberculosis than VIII. However, its terramycin counterpart, digestion mixture XII, exhibited greater toxicity with respect to recovery of tubercle bacilli on medium III and not on Petragnani's.

Terramycin mixtures in general proved unsuccessful due to the high degree of contamination of cultures planted from material digested by these solutions. Their relative toxicity to the tubercle bacillus was greater than their penicillin counterparts, as indicated by fewer positive cultures from negative concentrates, and by failure to give any positive medium III cultures when all Petragnani cultures were negative.

Discussion

The experimental evidence indicates that 2 concentrations of sodium hydroxide (4 and 6 per cent) are relatively rapid in their digestive action, confirming reports published by several workers. However even when the period of digestion with these reagents is carefully controlled, it was found that they possessed high toxicity for M. tuberculosis, as evidenced by the slow growth of this organism following exposure to them. Even the protective effect of mucus failed to reduce their adverse effect.

Lower concentrations of sodium hydroxide were not studied, since it was found that this digestive agent precipitated the protein material of sputa sediments. It was believed that the presence of this precipitate surrounding the individual tubercle bacilli would further increase the period of adjustment of each to the unaccustomed environment of the culture medium. Twenty-three per cent trisodium phosphate proved less toxic in preliminary studies and did not require carefully timed periods of digestion. It also showed less tendency to form precipitates with sputa constituents. Therefore sodium hydroxide was used in subsequent experimental work only as a standard for comparison.

It was not utilized in conjunction with wetting agents because sodium hydroxide "Triton" mixtures were found to be less stable over several months observation than is

trisodium phosphate in various concentration. Although it was not demonstrated experimentally, it would appear that the toxicity of sodium hydroxide would be multiplied considerabley by dispersal of the naturally occurring clumps of tubercle bacilli brought about by wetting agents. Other moderately toxic chemicals have been observed by Dubos and Youmans to be markedly bacteriocidal to this organism when dissolved in surface active agents, as reviewed previously.

Sodium hydroxide was considered unsatisfactory as a digestive agent also due to the resistance of many saprophytic fungi to its action. It shared this shortcoming with all of the agents described by previous workers which were studied by the author. Attempts to use it, in several concentrations, in the presence of "Dowicides" A, B, and F were unsuccessful, since such combinations proved extremely toxic to the tubercle bacillus.

One might speculate that addition in this way of more sodium ions caused decreased ionization of the Dowicide into sodium and phenate ions by shifting the equilibrium to the left to produce a more concentrated solution of the un-ionized sodium phenate compound. This might subsequently, due to its insolubility, while passing through the colloid state, be absorbed on the lipid coating of tubercle bacilli and act directly upon these organisms, resulting in a substantial increase in toxicity. The possibility of this occurring is opposed by observation of the apparent stabil-

ity of these "Dowicides" in a 6 per cent solution of sodium hydroxide, and by the lack of such an increase in their toxicity when in trisodium phosphate solutions. The latter compound also yields sodium ions when dissolved in water.

However, it was found that degree of alkalinity had little effect on the extent of toxicity observed with the "Dowicide"--sodium hydroxide mixtures. When 6 per cent concentrations were partially neutralized by addition of 1 normal hydrochloric acid, the resultant increase in hydrogen ion concentration did not diminish it. These pH values ranged from 7.5 to 10.0. The pH of 23 per cent trisodium phosphate is approximately 10.5.

"Antiformin", which proved even more rapid than sodium hydroxide in the destruction of contaminants, was found to be much too toxic to tubercle bacilli for routine digestion of sputa prior to culture.

Potassium hydroxide in the 1 concentration utilized (5 per cent) proved approximately equivalent in efficiency to the sodium hydroxide solutions studied. Oxalic acid treatment of sputum sediments did not effectively inhibit saprophytic growth on culturing the digested sputum sediments. It was, however, the least toxic of the chemical agents studied in this group (section I).

The success of cultural examinations in the laboratory diagnosis of tuberculosis depends on the preservation of tubercle bacilli and the elimination of contaminating microorganisms. The selection of techniques and reagents will vary with local factors such as the populace from which specimens are received, general character of specimens, and the number of tubercle bacilli usually encountered. A sanatorium, whose beds are occupied by far-advanced tuberculosis patients, will more readily be able to use, in its laboratory, a harsh digestive agent which will rapidly dissolve mucus than a public health laboratory which examines specimens collected from the general public. In the latter instance the risk of secondary contamination is increased, and many types of bacteria are able to multiply before the specimen reaches the laboratory. The rate of contamination is relatively high, while the number of specimens in which tubercle bacilli can be detected is low. Thus the average health department laboratory requires a digestive agent which will prevent saprophytic contamination of cultures but will not harm the few acid-fast bacilli that may be present in specimens.

In view of the experimental results obtained, sodium hydroxide, "Antiformin", potassium hydroxide, and oxalic acid do not entirely fulfill the requirements of the average

sanatorium nor those of the public health laboratory.

Trisodium phosphate, however, proved to be a step nearer the ideal digestive agent. Treatment of sputa sediments for 26 hours with this reagent destroyed all contaminants. It was found to be relatively non-toxic to tubercle bacilli since it yielded growth of these microorganisms in 12 days incubation of cultures inoculated with the treated sediments. The trisodium phosphate method does not require neutralization of the digested material before culturing, nor does it have to be carefully timed.

However, it did not rapidly break down mucoid specimens to a homogeneous suspension, nor did it prevent growth of fungi from sputa which contained these microorganisms.

Therefore the wetting agent "Triton X-100" was added to various strength trisodium phosphate solutions to endeavor to more rapidly reduce the mucoid film surrounding and protecting undesirable bacteria. By means of timed digestion experiments, it was found that periods of action for various relative concentrations of solutions of these 2 agents could be established whereby action was fairly rapid, but the tubercle bacillus was not affected adversely.

In routine work with sputa, some disagreement in results from study to study can be noted with respect to time required to remove or inhibit saprophytes and also the time required for positive cultures to develop. The significance of this should not be exaggerated, since routine clinical

specimens may be irregular at times so far as the distribution of small numbers of tubercle bacilli is concerned. The lack of homogeneity in sputa accounts for the fact that the more tubes planted, the more positive cultures will result, a phenomenon nearly independent of the digestion agent utilized.

The inadvisability of adding trisodium phosphate--"Triton X-100" solutions to sputum containers before they are given to patients for collection of specimens was demonstrated by difficulty in standardizing digestion mixture ingredient concentrations because patients often failed to return the jars promptly after collection of the specimen. The confusion on the part of the patient in regard to this liquid, and the hazard of more frequently spilled specimens due to the increased volume, also convinced the author that this means of accelerating digestion was unreliable.

The periods of digestion studied were selected as those which would be feasible for adaption to the routine of the public health or clinical laboratory.

Fungi in specimens treated for the maximum time were not inhibited by the trisodium phosphate--"Triton" mixtures. Thus the study of the action of "Dowicides" A, B, and F was undertaken. "Dowicide A", which was found to be effective as a fungicide in suitably low concentrations and is least

toxic of the 3 to M. tuberculosis, has been recommended by the United States Department of Agriculture as a disinfectant for surfaces contaminated by bovine tubercle bacilli. The apparent contradiction is difficult to explain, except on the basis of differences in cellular chemistry between bovine and human tubercle bacilli.

"Dowicides" B and F inhibited M. tuberculosis in the concentrations in which they were fungicidal. The tubercle bacillus greatly resembles the pathogenic fungi in many of its characteristics. It also customarily produces deeply seated infections beneath the surface epithelium so that tissue must be removed or destroyed to combat it effectively. Under the right conditions it will assume filamentous forms resembling the molds. It also is fairly resistant to an adverse environment which would kill most bacteria. Colonies of virulent tubercle bacilli on the classical media are hard, dry and brittle like those of many fungi. Thus it is difficult to find fungicides which will not harm this bacterium. "Dowicides" B and F are effective against fungi in higher dilutions than "Dowicide A". It, therefore, is not surprising that the former chemicals should prove too toxic to the tubercle bacillus for use in digestion mixtures, while the latter, through some unexplained selectivity, is satisfactory for inhibition of fungi present in sputa sediments.

More complex digestion mixtures were formulated as attempts were made to achieve solution which would efficient-

ly destroy contaminants in a convenient length of time. The concentrations of trisodium phosphate and wetting agents were therefore varied, and antibiotics and "Dowicide A" were added in various concentrations to improve the selectivity of the mixtures.

The ammonium carbonate--penicillin reagent used by Goldie in cultivation of tubercle bacilli from sputa proved more toxic to this organism than trisodium phosphate--"Triton X-100". Nevertheless, the value of penicillin was demonstrated by its presence in the mixtures studied, and it was hoped that it would prove sufficiently stable for use in digestion mixtures. It was subsequently found to possess greater stability over several months time than terramycin, with which it was compared.

"Triton A-20" appeared to reduce the lag phase of the tubercle bacillus which occurs during the period of the organisms adjustment to the cultural environment, since it reduced the incubation time necessary for macroscopic colony formation. Like "Dowicide A", it had never previously been used in digestion mixtures formulated by other workers. Dubos, however, had found it an improvement over "Tween 80" as a growth stimulant in liquid media.

Terramycin hydrochloride was also an innovation as an ingredient of a sputum digestant. It appeared promising in limited preliminary trials, but subsequent use routinely on a large scale revealed its lack of stability and slight-

ly greater degree of toxicity to tubercle bacilli, when compared with penicillin. Terramycin's relative incompatibility to alkaline environments, as well as its instability probably account for the greater degree of contamination of cultures resulting from its routine utilization over a period of several months.

It should be realized that the number of specimens treated with terramycin mixtures is much smaller than that of those digested with some of the solutions containing penicillin. Thus it would doubtless be advisable to conduct more extensive routine studies with the former antibiotic, keeping the digestion mixtures refrigerated when not in use.

Digestion mixture IX proved more toxic to these organisms than VIII, thus indicating that 100 units/ml. of penicillin G is not only unnecessary but also reduces the efficiency of digestion.

Digestion mixture VIII, containing 25 units/ml. of penicillin G, 10 per cent trisodium phosphate, 1.4 per cent "Triton A-20", and 0.03 per cent "Dowicide A", was found to be the most effective of all the agents and mixtures studied. Penicillin, in the presence of a wetting agent is slightly toxic to tubercle bacilli, so this mixture does not completely solve all problems involved in obtaining M. tuberculosis in pure culture from sputa. However it does in-

hibit the great majority of saprophytes (including fungi) and the toxicity of penicillin may be partially overcome by the stimulatory effect of "Triton A-20". Therefore when used to treat sediments for 24 hours at 37°C., digestion mixture VIII will prove more efficient than any of the other digestants studied.

Summary

The relative efficiency of sodium hydroxide, potassium hydroxide, "Antiformin", oxalic acid, ammonium carbonate--penicillin, and trisodium phosphate in specific concentrations as digestants of sputa prior to culture for M. tuberculosis was studied experimentally using specimens from tuberculosis patients. Twenty-three per cent trisodium phosphate proved most effective. It was subsequently modified extensively through addition of other chemicals, and these modifications used as sputum digestants. Fungi were effectively inhibited by 0.03 per cent "Dowicide A", which did not prove toxic to tubercle bacilli in this concentration. Surface active agents were also used in the formulated digestion mixtures to permit more rapid action of the other ingredients. "Triton A-20" was found to be the most effective of these when digestion periods were standardized. Prolonged exposure to digestion mixtures containing wetting agents is toxic to tubercle bacilli. Penicillin G and terramycin hydrochloride were used as digestion mixture ingredients. Penicillin G was superior in efficiency and stability. Of the several digestion mixtures formulated and/or studied, VIII, 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 M. tuberculosis and most effective in reducing cultural contamination.

APPENDIX

Culture media utilized in experimental studies:

Medium I

Petragnani Egg Medium
(Lemon modification)

Potato (peeled and cut into small pieces)	250.0 g.
Distilled water	300.0 ml.

Mix and autoclave at 121°C. for 45 minutes. Drain, and make up potato water to 450 ml. Add:

Bacto-skim milk (Difco)	45.0 g.
Potato starch	15.0 g.
Proteose peptone No. 3 (Difco)	3.0 g.

Stir to a smooth paste and heat in a double boiler for 2 hours, with frequent stirring. Cool to 58°C. Add the following mixture:

Eggs (whole)	12
Egg yolks	3
Glycerol	35.0 ml.
Malachite green (2.0% aqueous)	30.0 ml.

Mix thoroughly and filter through sterile gauze into a sterile distributing funnel. Distribute as aseptically as possible into sterile screw-capped culture tubes. Place in a slanted position in an inspissator or Arnold and heat at 80°C. for 55 minutes the first day and 2 hours, or more, the second day.

Medium II

Experimental Liquid Medium

Proteose peptone No. 3 (Difco)	2.0 g.
Disodium phosphate	2.5 g.
Monopotassium phosphate	1.0 g.
Magnesium sulfate	0.7 g.
Ferric ammonium citrate	0.1 g.
Yeast extract (Difco)	2.0 g.
Sodium silicate (meta)	0.2 g.
Triton A-20 (10% solution)	4.0 ml.
Distilled water	987.0 ml.

Adjust pH to 7.2.

Dispense in screw-capped culture tubes in 10 ml. portions. Sterilize by autoclaving at 121°C. for 20 minutes. Then add aseptically 1 ml. of the following to each tube:

Lacto-penase (Difco)	0.1 ml.
Serum albumin (bovine fraction)	5.0 ml.
Sodium chloride	0.85 g.
Distilled water	100.0 ml.

Sterilize by filtration through Seitz or porcelain filter. Final pH of medium: 7.0-7.2.

Medium III

Experimental Solid Medium

Protease peptone No. 3 (Difco)	2.0 g.
Disodium phosphate	2.5 g.
Monopotassium phosphate	1.0 g.
Magnesium sulfate	0.7 g.
Ferric ammonium citrate	0.1 g.
Yeast extract (Difco)	2.0 g.
Sodium silicate (meta)	0.2 g.
Agar	16.0 g.
Distilled water	975.0 ml.

Adjust pH to 7.2

While at 55°C., dispense in sterile screw-capped culture tubes in 10 ml. portions. Sterilize by autoclaving at 121°C. for 20 minutes. Add aseptically 1 ml. of the following solution to each tube:

Triton A-20 (10% aqueous solution)	1.5 ml.
Bacto-penase (Difco)	0.1 ml.
Oleic acid-albumin complex	40.0 ml.
Glucose	14.0 g.
Distilled water	45.0 ml.

Sterilize by filtration through Seitz or porcelain filter. The tubes are slanted for the medium to cool.

The oleic acid-albumin complex is prepared as follows:

(1) Dissolve 0.12 ml. of oleic acid (0.1 g.) in 10 ml. of N/20 sodium hydroxide by shaking with a rotary motion in a small flask.
(2) Add 5 ml. of this solution to 95 ml. of a neutral 5 per cent solution of bovine serum fraction V (albumin) in 0.85 per cent saline.
(3) Incubate at 56°C. for 30 minutes to inactivate lipase.

TABLE I. The results of digesting 20 similar sputum specimens with various chemical agents.

DIGESTION AGENT	% OF CULTURES CONTAMINATED			% CULTURES POSITIVE FOR *M. TUBERCULOSIS*			GROWTH TIME (DAYS)* OF *M. TUBERCULOSIS*		
Medium	I	II	III	I	II	III	I	II	III
6% Sodium hydroxide	0	15	10	85	95	90	32	29	32
4% Sodium hydroxide	5	15	20	95	85	80	26	17	22
"Antiformin"	0	15	5	50	40	55	42	38	40
5% Oxalic acid	10	45	30	80	60	70	22	14	19
5% Potassium hydroxide	5	15	15	100	70	85	22	15	17

*The growth time recorded is the number of days required on each medium for macroscopic colony formation.

TABLE 2. Treatment of equal portions of a seeded sputum specimen with various digestive agents, cultures planted after the recorded digestion period.

DIGESTION AGENT	DIGESTION PERIOD (HOURS)									TIME NECESSARY FOR COLONY FORMATION (DAYS)(4)
	0.16	0.34	0.50	1.5	2.5	6.0	12	24	26	
23% Trisodium phosphate	C(1)	C	C	C	C	C	C	U-C(2)	U(3)	12
23% Trisodium phosphate + 3% "Triton X-100"	C	C	C	C	C	U-C	U	U	U	11
4% Sodium hydroxide	C	C	C	U	U	U	U	U	U	16
6% Sodium hydroxide	C	C	U-C	U	U	U	U	U	U	20
5% Oxalic acid	C	C	C	U-C	U	U	U	U	U	13
5% Potassium hydroxide	C	C	C	C	U-C	U	U	U	U	13

(1) C indicates that all media were contaminated, (2) U-C, in most instances, Petragnani and medium III uncontaminated, medium II contaminated, (3) U indicates that all cultures were uncontaminated. (4) The number of days required for macroscopic colony development in medium II following the minimum digestion period yielding uncontaminated cultures is recorded for each agent.

TABLE 3. Percentages of "Dowicides" necessary to kill selected fungi.

FUNGUS	CHEMICAL AGENT (% REQUIRED FOR KILL)		
	"Dowicide A" (orthophenylphenol, sodium salt)	"Dowicide B" (2,4,5-Trichlorophenol, sodium salt)	"Dowicide F" (2,3,4,6-Tetrachlorophenol, sodium salt)
Monilia albicans	0.01	0.008	0.008
Monilia candida	0.03	0.06	0.02
Rhizopus migricans	0.01	0.02	0.009
Microsporon furfur	0.02	0.04	0.009
Poria lutecfibrata	0.01	0.007	0.001
Trichophyton interdigitale	0.01	0.004	0.001
Trichophyton rosaceum	0.02	0.008	0.006
Aspergillus flavus	0.02	0.01	0.03
Penicillium notatum	0.03	0.05	0.03

TABLE 4. Results of treatment of tubercle bacilli at 37°C. for 12 hours with specific "Dowicides" before culture.

COMPOUND	MEDIUM AND RESPECTIVE INCUBATION PERIOD					
"Dowicide A" %	Petragnani (I)	Time (days)	Solid experimental (III)	Time (days)	Liquid experimental (II)	Time (days)
0.01	G*	12	G	7	G	3
0.05	G	11	G	9	G	3
0.1	A**	42	G	9	G	5
0.15	A	42	A	42	G	10
"Dowicide B" %						
0.001	G	21	G	16	G	11
0.005	A	42	A	42	A	42
0.01	A	42	A	42	A	42
0.05	A	42	A	42	A	42
0.1	A	42	A	42	A	42
"Dowicide F" %						
0.001	G	17	G	11	G	8
0.005	A	42	G	19	G	17
0.01	A	42	A	42	A	42
0.05	A	42	A	42	A	42
0.1	A	42	A	42	A	42
None	G	11	G	6	G	3

* ** G symbolizes macroscopic colony formation, while A represents absence of microscopically detectable growth in the given time interval.

TABLE 5. The result of treating equal portions of a specimen having a Gaffky of V with various digestive agents.

DIGESTION AGENT	DIGESTION PERIOD (HOURS)									TIME REQUIRED FOR COLONY DEVELOPMENT (DAYS)(4)
	0.16	0.34	0.50	1.5	2.5	6	12	24	26	
23% Trisodium phosphate	C(1)	C	C	C	C	C	C	U-C(2)	U(3)	12
23% Trisodium phosphate + 3% Triton X-100	C	C	C	C	C	U-C	U	U	U	11
23% Trisodium phosphate + 1% Triton X-100	C	C	C	C	C	U-C	U	U	U	11
23% Trisodium phosphate + 0.1% Triton X-100	C	C	C	C	C	C	U	U	U	12
6% Sodium hydroxide	C	C	U-C	U	U	U	U	U	U	17
4% Sodium hydroxide	C	C	C	U	U	U	U	U	U	13
5% Oxalic acid	C	C	C	U-C	U	U	U	U	U	16
7% Ammonium carbonate + 10 units penicillin per. m.	C	C	C	C	C	U-C	U	U	U	14
17% Sodium tripolyphosphate + 3% Triton X-100	C	C	C	C	C	C	C	C	C	42* (none)
23% Sodium pyrophosphate + 3% Triton X-100	C	C	C	C	C	C	C	C	C	42* (none)
10% Trisodium phosphate + 1% Triton X-100	C	C	C	C	C	C	C	C	U-C	9
7% Trisodium phosphate + 1% Triton X-100	C	C	C	C	C	C	C	C	U-C	9
10% Trisodium phosphate + 1% Triton X-100 + 25 units penicillin/ml.	C	C	C	U-C	U	U	U	U	U	11

7% Trisodium phosphate + 1% Triton X-100 + 25 units penicillin/ml.	C	C	C	U-C	U	U	U	U	U	10

(1) C represents all cultures contaminated; (2) U-C -- 1 tube contaminated and 1 uncontaminated; (3) U--both tubes uncontaminated. (4) The number of days required for development of macroscopic colonies of M. tuberculosis is recorded for the first uncontaminated tube obtained for each agent.

*overgrown

TABLE 6. The results of dividing 100 sanatorium patients' sputa into 14 equal portions and treating each with the listed digestive agents.

DIGESTION AGENTS	PER CENT CONTAMINATION	TIME REQUIRED FOR COLONY DEVELOPMENT (DAYS)		
		High Gaffky sputa	Low Gaffky sputa	Sputa positive by concentrate
23% Trisodium phosp ate	18	8	12	24
23% Trisodium phosphate + 3% Triton X-100	3	8	11	22
23% Trisodium phosphate + 1% Triton X-100	12	9	12	22
23% Trisodium phosphate + 0.1% Triton X-100	14	10	12	28
6% Sodium hydroxide	5	13	17	29
4% Sodium hydroxide	8	10	13	21
5% Oxalic acid	37	14	16	27
7% Ammonium carbonate + 10 units penicillin/ml.	31	10	14	21
17% Sodium tripolyphosphate + 3% Triton X-100	100	-	-	-
23% Sodium pyrophosphate + 3% Triton X-100	100	-	-	-
10% Trisodium phosphate + 1% Triton X-100	5	7	9	14
7% Trisodium phosphate + 1% Triton X-100	8	6	9	12
10% Trisodium phosphate + 1% Triton X-100 + 25 units penicillin/ml.	0	7	11	16

7% Trisodium phosphate +1% Triton X-100 + 25 units penicillin/ml.	6	7	10	12

Three tubes of medium II were inoculated from each digested portion. The number of days required for development of macroscopic colonies of M. tuberculosis is recorded for the earliest tube of medium for each reagent.

TABLE 7. Results of treatment of the listed fungi individually with digestion mixtures and with 6 per cent sodium hydroxide.

FUNGUS	DIGESTION MIXTURE					6PER CENT NAOH	UNTREATED
	I	IV	VII	VIII	IX		
Monilia albicans	-Δ	-Δ	≠Δ	-Δ	-Δ	-Δ	≠Δ
Monilia candida	≠C	-Δ	≠Δ	-Δ	-Δ	≠C	≠Δ
Rhizopus nigricans	≠Δ	-Δ	-Δ	-Δ	-Δ	≠Δ	-C
Microsporon furfur	-Δ	-Δ	-Δ	-Δ	-Δ	-Δ	-C
Poria luteofibrata	≠Δ	-Δ	≠C	-Δ	-Δ	≠C	-C
Trichophyton interdigitale	≠C	-C	-Δ	-Δ	-Δ	≠Δ	-C
Trichophyton rosaceum	≠Δ	-Δ	≠Δ	-Δ	-Δ	≠Δ	-C
Aspergillus flavus	-Δ	-Δ	≠Δ	-Δ	-Δ	-Δ	-C
Penicillin notatum	≠C	-Δ	≠C	-Δ	-Δ	≠C	-C

Treated sediments cultured on medium III. Recovery of fungus: ≠, non-recovery of fungus: -, recovery of M. tuberculosis: Δ, non recovery of M. tuberculosis: C.

TABLE 8. The results of treating equal portions of a similar positive sputum with the given digestion mixtures.

DIGESTION MIXTURE	DIGESTION PERIOD (DAYS)									TIME REQUIRED FOR COLONY DEVELOPMENT (DAYS)
	1	1.5	2	2.5	5	8	12	24	48	
IV	C	C	C	C	C	C	U-C	U-C	U	9
VIII	C	C	C	C	C	C	U	U	U	7
IX	C	C	C	C	C	U	U	U	U	8
X	C	C	C	C	C	C	C	U	U	10
XI	C	C	C	C	C	C	U-C	U-C	U-C	9
XII	C	C	C	C	U-C	U	U	U	U	10
XIII	C	C	C	C	C	U	U-C	U	U	10
XIV	C	C	C	C	C	C	C	C	C	42* (none).
XV	C	C	C	C	C	C	C	C	U-C	16** (none)
XVII	C	C	C	C	C	C	C	C	U	9

C represents all cultures contaminated. U-C at least one tube contaminated. U all cultures contaminated. The number of days required for development of macroscopic colonies of M. tuberculosis is recorded for the first uncontaminated culture for each mixture.

* overgrown
** contaminated

TABLE 9. The time required for macroscopically visible colony formation on medium III following treatment of microscopically positive sputa sediment by the listed digestion mixtures.

DIGESTION MIXTURE	TOTAL SPECIMENS	PER CENT CONTAMINATION	TIME IN DAYS REQUIRED FOR DEVELOPMENT OF MACROSCOPIC COLONIES OF *M. TUBERCULOSIS*.		
			High Gaffky sputa	Low Gaffky sputa	Sputa positive by concentrate
IV	18	41	9	13	22
VIII	421	2	6	10	12
IX	669	0	8	11	15
X	31	3	10	13	24
XI	57	62	9	12	15
XII	84	6	10	14	22
XIII	31	6	10	13	22
XIV	57	84	none in 42*	none in 42*	31**
XV	27	46	16**	16	28**
XVII	27	69	9	13	20

* overgrown
** contaminated

TABLE 10. Comparison of cultural findings with microscopic examination of sputum specimens.

MIXTURE	TOTAL SPECIMENS	% Positive Conc. and Cult.	% Negative Conc. but Positive Cult.	% Positive Conc. but Negative Cult.	% Positive Conc. but Cult. Overgrown	% Negative Conc. and Cult.	% Neg. Conc. Pos. Experimental Medium, Neg. Petragnani
VIII	421	20.4	14.0	0	1.43	60.03	4.14
IX	669	11.2	3.29	3.44	0.93	79.42	1.72
X	31	9.72	0	0	10.3	79.98	0
XI	57	31.6	3.51	14.0	21.1	70.21	0
XII	84	38.2	3.58	8.34	2.38	47.50	0.67
XXIII	31	9.68	0	0	9.67	80.65	0
XIV	57	7.02	3.51	0	45.6	43.87	0
XV	27	14.8	0	0	22.2	63.00	0
XVII	27	7.42	0	0	29.6	62.98	0

Results are expressed in per cent of the total number of specimens digested with each mixture.

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