CONCENTRATION OF ACID-FAST BACILLI WITH WATER-INSOLUBLE LIQUIDS

by

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I. INTRODUCTION

To workers engaged in the laboratory study of tuberculosis, sensitive methods for detection, isolation and identification of the causative organism are of great importance.

The characteristics of the genus Mycobacterium are such that techniques for the isolation and identification of the most important member of the genus differ markedly from those used with other microorganisms. The laboratory procedures most widely used are: (a) microscopic studies of direct and concentrated smears, (b) culture, and (c) animal inoculation. Serological methods of diagnosis are not wholly satisfactory.

Although direct smears are usually made of sputum specimens, because of the difficulty of finding the bacilli, microscopic examinations of other pathologic material are seldom made. The major limitation of the microscopic examination is that it does not distinguish between tubercle bacilli and saprophytic and other non-pathogenic acid-fast bacilli.

The concentration of small numbers of tubercle bacilli from pathologic material into a final compact volume presents the problems of homogenizing the specimen, destroying contaminating organisms and collecting the bacilli. Homogenization

of specimens may be accomplished by a variety of chemicals. Desirable ones dissolve the undesired tissues and cellular debris, and at the same time, exert a bactericidal action upon contaminating microorganisms without affecting the staining properties or killing the Mycobacteria. Following homogenization, the material is concentrated either by sedimentation or by flotation.

A number of hydrocarbons have been used with excellent reports concerning their ability to concentrate tubercle bacilli. However, the toxic effect has limited these procedures to bacterioscopy.

The observation has been made that acid-fast bacilli which reach the interface of a water-oil emulsion quickly go into the oil phase. When non-acid-fast bacteria reach the interface of a water-oil emulsion, they remain at the interface or return to the water but never pass into the oil.

The possibility existed that flotation with a volatile oil might improve the concentration of the tubercle bacilli. When the volatile oil containing the bacteria is separated from the pathological material, it can be evaporated, leaving only the tubercle bacilli. This flotation method may eliminate the toxic effect common to all hydrocarbons which have been tried.

It was hoped that this study would yield a more efficient, cheaper, and simpler technique than those being

used at present. This would be of advantage to small institutions doing a few specimens daily and to large hospitals and health departments where many examinations are done daily.

II. REVIEW OF THE LITERATURE

The acid-fast characteristic of Mycobacterium tuberculosis and its appearance in stained smears in small groups or bundles have been utilized in microscopic examination. Although this is the least sensitive of the methods available, it holds an important place in the diagnosis of sputum because of its simplicity and speed. Cummings (1950) reported that acid-fast bacilli found in sputum samples are usually M. tuberculosis.

It has been shown that microscopic techniques are not sensitive enough to be reliable when small numbers of bacilli are present in the sample. Corper (1928) estimated that 100,000 tubercle bacilli per milliliter were necessary for a reasonable chance of finding the bacilli. Halloway and Cummings (1949) found the percent positives was too low for the amount of work involved in direct examination of gastric specimens. Although Mishulow, Melman and Romano (1934) and Spendlove, Cummings and Patnode (1949a) reported direct smears of choice particles may be as good as the concentration method in finding acid-fast bacilli, it is not always possible to examine fresh specimens. Pathological materials sent to state health departments may have been in transit three or four days by which time the choice particles have disappeared. Therefore, concentration is necessary for

microscopic examinations and also for culture and animal inoculations.

The first step in the preparation of the specimen for concentration is the homogenization and digestion with any of a variety of chemicals. The chemicals used must digest the organic matter and also destroy the contaminating microorganisms. Two different approaches have been used. One utilizes chemical substances which are very efficient in concentrating the bacilli but are so toxic to the tubercle bacilli that the specimen is good for microscopic examination only. In the other, chemicals which are not too toxic to the bacilli are used, thus making culture and animal inoculations possible.

The first attempts to concentrate specimens were by Biedert in 1896 and Mulhauser and Czaplewski in 1891 using 0.2% sodium hydroxide as the digestant. Further attempts at concentration were reported by Ellerman and Erlandsen (1908) using sodium carbonate and by Uhlenhuth and Xylander (1909)) using antiformin. Both these solutions were toxic to the tubercle bacilli and were restricted to microscopic examination. Antiformin, a product containing sodium hypochlorite, was used extensively until 1915 when Petroff (1915) reintroduced sodium hydroxide. This method became very popular because the concentrated specimen could be stained for microscopy, cultured or inoculated into animals. Corper and

Uyei (1927) reported the sodium hydroxide method to be decidedly superior and in general use as the most satisfactory method of obtaining primary cultures from contaminated sources.

Research was continued in two directions. Some sought compounds which would digest the specimen with less effort and at the same time would concentrate without altering the staining properties of the bacilli. This method would make use of microscopic examination. Petroff and Schain (1939) introduced the use of tergitol 08 in conjunction with sodium hydroxide. Petroff and Schain (1940) reported better success with javelle water (a hypochlorite solution) plus tergitol 08. The use of Chlorox, a commercial preparation of sodium hypochlorite, was introduced by Oliver and Ruesser (1942). Hypochlorites produced efficient concentration but were lethal to the bacilli. Reports have indicated the superiority of the concentrating ability of these hypochlorites (Cameron and Castles, 1945a, 1945b; Schain, Magdalin and Russo, 1942; Nagle, Lazarov and Willett, 1944; Tarshis and Lewis, 1949; Corper and Nelson, 1949).

Other researchers continued to seek compounds which would digest with less effort and still leave the bacilli viable for culture and animal inoculations. Corper and Uyei (1927, 1930) reported the use of mineral acids and oxalic acid. Hanks, Clark and Feldman (1938) introduced the

flocculation method using alum in conjunction with sodium hydroxide for sputum. In 1940, Hanks and Feldman (1940) introduced phosphate flocculation for urine specimens. Corper and Stoner (1946) reported the advantages of using 10% trisodium phosphate as a digestant. Although Beattie (1949) reported 10% trisodium phosphate was more lethal to the tubercle bacilli than some of the other methods, the reports by Van Vranken (1947), Mitchell, Jefferies and Stucker (1948), Mullahy (1950), Gifford, McKinley and Hunter (1951) have mentioned its superiority and advantages. Spendlove, Cummings and Patnode (1949b) and Peizer, Chaves and Widelock (1954) reported equal efficiency for sodium hydroxide and trisodium phosphate as digestants.

Hand shaking of the specimen is inadequate with all of the digestants mentioned (Smith, 1951). For thorough homogenization, a shaking machine as suggested by Steenken and Smith (1942) was advised.

According to Hawirko and Murray (1954), Couratte-Arnaude introduced the flotation technique in 1903 using ether. Ligroin was introduced by Lange and Nitsche (1909). In 1916, Krauss and Fleming (1916) introduced gasoline. Chloroform was reported in 1924 by Andrus and MacMahon (1924). Because chloroform is heavier than water, it is found on the bottom of the liquid but the idea is essentially the same. J. E. Pottenger reported the use of xylol (1931).

The last new hydrocarbon was reported in 1943 by Kelso and Galbraith (1943) who used toluene.

Pottenger (1948), Smith (1938a, 1938b) and Nagy (1939) claimed the hydrocarbon flotation technique to be a very efficient method. Using this method, they found a 60-80 fold advantage over the direct smears. However, the use of hydrocarbons lethal to the tubercle bacilli limited the examination to microscopy.

Hawirko and Murray (1954), using oils as flotation agents, reported that oil partition provides a reliable method of collection based on the hydrophobic and lipophilic properties of mycobacteria. This is true even when centrifugation fails to collect small numbers of bacilli.

Another flotation method was reported by Pelson (1930). This method made use of sodium chloride in increase the specific gravity of the specimen so that the bacilli would float on top. This method has not been of any particular value.

Many other concentration substances have been described and used. Some of them are: aluminum hydroxide cream (Saelhof, 1924), sodium carbonate-phenol (Greenfield and Anderson, 1919), sodium carbonate-phenol-autoclave (Raphael and Eldrige, 1920), alcohol precipitated protein as a sedimenting agent (Steenken, 1940), and calcium oxalate precipitation (Rappaport and Rosenknopf, 1948).

The use of enzymes have been reported by various workers. These include papain (Sullivan and Sears, 1939), pepsin (Gerundo, 1942) and trypsin (Haynes, 1942).

Various workers have found that ordinary centrifugal speeds do not completely sediment the tubercle bacilli. Hanks, Clark and Feldman (1938) used an alum flocculation technique and found bacilli in the supernatant fluid which is usually discarded. They reported there were instances where more bacilli were found in the supernatant fluid than in the sediment. Again in 1939, Hanks and Feldman (1939) reported tubercle bacilli cannot be efficiently concentrated by direct centrifugation. Hata, Venters and Cummings (1950) found sputum cultures made without centrifugation were about as efficient as those prepared after centrifugation. Robinson and Stovall (1941) compared various methods of sedimenting tubercle bacilli in sputum and found that only chloroform aided sedimentation to any degree. Klein, Maltz, Cummings and Fish (1952) concluded from their experiments that centrifugation of digested specimens at 3000 rpm for 15 minutes is not a very efficient method for concentrating tubercle bacilli.

The above reports may possibly be due to the specific gravity of the tubercle bacillus. Silverstope (1948) determined the specific gravity of tubercle bacilli and found them to range from 1.07 to 0.79, with a mean value just

below 1.00. The specific gravity of 4% sodium hydroxide is 1.04 and that of 10% trisodium phosphate is more than 1.06. The specific gravity of the tubercle bacilli must be greater than that of the material in which they are suspended if they are to be precipitated by centrifugation.

Various authors have claimed special merits and efficiency for their own modifications of the concentration technique. Sodium hydroxide and trisodium phosphate appear to be the most popular materials today because contamination is lessened, digestion of the specimen is good, and the concentrated material may be stained, cultured or inoculated into animals.

The ideal concentration method would then involve a substance which has good digestion and decontamination powers, can concentrate the material as the hydrocarbon flotation method claimed to do, and leaves a residue for culture and animal inoculation without loss of viability of the organisms.

III. MATERIALS AND METHODS

A. The Organisms.

The following stock strains of Mycobacteria were used as test organisms: *M. phlei*, *M. smegmatis*, Birkhaug strain of BCG, and an attenuated human strain, H37Ra. All cultures were obtained from the Communicable Disease Center, Atlanta, Georgia. Subcultures were made monthly onto Lowenstein-Jensens media. When bacilli were used from broth cultures, TB Broth Base (Bacto) with Bacto Dubos Medium Albumin was used.

B. Culture Medium.

The culture medium used in the experiments was the Jensen modification of Lowenstein's medium. It contained the following:

		1 flask	
A.	salt solution		
	monopotassium phosphate	2.4	gm.
	magnesium sulfate	0.24	gm.
	magnesium citrate	0.6	gm.
	asparagine	3.6	gm.
	glycerol	12.0	gm.
	distilled water	600.0	ml.
B.	potato flour	30.0	gm.
C.	homogenized whole eggs	1000.0	ml.
D.	malachite green, 2% aqueous	20.0	ml.

30 grams of potato flour was added to one flask of salt solution and the mixture was autoclaved at 121° C. for 30 minutes.

Two dozen large, grade AA eggs were cleaned by scrubing in a 5% soap and soda solution. They were then left in the soap and soda solution for 30 minutes after which they were rinsed with cold water until the water was clear. The eggs were broken into a sterile flask, beaten with a sterile egg beater and filtered through three layers of sterile gauze. The homogenized eggs were added to one flask of the potato-flour-salt solution mixture which had previously been cooled to room temperature. To this was added 20 ml. of a 2% aqueous solution of malachite green. After thorough mixing, the medium was left standing for one hour at room temperature.

The medium was tubed into sterile one ounce prescription bottles, 7 to 8 ml. per bottle. The medium was solidified by inspissation.

C. Solvent.

The normal hexane used extensively in the experiments was commercial grade n-hexane manufactured by Phillips Petroleum Company.

D. Toxicity of Solutions.

To 2 cc. of the solution to be tested, a small loop of bacilli grown on Lowenstein-Jensen media was added. The bacilli-clumps were broken as much as possible with a wire loop and then with a sterile glass rod. After the desired contact period with the solution to be tested, a sample was

taken and inoculated onto media to serve as a control. Two cc. of n-hexane was added to the bacilli-water mixture and this was shaken for an additional 10 minutes. The content was poured into a sterile separatory funnel. Within 5 minutes 2 distinct layers formed as the n-hexane separated from the water. The solution was separated into three portions: (1) water, (2) n-hexane and water at the miniscus, and (3) n-hexane. The n-hexane was pipetted from the top of the separatory funnel to prevent any washing action which might pick up bacilli sticking to the glass surface. A capillary pipetteful (approximately 0.5 cc.) of each solution was seeded onto media. After washing the media with the solution three or four times, the excess n-hexane was withdrawn and discarded.

F. Determination of Optimal Shaking Speed.

A small loop of bacilli was mixed with 10 cc. of sterile water on a mechanical shaker for 10 minutes using an International centrifuge equipped with a shaker head. One-half cc. of this suspension was pipetted into four bottles (a, b, c, d) containing 10 cc. of sterile water. The four bottles were shaken for 5 minutes, after which a capillary pipetteful was seeded onto media to serve as controls. Bottle (a) was shaken vigorously by hand for one minute, rested two minutes, shaken 2 minutes, rested 2 minutes and

then shaken for an additional one minute. Bottle (b) was shaken at 9 rpm for 10 minutes on the converted centrifuge. At this speed the two layers did not mix well. Bottle (c) was shaken at 12 rpm for 10 minutes. Agitation and mixing of the 2 layers were good. Bottle (d) was shaken at 15 rpm for 10 minutes. This involved very vigorous shaking.

After shaking, the solutions were poured into separatory funnels. After 5 minutes, the two portions separated. Many times, the n-hexane layer contained two portions: the upper layer which was clear and the lower layer which was milky and bubbly. Therefore, the n-hexane layers were checked for bacilli in both the upper and lower layers. The different layers of n-hexane was noticed only during this experiment.

G. Determination of Optimal Shaking Time.

A small loop of bacilli was introduced into a screw-capped bottle containing 10 cc. of sterile water. This was shaken vigorously for 10 minutes on the mechanical shaker. One-half cc. of this mixture was pipetted into three bottles containing 10 cc. of sterile water. This was then shaken for 5 minutes. A sample was taken and seeded onto media to serve as a control. Two cc. of n-hexane was added to each bottle and the bottles were shaken by hand for the desired times. A capillary pipetteful of the material was then seeded onto media.

H. Value of Multiple Extractions.

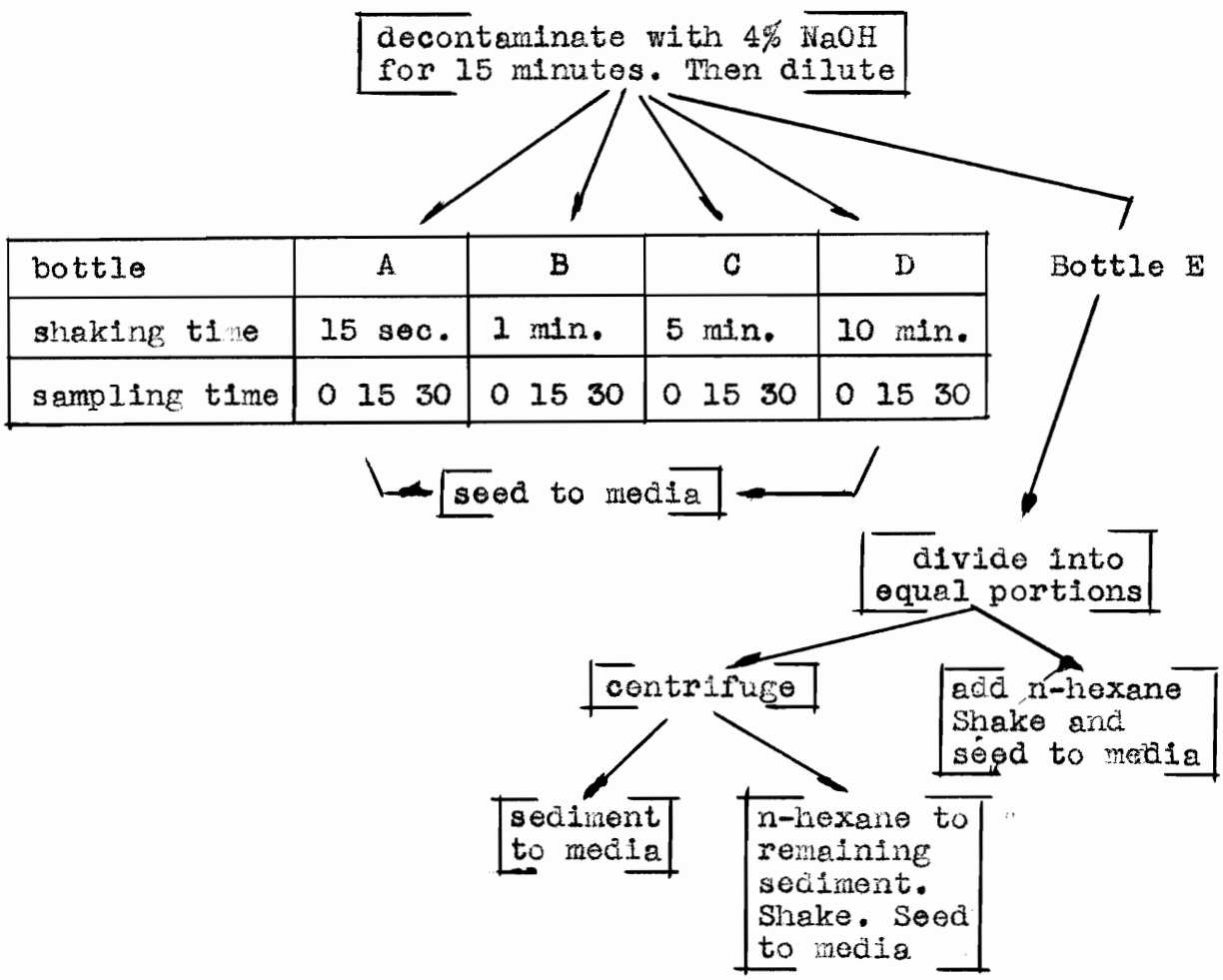
A small loop of bacilli was added to 10 cc. of sterile water in a screw-capped bottle. Two cc. of n-hexane was added. The suspension was then shaken for two minutes. The n-hexane was seeded onto media. Another 2.0 cc. of n-hexane was added and shaken as before. This procedure was repeated three times. Following each shaking, the n-hexane was seeded onto media. The excess n-hexane was withdrawn and discarded.

I. Comparison of Sampling Time and Shaking Time From Suspected Tuberculous Materials.

To the specimen, an equal volume of 4% sodium hydroxide was added. The sample was homogenized on the mechanical shaker for 10 minutes. It was allowed to remain at room temperature for an additional 10 minutes. The sample was centrifuged at 3000 rpm for 15 minutes. The supernatant fluid was discarded. Four per cent hydrochloric acid containing phenol red was added drop by drop to the sediment until it was slightly acid (yellow). It was back titrated with 4% sodium hydroxide until the sediment just turned to a persistent pink color. The sediment was then inoculated onto culture media. Ten cc. of sterile water and 1.0 cc. of n-hexane were then added to the sediment and the mixture was shaken for the desired time. The n-hexane layer was seeded to media after the separation of the two layers. Any excess n-hexane was withdrawn and discarded.

J. Comparison of Sampling Time and Shaking Time From Tuberculous Materials.

These specimens were received from patients previously found to harbor the tubercle bacillus. See the flow sheet below as to the method.



IV. RESULTS

A. Preliminary Investigations With *M. phlei*.

The initial experiments were conducted with *M. phlei* because of its fast rate of growth and its bright orange pigment. The first experiment was conducted to determine whether or not a substance could be found which could concentrate the bacilli by the flotation method and still not kill them.

The first series of chemical reagents used was: acetonitrile, n-hexane, amyl alcohol, butyl alcohol, amyl acetate, benzene and anisol. They all had in common the property of immiscibility with water. Physiological saline was used for the control. After 10 days of growth, all tubes were negative except n-hexane and the saline control. Good growth appeared with both solutions. This indicated that n-hexane might be the concentrating agent desired.

Comparison of the toxicity of n-hexane with 4% sodium hydroxide, 10% trisodium phosphate and 5% sulphuric acid was determined. After a contact period of five, fifteen and thirty minutes, a capillary pipetteful (approximately 0.5 cc.) of the test solution was seeded onto media. Growth was very heavy after 10 days following exposure to 10% trisodium phosphate, n-hexane and the saline control. The above solutions did not need neutralization. Four percent sodium

hydroxide and 5% sulphuric acid were negative in all three exposure periods. However, since these solutions had to be neutralized, a second experiment was conducted so that the final volume of all the solutions would be the same. The results from this experiment showed essentially the same thing. Ten percent trisodium phosphate, n-hexane and the saline control had heavy growth whereas only 2 colonies appeared with 5% sulphuric acid and 8 colonies appeared with 4% sodium hydroxide.

The classification of growth used throughout the experiments are as follows:

////+	growth throughout the media
///+	3/4 of media with growth
//+	1/2 of media with growth
/+	1/4 of media with growth
0-40	actual count of the colonies.

Among hydrocarbons which have been used in previous flotation techniques are chloroform, xylene, benzene and ether. The toxic effect of these hydrocarbons upon M. phlei was determined. Ten percent trisodium phosphate was used as the control. The growth was observed for seven days. The results are summarized in Table 1. Although the control showed very heavy growth, there occurred only sparse growth in ether and benzene. After one hour contact, all hydrocarbons were found to be toxic except n-hexane which showed

TABLE 1. TOXICITY OF VARIOUS HYDROCARBONS UPON M. PHLEI. 7 DAYS GROWTH

exposure time	chloro-form	xylene	benzene	ether	n-hexane	10% Na_3PO_4
15 min.	0	0	1 col.	2 col.	++++	++++
30 min.	0	1 col.	0	2 col.	+++	
1 hour	0	0	0	0	+++	
2 hour	0	0	0	0	+++	++++
20 hours	0	0	0	0	++	1 col.

0 = no growth

better growth in 24 hours than the 10% trisodium phosphate control.

Following the information that n-hexane was only mildly toxic to M. phlei, it was necessary to determine whether n-hexane would be a good concentrating agent. A suspension of M. phlei was made from bacilli growing on Lowenstein-Jensen media. Dilutions were made ranging from 10^{0} to 10^{-7}. The results indicated that concentration could be achieved. This fact became more obvious as the dilutions were increased. The results are summarized in Table 2.

Because of its concentrating ability and mild toxicity with M. phlei, it was decided to use n-hexane for further studies with M. smegmatis, BCG and H37Ra.

B. Toxicity of the Solutions.

M. smegmatis was mildly affected by the exposure to n-hexane and 10% trisodium phosphate but the amount of recoverable bacilli was decreased after exposure to 4% sodium hydroxide for a period of 20 minutes (Table 3).

Table 4 shows that H37Ra and Table 5 shows that BCG were mildly affected by the action of n-hexane.

In comparing n-hexane with the other hydrocarbons, it was found that chloroform, xylene, benzene and ether were very toxic to M. smegmatis, BCG and H37Ra. No growth was

TABLE 2. CONCENTRATION ABILITY OF n-HEXANE ON VARIOUS DILUTIONS OF M. PHLEI. 7 DAYS GROWTH

Dilutions	n-Hexane	Water	Control
10^{0}	++++	++	++++
10^{-2}	++++	++	+++
10^{-4} *	++	+	++
10^{-5}	++++	6 col.	++
10^{-6}	++++	2 col.	++
10^{-7}	++	3 col.	14 col.

* Leaked in shaker

TABLE 3. TOXICITY OF n-HEXANE, 10% Na_3PO_4 AND 4% NaOH TO M. SMEGMATIS. 7 DAYS GROWTH.

	exposure time				
	15 min.	30 min.	1 hour	2 hour	20 hour
n-hexane	+++	+++	++	+	+
10% Na_3PO_4	++++	++++	++++		
4% NaOH	1 colony after 20 minutes exposure				

TABLE 4. TOXICITY OF n-HEXANE, 10% Na_3PO_4 AND 4% NaOH TO H37Ra. 28 DAYS GROWTH.

	exposure time				
	15 min.	30 min.	1 hour	2 hour	20 hour
n-hexane	++	+++	+++	+	+
10% Na_3PO_4	+	6 col.	18 col.		
4% NaOH	+ after 20 minutes exposure				

TABLE 5. TOXICITY OF n-HEXANE, 10% Na_3PO_4 AND 4% NaOH TO BCG. 28 DAYS GROWTH.

	exposure time				
	15 min.	30 min.	1 hour	2 hour	20 hour
n-hexane	+++	++	+	+	+
10% Na_3PO_4	+	6 col.	18 col.		
4% NaOH	++ growth after 20 minutes exposure				

found even after 5 minutes exposure to the hydrocarbons. The results showed that the hydrocarbons were more toxic to the other mycobacteria than to M. phlei.

C. Sampling Level.

Experiments were carried out to determine where the bacilli might be found, i.e. the lower layer, middle layer, or throughout the n-hexane. This determination was made with a sample having very many bacilli and another sample which had only a few bacilli. It was necessary to know if there would be any difference when the amount of bacilli present was different. As was expected, the experimental results showed bacilli to be most readily found at the juncture of the water and n-hexane. It was expected because it was already known that bacilli would go to the n-hexane and because the specific gravity of the tubercle bacilli is near 1.0 (Silverstope, 1948) and the specific gravity of n-hexane is .687.

D. Determination of Optimal Shaking Speed.

During the experiments with M. smegmatis, BCG and H37Ra, there occurred a consistent decline in the number of recoverable bacilli. The shaking time and shaking speed were thought to influence the reaction so these factors were evaluated. M. phlei was used as the experimental organism

because of its fast rate of growth.

In determining optimal shaking speeds, it was decided to check the reaction obtained with shaking of the specimen by hand and using three different speeds on the mechanical shaker (converted centrifuge). The actual speeds were not determined. From visual observations and the speeds which the centrifuge was turned on, it was shown that the following occurred:

hand shaking	moderate shaking and mixing.
9 rpm (900)	no agitation, very little mixing of n-hexane and water.
12 rpm (1200)	good agitation with thorough mixing.
15 rpm (1500)	vigorous agitation with thorough mixing.

In this experiment, it was noticed that n-hexane had two distinct layers, a clear portion at the top and a bubbly, milky lower layer. It was decided to check both the upper and lower layers for bacilli.

The results indicated vigorous hand shaking for one minute was as good as shaking on a mechanical shaker with moderate and vigorous shaking. The material was seeded immediately after shaking. The experiment revealed that the greatest concentration of bacilli were found at the lowest portion of n-hexane. The results are similar to that found in Experiment C.

When the samples were allowed to sit for 10 minutes after shaking, there was a more marked difference between the n-hexane and water. This indicated that allowing the solution to sit for 10 minutes after shaking increased the concentration potential of n-hexane.

In one experiment to determine optimal shaking speed, the inoculum was so diluted that all tubes were negative except the n-hexane tube which showed a few colonies.

In determining optimal shaking speed with M. smegmatis, the sample was divided into three portions (a, b, c) and shaken as follows:

bottle a hand shaken for one minute.

bottle b 12 rpm, 5 minutes on the shaker.

bottle c 15 rpm, 5 minutes on the shaker.

No determinations were done with 9 rpm because previous experiments has shown actual mixing of the fluids was necessary to transfer the bacilli from the water phase to n-hexane. Actual visual speed showed very little agitation or mixing at 9 rpm.

E. Determination of Optimal Shaking Time.

When vigorous shaking by the mechanical shaker was tried, there was leakage of the material quite regularly. This occurred even though the lids were screwed on as tightly as possible. As the concentration of bacilli were found to

be equally effective with shaking by hand, the optimal shaking times were determined by the hand shaking method. The results from the experiments with M. phlei showed no apparent difference in concentration of the bacilli by shaking by hand for one or two minutes.

F. Value of Multiple Extractions.

The results with M. phlei and M. smegmatis showed heavy concentration with each of three extractions. Although the first extraction produced as much or more than the subsequent extractions, the other extractions still showed ++++ growth when the controls showed only + growth. This would indicate the possibility of n-hexane having a limited reacting surface. (See Table 6a).

Samples of H37Ra and BCG showed again that the initial extractions give the most concentration. However, as the number of bacilli is diminished, the concentrating potential appears to diminish also. (Table 6b).

G. Hand Shaking of Suspected Tuberculous Specimens.

Following the routine treatment of suspected tuberculous specimens, they were shaken by hand for one minute, rested 2 minutes, shaken one minute, rested 2 minutes and then shaken for an additional minute. A total of 42 specimens were studied. Of these specimens, there were 2 gastric, 2 urine and 39 sputum. Thirteen were positive for

TABLE 6a. MULTIPLE EXTRACTIONS WITH M. PHLEI AND M. SMEGMATIS. 7 DAYS GROWTH.

extraction	M. phlei	M. smegmatis
1	++++	++++
2	++++	++++
3	++++	++++

TABLE 6b. MULTIPLE EXTRACTION WITH BCG AND H37Ra. 28 DAYS GROWTH.

extraction	sample	BCG	H37Ra
1	n-hexane water	++++ ++	++++ +
2	n-hexane water	++++ ++	+++ +
3	n-hexane water	+++ ++	++ +
4	n-hexane water	++ 20 col.	contam. +

tubercle bacilli with the n-hexane treatment and only 11 were positive after 4% sodium hydroxide treatment. The results are tabulated in Table 7.

Of the 42 specimens examined, only 2 did not agree. The n-hexane treated specimens in both cases had 2 colonies whereas the 4% sodium hydroxide method showed none. Both specimens positive by the n-hexane treatment were from tuberculosis patients known to be harboring the bacilli.

Hand shaking with n-hexane produced eugonic colonies (5-7 mm.) when only a few colonies were found. When only a few colonies were found after 4% sodium hydroxide treatment, the colony size remained the usual size (1-2 mm.).

H. Use of the Mechanical Shaker on Suspected Tuberculosis Specimens.

The use of hand shaking gave encouraging results. However, it became tiresome to shake the specimens. Up to this time, there appeared leakage of the specimen when it was shaken so that thorough mixing of the sputum concentrate with the n-hexane occurred.

It was decided to use rubber liners in place of the plasticized paper liners previously used. Preliminary work with rubber liners using M. phlei gave no evidence of leakage at the highest mixing speeds previously used.

The samples for the first series of determinations were shaken for 10 minutes on the mechanical shaker set at

TABLE 7. HAND SHAKING OF SUSPECTED TUBERCULOUS MATERIAL. (ONLY POSITIVE SPECIMENS TABULATED).

laboratory number	n-hexane	4% NaOH routine
463 (g)	1 lg col.	1 sm. col.
469	++++	++++
470	35 col.	40 col.
472	10 col.	6 col.
475 (g)	3 lg col.	4 sm. col.
523	++++	++++
555	++++	++++
556	++++	++++
557	++++	++++
565	2 lg. col.	neg.
568	2 lg. col.	neg.
570	++++	++++
571	++++	++++

(g) gastric (sm) small (lg) large

15 rpm. For this study, 139 specimens were used. Only 12 were found to be positive for tubercle bacilli with the 4% sodium hydroxide method. Only 8 were positive with the n-hexane treatment. There were no eugonic colonies. Of the 139 specimens, there were 6 gastric, 1 urine, 1 spinal fluid and 131 sputum. The results are summarized in Table 8.

The results using n-hexane on the basis of this experiment did not appear fruitful. In almost every case, the number of bacilli found were small with the n-hexane method. The loss of bacilli may possibly be attributed to the fact that the increased time and speed of agitation may have caused the quicker death of the bacilli. Previous toxicity tests with n-hexane were determined without agitation. The toxic effect of n-hexane may have been increased by shaking.

Of 44 specimens shaken this time for 5 minutes, 9 were positive with n-hexane, 11 were positive with 4% sodium hydroxide. As can be seen in Table 9, the results indicated that the method was improved by a few variations. Most noticeable was the fact that the n-hexane method was producing heavier growth. The main discrepancies occurred with numbers 621 and 623. Notes jotted down at the time of the experiment showed four specimens (including numbers 621 and 623) remained in contact with the n-hexane for a period of 4 hours after the preliminary treatment. The results indicated that if single bacilli were exposed to n-hexane for

TABLE 8. 10 MINUTES SHAKING OF SUSPECTED TUBERCULOUS MATERIAL. (ONLY POSITIVE SPECIMENS TABULATED).

laboratory number	n-hexane	4% NaOH routine
724	+++	+++
775	neg.	+
834	neg.	+
879	1 col.	++++
919	neg.	3 col.
002	+++	+++
056	neg.	8 col.
138	++	+++
172	neg.	+
251	++	+++
262	1 col.	4 col.

TABLE 9. 5 MINUTES SHAKING TIME OF SUSPECTED TUBERCULOUS MATERIAL. (ONLY POSITIVE SPECIMENS TABULATED).

laboratory number	n-hexane	4% NaOH routine
473	+++	++
558	++++	++
587	++++	++++
588	+++	++
603	+++	++++
604	1 col.	++
605	<u>++++</u>	++++
606	<u>++++</u>	++++
621	neg.	+++
623	neg.	++
628	++++	++

<u>++++</u> more growth than ++++

4 hours, it would become quite susceptible to the toxic effects. The toxic factor may be due to the shaking with n-hexane and leaving for four hours or it may be the 4% sodium hydroxide-n-hexane treatment making the bacilli more susceptible to the prolonged n-hexane treatment. The effect of 100 units of penicillin per cubic centimeter of the concentrated bacilli may be another factor.

I. Comparison of Sampling and Shaking Times From Suspected Tuberculous Specimens.

A variety of times and waiting periods were studied with pretreated specimens.

Of 47 specimens studied at 1 minute shaking and 30 minutes rest, 4 were found positive with n-hexane and 3 were positive with 4% sodium hydroxide. However, the results were felt to be within experimental error as number 593-n-hexane had only one colony and the routine had none.

Forty specimens were studied with 1 minute shaking and 15 minutes rest. The n-hexane concentration gave 8 positives while the 4% sodium hydroxide method showed 9 positives. There were no apparent difference between the two.

With 15 seconds shaking and 15 minutes rest, 7 were found positive with n-hexane and 9 were positive with 4% sodium hydroxide. A total of 40 specimens were studied.

Forty specimens were studied at 15 seconds shaking and 30 minutes rest. The n-hexane showed 13 positives while 4%

sodium hydroxide showed 14. However, this shaking time and speed plus 30 minutes waiting before seeding to media gave different results. Whereas the routine had the usual colony characteristics, all 13 positives with n-hexane showed eugonic growth. Eugonic colonies had been found with hand shaking for 2 minutes and 15 seconds shaking plus 15 minutes rest but they did not grow as eugonic colonies consistently.

J. Comparison of Sampling and Shaking Times From Previously Diagnosed Tuberculosis Patients.

Because the previous specimens were studied from samples already concentrated with 4% sodium hydroxide with potential injury to the bacilli, it was decided to study specimens from patients formerly diagnosed as having active tuberculosis. The dilution of the specimens were based upon the approximate number of bacilli found in their last specimen to the laboratory.

The sputums were decontaminated with 4% sodium hydroxide for 15 minutes. The material was diluted and then treated according to the protocol discussed under Materials and Methods, page 16.

Four specimens were not summarized in Table 10 because one was negative (diluted too far) and three others showed ++++ growth in every tube. Sample number 1 and number 4 appeared to give most bacilli when checked immediately following n-hexane shaking. Samples numbered 2 and 3

TABLE 10. COMPARISON OF SAMPLING AND SHAKING TIMES FROM PATIENTS PREVIOUSLY DIAGNOSED FOR TUBERCULOSIS.

shaking time	resting time (Min.)	A	B	C	D
15 sec.	control	25 col.	++	16 col.	+
	0	+	++	33 col.	++
	15	+	+++	+	+
	30	+	++	30 col.	+
1 min.	control	+	++	30 col.	+
	0	++	+++	33 col.	+++
	15	+	+++	34 col.	++
	30	+	++	+	++
5 min.	control	+	++	26 col.	+
	0	++	+	+	+++
	15	+	++	+	+
	30	+	+++	+	+
10 min.	control	8 col.	++	22 col.	+
	0	++	++	41 col.	++
	15	+	+	+	neg.
	30	+	++	+	++
control		+	++++	+	
sediment		++	++++	43 col.	
n-hexane		+	++++	+	
sediment-hexane		26 col.	++++	++	

appeared to give most bacilli after 15-30 minutes wait. The results were not conclusive as to the desired shaking and waiting times.

From bottle E, the results were again not conclusive. Where the control had ++++ growth, both the sediment and n-hexane had ++++ growth. However, where only + growth was observed with the controls, the sediment had greater growth in one sample and the n-hexane had greater growth in the other.

V. DISCUSSION

A search for a cultural test which can detect small numbers of tubercle bacilli is a given sample was conducted. The use of hydrocarbon flotation of the tubercle bacilli has been reported to be a more efficient method than that of centrifugation or flocculation methods. When hydrocarbon flotation was used as the concentrating technique, growth could not be obtained due to the toxicity of the hydrocarbons. The material had to be stained on a glass slide and the presence or absence of acid-fast bacilli determined. Of the present diagnostic methods, microscopy is the least efficient. Therefore, it would be desirable to achieve concentration and have cultural evidence as well.

Preliminary studies using olive oil as the water-insoluble liquid showed tubercle bacilli could be found in the oil. However, it was found that only 25% of the bacilli grew on culture when compared with the control. The above results were obtained with machine shaking for 10 minutes. No bacilli could be found when shaken by hand for 2 minutes. There was difficulty in spreading the oil onto media and the oil would not evaporate away. The oil that was left in contact with the media appeared to hinder the growth of the tubercle bacilli.

In the flotation technique, it was desirable to find a liquid which would meet the following characteristics:

1. immiscibility with water
2. specific gravity less than 1.0
3. volatile
4. not toxic to mycobacteria
5. cheap and efficient

In the first series of solutions tested, it was found that n-hexane was not very toxic to M. phlei. The other water-immiscible liquids used (acetonitrile, amyl alcohol, butyl alcohol, amyl acetate, benzene and anisol) were all quite toxic to the bacilli.

Using M. phlei as the test organism, it was found that large numbers of bacilli gave evidence that n-hexane was not an efficient concentrating agent. Bacilli could be found in both layers. When the concentrating ability of n-hexane was determined with various dilutions of M. phlei, dramatic results were obtained. As the dilutions were increased, the water portion (water left after n-hexane treatment) had the least growth, the control showed little growth, and the n-hexane tube contained the heaviest growth. This indicated that a given volume of n-hexane may have the capacity to attract only a given number of bacilli when they are in great numbers. It is possible that n-hexane had attracted all it could per unit volume with more bacilli still available.

Up to the present time, no mention has been made in the literature concerning the use of n-hexane as a possible concentrating agent. If the flotation technique was superior to centrifugation as suggested by some authors, it was felt

that n-hexane might prove to be the successful agent.

Experiments were conducted to determine the optimal sampling level and the toxicity of a variety of solutions on M. smegmatis, BCG and H37Ra. It was decided from these experiments that n-hexane should be used in further tests. It was also found that the bacilli could be found most advantageously at the bottom of the n-hexane layer. The concentrating ability of n-hexane was easily demonstrated with M. phlei and M. smegmatis. With BCG and H37Ra discrepancies in results were obtained.

Because of the discrepancies, it was decided to recheck the toxicity of n-hexane upon M. phlei, M. smegmatis, BCG and H37Ra. It was again found that n-hexane was not very toxic after an exposure period of 2 hours. However, evidence of toxic reactions were found after 24 hours exposure. The only difference between the toxicity studies and the study of the concentrating ability of n-hexane was the shaking needed for concentration. It appeared from these experiments that n-hexane plus shaking did not injure M. phlei and M. smegmatis but was injurious to BCG and H37Ra.

Toxicity studies with 4% sodium hydroxide, 5% sulphuric acid and 10% trisodium phosphate showed the bacilli were affected least by 10% trisodium phosphate for an hour contact period. Four percent sodium hydroxide and 5% sulphuric acid were more injurious to M. phlei and M. smegmatis than with

BCG and H37Ra.

Toxicity studies with hydrocarbons previously reported for the flotation methods were determined. All hydrocarbons studied (xylene, benzene, chloroform and ether) showed toxic effects after 10 minutes exposure to M. phlei, M. smegmatis, BCG and H37Ra. Of the hydrocarbons studied, the toxic limits were reached without shaking the specimens.

In determining optimal shaking speed and optimal shaking time, it was found that hand shaking of the specimen was just as effective as the use of a shaking machine. The shaking machine used was a converted centrifuge and the actual shaking speed was not determined. From visual observations, it was found that vigorous hand shaking was equivalent to about 11 rpm on the scale of the International centrifuge. Shaking at lower speeds gave inferior results. Shaking at a higher speed gave results similar to that of hand shaking. However, when shaken very vigorously (equivalent to 16 rpm) leakage of the n-hexane occurred. This was a constant feature and proved to be a potential health hazard. The lids were screwed as tightly as possible but the leakage continued. It was decided to use the hand shaking method to avoid the hazards of the mechanical shaker. It soon became apparent that hand shaking would not be feasable. It was very tiresome to shake the specimens, especially if there were more than three. The optimal hand-shaking time

appeared to be 2 minutes.

In using n-hexane, it was noticed that eugonic colonies appeared quite frequently. In fact, when a positive specimen was treated with 4% sodium hydroxide and then centrifuged, the growth from the neutralized sediment showed small colonies. If this same sediment was treated with n-hexane, the resulting colonies were invariably larger. There were no appreciable difference in the type of growth when there were numerous colonies.

Although the toxicity studies with M. phlei and M. smegmatis showed n-hexane to be mildly toxic to the bacilli, this was not true for H37Ra and BCG. The variable results came about because all the n-hexane was seeded onto media and allowed to evaporate away. It later became apparent that the discrepancies arose because shaking of the specimens with n-hexane had a deleterious effect upon BCG and H37Ra even though shaking with n-hexane did not readily affect the saprophytes. This factor might indicate a difference in surface configuration of the bacilli.

Summing up the findings, it became apparent that BCG, H37Ra and the pathogenic human tubercle bacilli all reacted in the same way. They shared the following characteristics: (1) They readily go to n-hexane, (2) increased shaking with n-hexane caused a decrease in the survival rate, (3) 4% sodium hydroxide is slightly toxic when compared with the

saprophytes, (4) 3% hydrochloric acid was slightly toxic, and (5) all the hydrocarbons tested were toxic except n-hexane. The saprophytes behaved in the following manner: (1) go readily to n-hexane, (2) shaking with n-hexane was not very toxic, (3) 4% sodium hydroxide was quite toxic, (4) 3% hydrochloric acid was quite toxic, and (5) all the hydrocarbons tested were toxic with a few exceptions on a time basis (Table 1, page 19) with no appreciable effect by shaking with n-hexane.

With the aid of Mr. Donald Forsgren (1956), a chemist for the Utah State Department of Health, the following facts became known: (1) wettability by n-hexane indicates fatty or waxy groups surrounding the bacilli with waxes being more readily wet by n-hexane than fats, (2) susceptibility to shaking with n-hexane indicated chemical groups that can become detached by the combined solvation effect of n-hexane and agitation. Resistance to n-hexane on shaking indicates low solubility of the outer layer in n-hexane. It appears that mechanical agitation cannot affect the removal of sufficient groups to disturb the organism. This is typical of straight chain fats. (3) slight toxicity to 4% sodium hydroxide indicates strong saponification factor. (4) low 3% hydrochloric acid toxicity indicates lack of or few amino groups in the bacterial layer. Susceptibility to 3% hydrochloric acid indicates greater numbers of active amino

groups.

With the above factors in mind, it is possible to visualize BCG, H37Ra and the human tubercle bacilli as in Figure A and the saprophytes as in Figure B (page 45).

It is tempting to give another possibility to the concept of the pathogenicity of tubercle bacilli. If we visualize the tubercle bacilli with esters on their outer surfaces, then the esterases in the circulation of the individual takes on importance. When due to stress and strain or poor nutrition, it is possible that the esterase level in the blood decreases to the point at which all of the esters of the tubercle bacilli will not be neutralized. Infection would then set it. This would not necessarily depend upon the "virulent mechanism" of the bacilli but actually to its surface configuration and to the resistance of the individual. The surface configuration may involve a specific ester on the metabolizing bacilli.

It has been said that the low saponification rate is typical of waxes and seems to be associated with the tubercle bacilli. Esterases would not be too effective against a coating of this type. This may mean that when the esterase level drops to the point where infection sets in, just the building up of the esterase level would not help. It would be necessary to kill the available bacilli also. The therapy for tuberculosis patients is essentially one of killing the

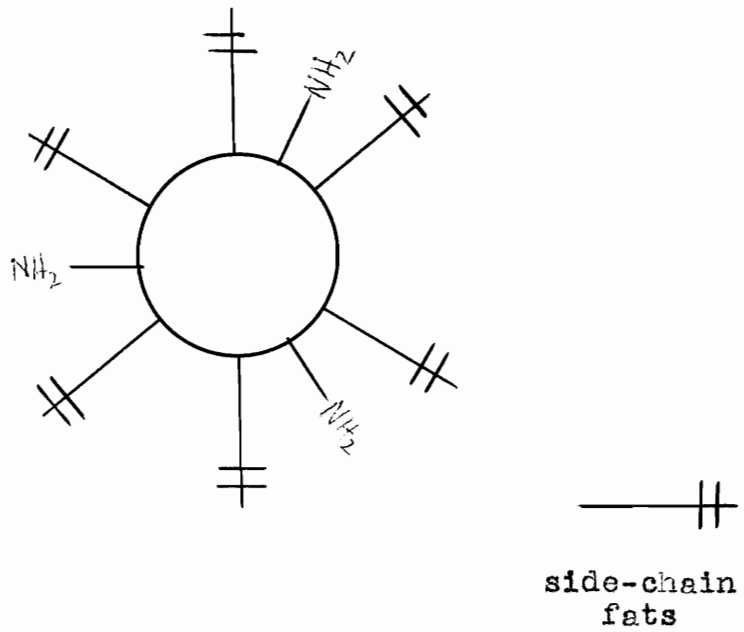


FIGURE A

Presumptive Diagram of BCG, H37Ra and Human Strain

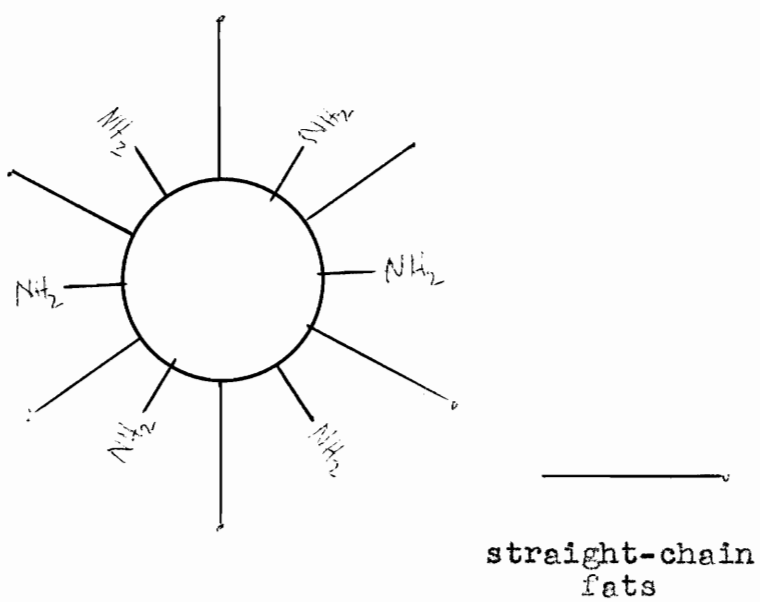


FIGURE B

Presumptive Diagram of the Saprophytes

bacilli present and to bring the patient back to health with proper nutrition.

The saprophytes, which appear to have more amino groups, would not readily cause disease because of the presence of specific esterases in the circulation and because the body defense mechanisms affecting hydrolysis of the protein would also be present.

The variable pathogenicity of the bacilli to different animals might be explained from these factors. It is possible that specific esterases are present in the circulation. Rabbits are very resistant to human tubercle bacilli but are susceptible to the bovine strain. Possibly the rabbit has an abundance of esterases for the human tubercle bacilli. Isolation of specific esterases and the determination of their normal levels in various species of animals could furnish valuable information concerning the validity of this hypothesis.

It would have been helpful if the pathogenicity of the bacilli, before and after n-hexane treatment, could have been determined.

The normal hexane used in these experiments were from petroleum products. Possibly, different results may be obtained by using synthetic normal hexane.

To determine if the action of n-hexane on the bacilli was due to a physical or chemical change would be helpful.

It would be possible to determine the change in the n-hexane layer by infra-red absorption spectrum. If the action of n-hexane is due only to a physical change there would be no change in the n-hexane after shaking with the bacilli.

It is possible, as stated in the book by the American Foundation (1955) that "Virulence as applied to the tubercle bacilli is acquiring meaning in terms of specific morphological and metabolic characteristics possessed by some organisms but not by others."

VI. SUMMARY

1. A study of the feasibility of the flotation method for concentrating acid-fast bacilli with water-insoluble, volatile liquids was conducted.

2. Commercial grade n-hexane, manufactured by the Phillips Petroleum Company, was found to have the characteristics of the desired solution.

3. The toxic effect shown by n-hexane was mild to M. phlei, M. smegmatis, BCG and H37Ra.

4. Shaking of the bacillary suspension produced toxic changes in BCG and H37Ra. M. phlei and M. smegmatis were not affected by the shaking with n-hexane.

5. Concentration of the bacilli into the n-hexane was achieved with shaking of the bacillary suspension with n-hexane.

6. When the suspension was shaken for 15 seconds and allowed to sit for 30 minutes before inoculation onto media, eugonic growth (5-7 mm) grew readily. When the suspensions were shaken vigorously for 10 minutes, the size and numbers of the colony diminished.

7. A presumptive diagram of the possible configuration of the tubercle bacilli and the saprophytes is presented.

8. A concept of the pathogenicity of the tubercle bacilli based upon an ester-esterase complex is suggested.

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CONCENTRATION OF ACID-FAST BACILLI WITH WATER-INSOLUBLE LIQUIDS

by

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An abstract of a thesis submitted to the faculty of the University of Utah in partial fulfillment of the requirements for the degree of

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1956

A study of the feasibility of the flotation method for concentrating acid-fast bacilli with some volatile, water-insoluble liquids were conducted. Although most of the solutions tested were toxic to the test organism (M. phlei), n-hexane did not show these effects. In using n-hexane in further tests with M. phlei, M. smegmatis, BCG and H37Ra, it was found that this hydrocarbon exerted very little deleterious effects as judged from the growth on Lowenstein-Jensen media. The toxic effects appeared with the shaking of the specimens with n-hexane. The saprophytes (M. phlei and M. smegmatis) showed very little effect from being shaken with n-hexane. BCG and H37Ra was very susceptible to being shaken with n-hexane.

The toxic effects were more pronounced when the specimens were shaken with n-hexane and all the n-hexane seeded to media and allowed to evaporate away. While the source of the toxic effect is unknown, the experiments showed it may possibly be due to the shaking with n-hexane, the prolonged contact of n-hexane with the bacilli after shaking of the specimen or the action of penicillin and n-hexane after shaking the specimen.

A variety of shaking times and shaking speeds were studied with suspected and known tuberculous specimens. The specimens were concentrated by the 4% sodium hydroxide-centrifugation method. When the sediment was seeded to

media, the usual type of colonies grew. However, when the sediment was shaken vigorously for 15 seconds with n-hexane, eugonic colonies grew. Some eugonic colonies appeared when the specimen were shaken by hand for 2 minutes. Generally speaking, as the shaking time and shaking speeds were increased, the amount of recoverable bacilli diminished.